RESEARCH ARTICLE SUMMARY

EPIGENETICS

Epigenetic inheritance uncoupled from sequence-specific recruitment

Kaushik Ragunathan, Gloria Jih, Danesh Moazed*

INTRODUCTION: Changes in histone post-translational modifications are associated with epigenetic states that define distinct patterns of gene expression. Whereas sequence-specific DNA binding proteins play essential roles in establishing an epigenetic state, their contributions to maintenance remain unclear. Previous attempts to separate the inheritance of epigenetic states from sequence-specific establishment suggest that specific DNA sequences and DNA binding proteins are continuously required for epigenetic inheritance. Moreover, in addition to DNA binding proteins, the establishment and maintenance of epigenetic states involves self-reinforcing interactions between histone modifications and RNA interference (RNAi) or DNA methylation. Therefore, whether histone-based mechanisms can transmit epigenetic memory independently of specific DNA sequences remains unknown.

RATIONALE: The fission yeast *Schizosaccharomyces pombe* contains chromosomal domains that share many features with heterochromatin in multicellular eukaryotes, such as methylation of histone H3 lysine 9 (H3K9), catalysis by the human Suv39h homolog Clr4, association with HP1 proteins (Swi6 and Chp2), and histone hypoacetylation. We developed an inducible system for heterochromatin establishment in *S. pombe* by fusion of the Clr4 methyltransferase catalytic domain to the bacterial tetracycline repressor (TetR) protein. To generate a reporter locus, we introduced 10 tetracycline operators upstream of the normally expressed *ade6*$^+$ gene (*10XtetO-ade6*$^+$). The silencing of *ade6*$^+$ results in the formation of red or pink colonies upon growth on medium with limiting adenine concentrations. This system allowed us to determine whether heterochromatin, once established, could be maintained after tetracycline-mediated release of the TetR-Clr4 initiator (TetR-Clr4-I) from DNA.

RESULTS: Cells containing the reporter gene in combination with the expression of TetR-Clr4-I formed pink colonies on low-adenine medium lacking tetracycline, indicating *ade6*$^+$ silencing. The establishment of heterochromatin resulted in high levels of H3K9 methylation (H3K9me), which was subsequently lost upon tetracycline-induced release of TetR-Clr4-I within ~10 cell divisions, resulting in the appearance of white colonies. Whereas perturbations to pathways that altered the rate of histone exchange or eliminating competition from endogenous heterochromatic loci had subtle effects on epigenetic inheritance of *ade6*$^+$ silencing, deletion of the putative JmjC domain–containing demethylase Epe1 resulted in cells that retained *ade6*$^+$ silencing for >50 generations after tetracycline-induced release of TetR-Clr4-I or deletion of the TetR module. Furthermore, the chromodomain of Clr4, which is involved in recognition of the H3K9me mark, was indispensable for maintenance, suggesting that a direct "read-write" mechanism mediated by Clr4 propagates histone modifications and allows histones to act as carriers of epigenetic information. This mechanism allows epigenetic states to be inherited during mitosis and meiosis and is also critical for maintaining low levels of H3K9me at native pericentromeric repeats.

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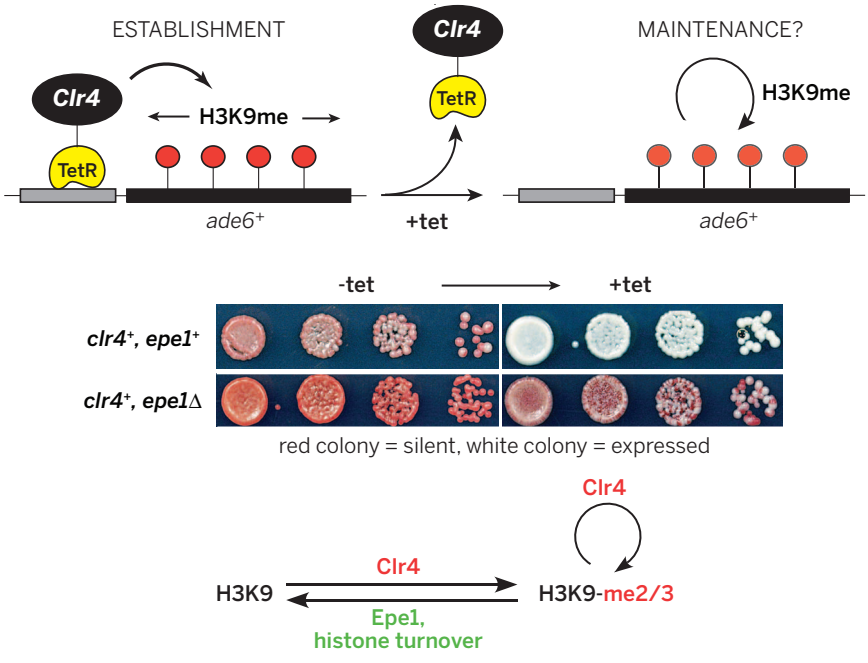
CONCLUSION: Our findings indicate that even in the absence of any coupling to other positive-feedback loops, or in the absence of sequence-dependent initiation signals, H3K9me defines a silent state that can be epigenetically inherited. Maintenance of the OFF state is determined by the balance between the rate of H3K9me by the Clr4 reader-writer module and the loss rate due to demethylation by an Epe1-dependent mechanism, transcription-coupled nucleosome exchange, and dilution of histones during DNA replication. The regulation of histone demethylation activity may play a broad role in determining the reversibility of epigenetic states. ■



H3K9me defines a silent state that can be epigenetically inherited. A direct read-write mechanism involving the Clr4 H3K9 methyltransferase propagates histone modifications and allows histones to act as carriers of epigenetic information in the absence of any input from the DNA sequence, DNA methylation, or RNAi. Epe1, a putative demethylase, and other transcription-associated histone turnover pathways modulate the rate of decay of the epigenetic state.

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RESEARCH ARTICLE

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Epigenetic inheritance uncoupled from sequence-specific recruitment

Kaushik Ragunathan, Gloria Jih, Danesh Moazed*

Changes in histone posttranslational modifications are associated with epigenetic states that define distinct patterns of gene expression. It remains unclear whether epigenetic information can be transmitted through histone modifications independently of specific DNA sequence, DNA methylation, or RNA interference. Here we show that, in the fission yeast *Schizosaccharomyces pombe*, ectopically induced domains of histone H3 lysine 9 methylation (H3K9me), a conserved marker of heterochromatin, are inherited through several mitotic and meiotic cell divisions after removal of the sequence-specific initiator. The putative JmjC domain H3K9 demethylase, Epe1, and the chromodomain of the H3K9 methyltransferase, Clr4/Suv39h, play opposing roles in maintaining silent H3K9me domains. These results demonstrate how a direct "read-write" mechanism involving Clr4 propagates histone modifications and allows histones to act as carriers of epigenetic information.

An individual cell can give rise to progeny with distinct patterns of gene expression and phenotypes without change in its DNA sequence. In eukaryotic cells, a major mechanism that gives rise to such phenotypic or epigenetic states involves changes in histone posttranslational modifications and chromatin structure (*1*, *2*). The basic unit of chromatin is the nucleosome, which is composed of 147 base pairs (bp) of DNA wrapped twice around an octamer composed of histones H2A, H2B, H3, and H4 (*3*). The highly conserved basic N termini and, to a lesser extent, the globular domains of histones contain a variety of posttranslational modifications that affect nucleosome stability or provide binding sites for effectors that activate or repress transcription (*4*–*8*).

It has been established for nearly four decades that parental histones are retained and randomly distributed to newly synthesized daughter DNA strands during DNA replication (*9*–*12*). It was therefore logical to propose that combinations of histone modifications, sometimes referred to as a "histone code," are responsible for epigenetic memory of gene expression patterns (*13*, *14*). However, previous attempts to separate sequence-specific establishment from maintenance have failed to provide unambiguous support for a purely histone-based inheritance mechanism in systems that display stable epigenetic expression states (*2*, *15*, *16*). Most notably, silencers (i.e., DNA sequences that mediate the establishment of hypoacetylated domains of silent chromatin in the budding yeast *Saccharomyces cerevisiae*) are continuously required for maintenance of the silent state (*17*, *18*). Similarly, the *Drosophila* Polycomb response element, which acts analogously to the yeast silencer, is continuously required for maintenance of domains of histone H3 lysine 27 (H3K27) methylation and reporter gene silencing (*19*, *20*). The question therefore remains as to whether histones can act as carriers of epigenetic information in the absence of any input from the underlying DNA sequence.

The fission yeast *Schizosaccharomyces pombe* contains extensive domains of heterochromatin at its pericentromeric DNA repeats, subtelomeric regions, and the silent mating type loci (*21*). These domains share many features of heterochromatin in multicellular eukaryotes, such as H3K9 methylation (H3K9me), which is catalyzed by the human Suv39h homolog Clr4; association with HP1 proteins (Swi6 and Chp2); and histone hypoacetylation. Furthermore, *S. pombe* heterochromatin displays epigenetic inheritance properties in which cells containing a reporter gene inserted within heterochromatin display variegating reporter gene expression (*22*). The ON and OFF states of such reporter genes can be stably transmitted in cis through both mitotic and meiotic cell divisions (*23*, *24*). However, because these observations of epigenetic inheritance were made at native sequences, contributions arising from sequence-specific elements that stabilize heterochromatin could not be ruled out (*2*). To determine whether heterochromatin maintenance can be separated from the sequences that initiate its establishment, we developed a system for inducible heterochromatin establishment in *S. pombe* by fusion of the Clr4 methyltransferase catalytic domain to the bacterial tetracycline repressor (TetR) protein. This allowed us to establish an extended heterochromatic domain and study its initiator-independent maintenance either by tetracycline-mediated release of TetR-Clr4 from DNA or after deletion of the TetR module. Our results indicate that domains of H3K9me can be inherited for >50 generations in the absence of sequence-specific recruitment and define central roles for the putative demethylase, Epe1, in the erasure of H3K9me and the chromodomain of the Clr4 methyltransferase in its maintenance.

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Inducible establishment of heterochromatin

Silent chromatin domains can be established by ectopic recruitment of histone-modifying enzymes to chromatin via fusion with heterologous DNA binding proteins (*25*, *26*). To create an inducible system for heterochromatin formation, we fused a Clr4 protein lacking its N-terminal chromodomain (required for binding to methylated histone H3K9) while retaining its enzymatic methyltransferase activity to the bacterial TetR protein (designated "TetR-Clr4-I" for TetR-Clr4 initiator) (Fig. 1A). The TetR DNA binding domain facilitates protein targeting to a locus that harbors its cognate DNA binding sequence, and this recruitment activity is abrogated by the addition of tetracycline ($TetR_{off}$ system) (*27*). We generated cells in which TetR-Clr4-I replaced the wild-type (WT) Clr4 (*TetR-clr4-I*) or in which WT Clr4 was intact and TetR-Clr4-I was inserted at another locus (*TetR-clr4-I, clr4*$^+$). Comparisons between strains with or without *clr4*$^+$ allowed us to evaluate the contribution of the Clr4 chromodomain to establishment and/or maintenance. To generate a reporter locus, we replaced the euchromatic *ura4*$^+$ locus with an *ade6*$^+$ gene containing 10 tetracycline operators immediately upstream of the promoter (*10XtetO-ade6*$^+$) (Fig. 1A). *ade6*$^+$ provides a convenient visual reporter, as its silencing results in formation of red or pink colonies upon growth on medium with limiting adenine concentrations (*22*).

As shown in Fig. 1B, cells containing the *10XtetO-ade6*$^+$ reporter in combination with the expression of the TetR-Clr4-I fusion protein, but not those containing the fusion protein alone or the reporter alone, formed pink colonies on low-adenine medium lacking tetracycline (–tet). Consistent with previous observations (*26*), silencing did not require *clr4*$^+$—suggesting that the chromodomain of Clr4 was not required for de novo heterochromatin establishment (Fig. 1B)—but depended on HP1 proteins (Swi6 and Chp2) and histone deacetylases (Clr3 and Sir2), which act downstream of H3K9me (fig. S1A). Colonies grown in the absence of tetracycline were then plated on medium containing tetracycline (+tet) to determine whether the silent state could be maintained upon release of TetR-Clr4-I from DNA. Tetracycline-dependent release of TetR-Clr4-I from *tetO* sites resulted in the loss of silencing, as indicated by the formation of white colonies (Fig. 1B, +tet). Chromatin immunoprecipitation (ChIP) experiments verified that tetracycline addition resulted in release of TetR-Clr4-I from the *10XtetO* sites, as the TetR ChIP signal in the presence of tetracycline was near background levels similar to that observed for cells lacking TetR-Clr4-I (Fig. 1C). Furthermore, ChIP combined with high-throughput sequencing (ChIP-seq) and ChIP

quantitative polymerase chain reaction (qPCR) experiments showed that a 40- to 50-kb domain of H3K9 di- and trimethylation (H3K9me2 and -me3) encompassing the *10XtetO-ade6*$^+$ region was completely lost 24 hours (~10 cell divisions) after the addition of tetracycline to the growth medium (Fig. 1D and fig. S1, B to D). We obtained similar results with cells that carried a WT copy of *clr4*$^+$ in addition to *TetR-clr4-I* (Fig. 1D and fig. S1D, lower two rows). These results demonstrate that a domain of H3K9me and the associated silent state are reversed within 10 cell divisions after release of the sequence-specific initiator from DNA.

Inheritance uncoupled from sequence-specific initiation

A minimal mechanistic requirement for inheritance of a domain containing H3K9me marks involves the recognition of preexisting H3K9 methylated histones coupled to the modification of newly deposited histones. The loss of a ~45-kb domain of H3K9 dimethylation after release of TetR-Clr4-I (Fig. 1D) may either be due to nucleosome exchange processes associated with transcription and chromatin remodeling or erasure of the methyl mark by a demethylase. Alternatively, the rapid decay of methylation in this domain may be facilitated by competition between the ectopic locus and native heterochromatic domains for a limiting pool of proteins that play critical roles in maintenance.

To test these different scenarios, we constructed *TetR-clr4-I, 10XtetO-ade6*$^+$ cells (with or without *clr4*$^+$) that carried deletions for genes involved in various chromatin-maintenance pathways (*28*–*30*). The deletion of *epe1*$^+$, which encodes a putative histone H3K9 demethylase (*30*), did not affect the establishment of silencing, as indicated by the appearance of red colonies on –tet medium for both *TetR-clr4-I, epe1*Δ cells and *TetR-clr4-I, clr4*$^+$*, epe1*Δ cells (Fig. 2A, left side). In contrast to *epe1*$^+$ cells, *epe1*Δ cells formed red, white, and sectored colonies in the presence of tetracycline, indicating that the silent state was maintained after release of the initiator and that this phenotype was observed only in the case of cells that also contained a WT copy of *clr4*$^+$ (Fig. 2A, right side; compare last two rows with first two rows). ChIP experiments verified that tetracycline addition promoted the release of TetR-Clr4-I from *tetO* sites (Fig. 2B). Consistent with the colony color–silencing assays, ChIP-seq and ChIP-qPCR experiments also indicated that the large domain of H3K9 di- and trimethylation surrounding the *10XtetO-ade6*$^+$ locus was lost in *TetR-clr4-I, epe1*Δ cells but was maintained in *TetR-clr4-I, clr4*$^+$*, epe1*Δ cells (Fig. 2C and fig. S2, A to C) 24 hours after the addition of tetracycline. Within this domain, the expression of several other transcription units was silenced on –tet medium, and this silencing was maintained for several hours after tetracycline addition (fig. S3).

Maintenance of the silent state in *epe1*Δ cells required the HP1 proteins (Swi6 and Chp2) and the Clr3 and Sir2 histone deacetylases (Fig. 3A) but not the Dicer ribonuclease (Dcr1) or the Argonaute protein (Ago1) (Fig. 3, B and C). Thus, maintenance requires the machinery that acts downstream of H3K9me but occurs independently of an RNA interference (RNAi)–based mechanism. Consistent with the idea that Epe1 acts as a demethylase, the replacement of *epe1*$^+$ with alleles containing active-site mutations (*epe1-K314A* and *epe1-H297A*) (*30*, *31*) displayed a maintenance phenotype similar to its deletion (*epe1*Δ) (Fig. 3D). Furthermore, consistent with a requirement for the chromodomain of *clr4*$^+$ in maintenance (Fig. 2A), the replacement of *clr4*$^+$ with a *clr4-I*Δ allele (which lacks the chromodomain) resulted in the formation of only white colonies on +tet medium (Fig. 3E). Therefore, rather than differences in *clr4*$^+$ dosage, the appearance of red or sectored colonies depends on the presence of Clr4 with an intact chromodomain.

In an attempt to identify other features of chromatin that could be important for maintenance, we deleted two genes that are associated with transcription: *mst2*$^+$, which encodes a histone acetyltransferase (*32*), and *set1*$^+$, which encodes a histone H3K4 methyltransferase (*33*). In both cases, we observed establishment that was stronger than that of the wild type (–tet), as well as weak maintenance (+tet) effects (fig. S4, A and

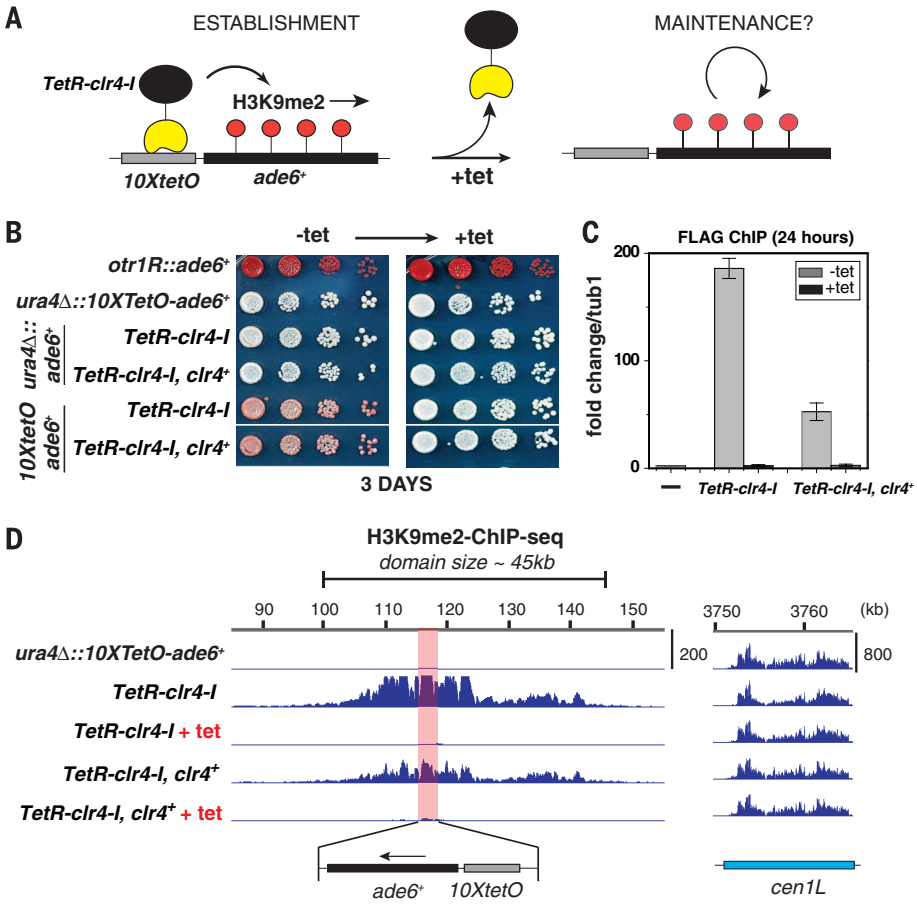

Fig. 1. Ectopic heterochromatin is lost after sequence-specific establishment upon tetracycline addition. (**A**) Diagram of experimental scheme for TetR-Clr4-I (TetR-Clr4-I)–mediated H3K9me at the *ura4Δ::10XtetO-ade6*$^+$ locus. Tetracycline (tet) promotes the release of TetR-Clr4-I from *tetO* sites so that initiator-independent maintenance could be tested. (**B**) Test of *ade6*$^+$ silencing on low-adenine medium in the absence (-tet) and presence (+tet) of tetracycline. Silencing of *ade6*$^+$ results in formation of red colonies. Centromeric *ade6*$^+$ (*otr1R::ade6*) served as a positive control; the target locus alone (*ura4Δ::10XtetO-ade6*$^+$) and *ura4Δ::ade6*$^+$ cells served as negative controls. (**C**) ChIP-qPCR experiments assess the association of FLAG-tagged TetR-Clr4-I with the *10XtetO-ade6*$^+$ locus in the presence and absence of tetracycline. The dash on the *x* axis indicates background ChIP signal from cells that did not express TetR-Clr4-I. In the presence of tetracycline, TetR-Clr4-I occupancy is close to background levels. Error bars indicate SD. (**D**) ChIP-seq experiments show that TetR-Clr4-I induces a de novo H3K9me2 domain that surrounds the *tetO-ade6*$^+$ locus (highlighted in red) for ~20 kb on either side, in cells with or without *clr4*$^+$, which is lost 24 hours after tetracycline addition. H3K9me2 ChIP-qPCR data and H3K9me3 for samples in (D) are presented in fig. S1. Chromosome 3 (site of insertion of *10XtetO-ade6*$^+$) and chromosome 1 [centromere 1 left (*cen1L*)] coordinates are shown above the tracks, and read numbers (per million) are indicated on the right. H3K9me2 at DNA repeats of *cen1L* serves as an internal control for the ChIP-seq data.

D). Although there were no obvious effects on colony color after 3 days of growth on tetracycline-containing medium, we observed clear persistence of H3K9me coupled with the release of TerR-Clr4-I in *mst2*Δ cells 24 hours after the addition of tetracycline (fig. S4, B and C). We then tested whether destabilizing endogenous heterochromatic regions could release factors that affect maintenance by deleting *pozI*$^+$, a DNA binding protein required for heterochromatin formation at telomeres (*34*), or *dcr1*$^+$, which is required for RNAi-dependent heterochromatin formation at centromeres (*35*) (fig. S5). In these mutants, effects on colony color were absent (*dcr1*Δ, fig. S5A) or subtle (*poz1*Δ, fig. S5D), but we clearly observed persistence of H3K9me 24 hours after release of TetR-Clr4-I in both deletion backgrounds (fig. S5, B, C, E, and F).

These results indicate that a domain of H3K9 methylation and its associated silent state can be maintained through mitotic cell divisions in the absence of sequence-specific initiation. The decay rate of this epigenetic state is primarily influenced by the erasure of the methyl mark by the putative demethylase Epe1 and, to a lesser extent, by pathways that promote transcription or heterochromatin assembly at endogenous loci. We note that the efficiency of maintenance does not correlate with the strength of the initial silenced state (establishment), indicating that perturbations to each pathway results in the release or recruitment of subsets of proteins that make different contributions to the establishment and/or maintenance of heterochromatin.

Because the different deletions essentially altered the rate of decay of ectopic H3K9me, we hypothesized that epigenetic maintenance at the ectopic locus might also exist in WT cells over the time scales of a few cell divisions. To observe the decay of silent chromatin with higher time resolution, we generated cells in which a green fluorescent protein (GFP) reporter was silenced by TetR-Clr4-I. We modified the endogenous *ura4*$^+$ gene to encode a Ura4-GFP fusion protein. In addition, we inserted the same *10XtetO* sites used in the earlier experiments immediately upstream of a 114-bp fragment of the *ura4*$^+$ promoter (Fig. 4A). As determined by fluorescence-activated cell sorting (FACS) analysis, *10XtetO-ura4-GFP* was silenced in a TetR-Clr4-I–dependent manner in the presence or absence of *clr4*$^+$. To assess decay rates, we transferred cells that were grown in medium lacking tetracycline (GFP OFF cells) to medium containing tetracycline and harvested samples at 0, 3, 6, 9, 12, 15, 23, 26, 32, 36, 39, 50, 60, 77, and 100 hours for FACS analysis. FACS data for a subset of these time points are presented in Fig. 4, B to E, and the results for all time points are plotted in Fig. 4F. In general, upon tetracycline addition, the pattern of silencing of the *10XtetO-ura4-GFP* reporter in either *epe1*$^+$ or *epe1*Δ over ~2 days was consistent with the *10XtetO-ade6*$^+$ silencing results (Fig. 4, B and C). With the higher time resolution and sensitivity of detecting phenotypic expression states, we found that the OFF state persists in ~10% of the *TetR-clr4-I, clr4*$^+$*, epe1*$^+$ cells for up to 40 hours (~20 cell divisions) after transfer to tetracycline-containing medium (Fig. 4, D and F), whereas cells containing *TetR-clr4-I, clr4*$^+$*, epe1*Δ displayed the most stable maintenance patterns with ~60% of the cells maintaining the OFF state, even after 100 hours of growth (~50 cell divisions) in tetracycline-containing medium (Fig. 4E). These observations indicate that epigenetic maintenance was not unique to *epe1*Δ cells and that its detection was normally masked by the rapid erasure of H3K9me marks by Epe1. Although the decay rate for cells containing *TetR-clr4-I, epe1*$^+$ was rapid (<6 hours), the deletion of Epe1, even in this background, resulted in a slower decay rate (Fig. 4E). We observed the GFP OFF state in *TetR-clr4-I, epe1*Δ cells lacking *clr4*$^+$, 6 hours after transfer to tetracycline medium with a complete shift to the ON state occurring only at ~23 hours after transfer (Fig. 4C). In these cells, upon elimination of Epe1, the decay rate is probably defined primarily by the dilution of modified histones, which may maintain epigenetic states for a few generations in the absence of Clr4-mediated reestablishment.

It may be argued that maintenance of H3K9me and the silent state in the initiator-based experiments might arise from low-affinity binding of TetR-Clr4-I to DNA in the presence of tetracycline (*27*). We sought to unequivocally rule out any role for sequence-dependent initiation in the inheritance we observed by using homologous recombination to replace *TetR-clr4-I* with *clr4-I*Δ, which harbors a deletion of the TetR DNA binding domain (Fig. 5A). After transformation to replace *TetR-clr4-I* with *clr4-I*Δ and plating on selective low-adenine medium (Fig. 5A), we obtained red, sectored, and white colonies, which were tested and confirmed for the replacement event by allele-specific PCR (fig. S6). The isolation of red *clr4-I*Δ*, epe1*Δ cells confirmed that the silent state could be maintained in the complete absence of sequence-dependent Clr4 recruitment to DNA. Furthermore, the plating of red *clr4-I*Δ*, epe1*Δ cells on low-adenine medium produced red, sectored, and white colonies (Fig. 5B), whereas plating of white *clr4-I*Δ*, epe1*Δ isolates produced only white colonies (Fig. 5C). In the absence of the initiator and no other means of reestablishment, the loss of the silent state was an irreversible event (Fig. 5C). Consistent with the deletion of the TetR domain, ChIP experiments showed a complete loss of the TetR occupancy signal at the *10XtetO-ade6*$^+$ locus (Fig. 5D). On the other hand, we detected high levels of H3K9 dimethylation at the *10XtetO-ade6*$^+$ locus in red but not white isolates (Fig. 5E). We conclude that, once

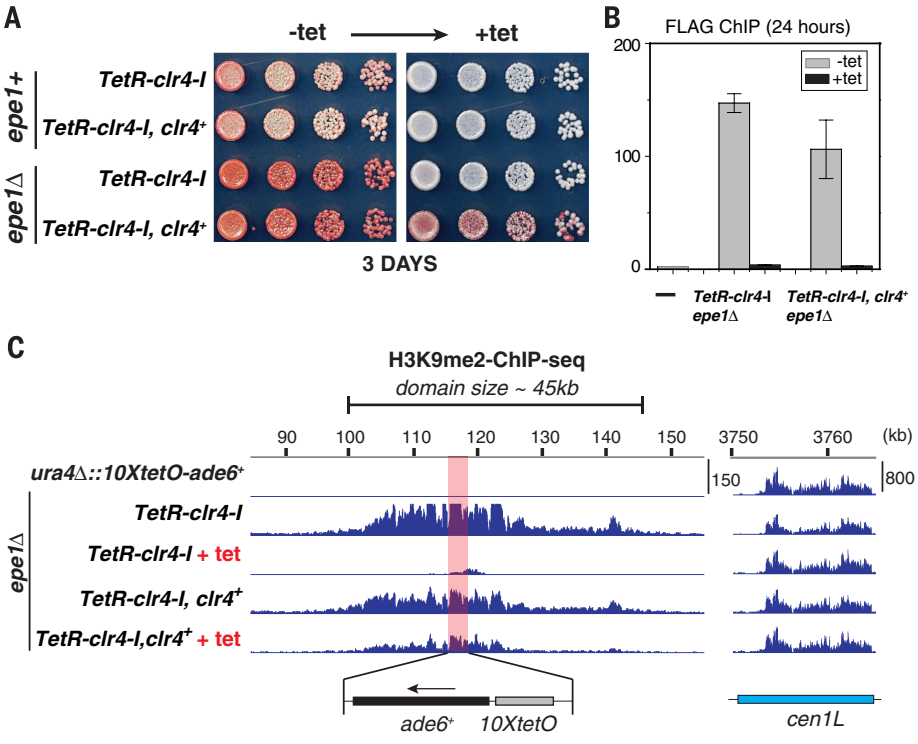

Fig. 2. Deletion of *epe1*$^+$ allows maintenance of heterochromatin after release of TetR-Clr4-I. (**A**) Color-silencing assays showing that in *TetR-clr4-I, clr4*$^+$*, epe1*Δ cells, silencing is maintained on +tet medium. (**B**) ChIP experiments showing that TetR-Clr4-I is released from *tetO* sites in +tet medium. Error bars indicate SD. (**C**) ChIP-seq experiments showing that in *epe1*Δ cells, H3K9me2 is maintained 24 hours after tetracycline addition in a *clr4*$^+$-dependent manner. H3K9me2 ChIP-qPCR and H3K9me3 ChIP-seq data for samples shown here are presented in fig. S2. Chromosome 3 (site of insertion of *10XtetO-ade6*$^+$) and chromosome 1 (*cen1L*) coordinates are shown above the tracks, and read numbers (per million) are indicated on the right. H3K9me2 at DNA repeats of *cen1L* serves as an internal control for the ChIP-seq data.

assembled, silent chromatin and histone H3K9me at the *10XtetO-ade6*$^+$ locus can be maintained in the complete absence of the sequence-specific recruitment. To determine whether the initiator-independent silent state could also be inherited through meiosis, we crossed red haploid cells of opposite mating type, which lacked the TetR DNA binding domain (*clr4-IΔ*, *clr4*$^+$, *epe1Δ*), to obtain diploid cells (Fig. 6A). These diploid cells were then sporulated, and after tetrad dissection, the resulting haploid progeny were plated on low-adenine medium. As shown in Fig. 6B, the resulting haploid cells formed mostly red or sectored colonies, indicating that the silent state was also inherited through meiotic cell divisions.

Inheritance of H3K9me at native heterochromatin

We next determined the extent to which epigenetic maintenance mechanisms akin to what is observed for the ectopic locus might also operate at native *S. pombe* pericentromeric repeats. Although RNAi is required for silencing of reporter genes that are inserted within pericentromeric repeat regions, deletion of RNAi components does not entirely eliminate H3K9me and silencing (*36*, *37*) (Fig. 7A). Residual H3K9me in RNAi deletions might arise from either epigenetic maintenance mechanisms or weak RNAi-independent establishment signals within centromeres (*38*). To test for the presence of RNAi-independent signals that may operate at pericentromeric repeats, we determined whether H3K9me could be established de novo at the repeats in cells with deletions of RNAi factors *dcr1*$^+$ or *ago1*$^+$. To perform this experiment, we reintroduced *clr4*$^+$ into *clr4Δ*, *clr4Δ ago1Δ*, or *clr4Δ dcr1Δ* cells (Fig. 7B) and used ChIP-seq and ChIP-qPCR to quantify H3K9 dimethylation levels. As shown in Fig. 7C, the reintroduction of *clr4*$^+$ into *clr4Δ* cells fully restored H3K9 dimethylation at the pericentromeric *dg* and *dh* repeats of chromosome 1. In contrast, *clr4*$^+$ reintroduction into *clr4Δ ago1Δ* or *clr4Δ dcr1Δ* double-mutant cells failed to promote any H3K9 dimethylation (Fig. 7, C and D). These results show that RNAi is the primary mechanism for sequence-specific establishment of pericentromeric H3K9me domains and suggest that the H3K9me observed at these repeats after deletion of RNAi components results from epigenetic maintenance.

Our ectopic heterochromatin experiments established a role for the chromodomain of Clr4 in epigenetic inheritance of H3K9me (Figs. 2 to 5). Consistent with the hypothesis that RNAi-independent H3K9me at pericentromeric repeats is maintained by epigenetic mechanisms, residual H3K9me at the centromeric *dg* repeats was abolished in *ago1Δ clr4-W31G* double-mutant cells (Fig. 7E). Clr4-W31G contains a mutation in the chromodomain that attenuates binding to H3K9me (*39*). The complete loss of H3K9me in *ago1Δ clr4-W31G* double mutant cells therefore suggests that the residual H3K9me marks are maintained by a mechanism that involves direct chromodomain-dependent recruitment of Clr4 to preexisting marks. In further support of this hypothesis, the introduction of an additional copy of WT *clr4*$^+$, but not the *clr4-W31G* mutant, into *ago1Δ* cells boosted the residual H3K9me levels approximately threefold (Fig. 5F). Together, these results support a direct "read-write" mechanism in which Clr4 binds to preexisting H3K9-methylated nucleosomes and catalyzes the methylation of H3K9 on newly deposited nucleosomes to maintain heterochromatin independently of the initial signals that induced methylation. It is noteworthy that, in RNAi mutant cells, deletions of *epe1*$^+$, *mst2*$^+$, and *pzl1*$^+$ boost H3K9me levels and restore silencing to varying degrees at the pericentromeric repeats (*28*–*30*). Furthermore, consistent with its role in H3K9me inheritance, the chromodomain of Clr4 is also required for the RNAi-independent spreading of H3K9me at the mating type locus (*40*). Our results suggest that the epigenetic maintenance of H3K9me, rather than alternative establishment pathways, is primarily responsible for RNAi-independent silencing in these deletion backgrounds.

Discussion

Our findings on mitotic and meiotic inheritance of H3K9me and silent chromatin in the absence of sequence-specific recruitment—and without requirement for a small RNA (sRNA) positive-feedback loop associated with RNAi, or other known modification systems such as DNA CpG methylation—strongly suggest that histones modified by H3K9me can act as carriers of epigenetic information. This conclusion is supported by (i) our demonstration that deletion of the fission yeast putative histone H3K9 demethylase, *epe1*$^+$, stabilizes the epigenetic OFF state and allows its transmission through >50 cell divisions and (ii) the requirement for the Clr4 methyltransferase chromodomain, a domain that recognizes

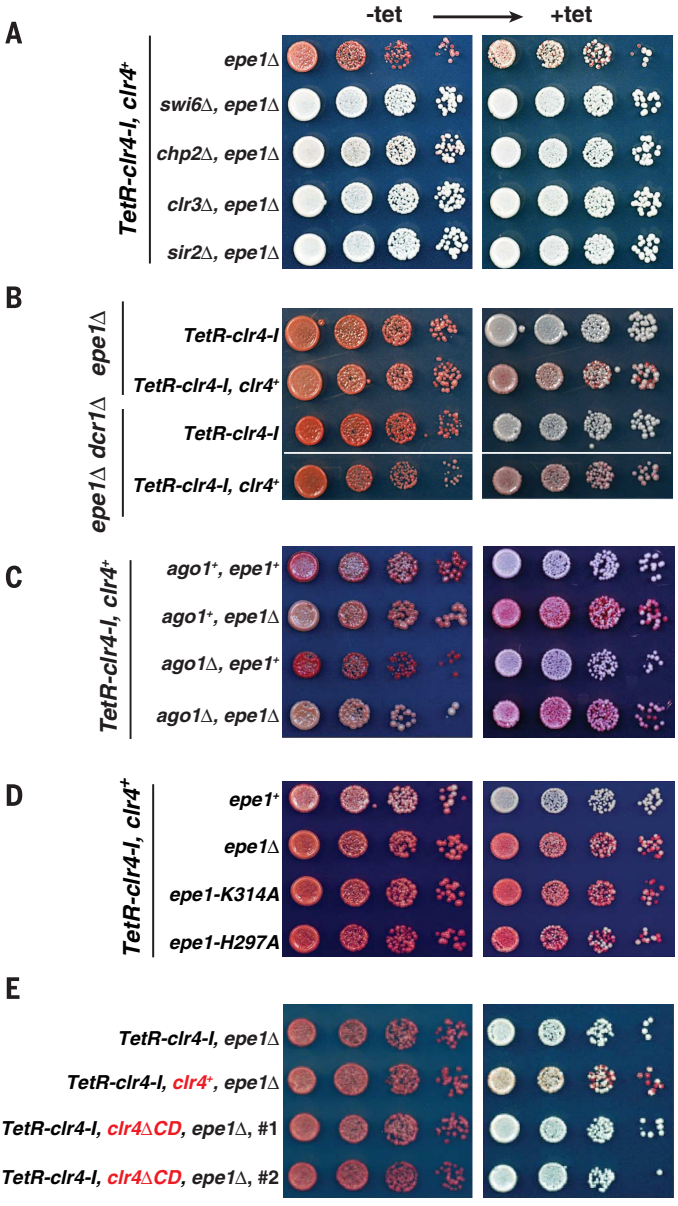

Fig. 3. Requirements for maintenance of the initiator-independent silent state. (**A**) Establishment and maintenance require HP1 proteins (Swi6 and Chp2) and histone deacetylases (Clr3 and Sir2). (**B**) Maintenance of ectopic silencing in *epe1Δ* cells does not require Dicer (Dcr1), as indicated by the growth of red *dcr1Δ* cells on +tet medium. (**C**) Maintenance of ectopic silencing in *epe1Δ* cells does not require Argonaute (Ago1), as indicated by the growth of red *ago1Δ* cells on +tet medium. (**D**) Either deleting *epe1* (*epe1Δ*) or mutations in its active site (*epe1-K314A* or *epe1-H297A*) allow maintenance of the off state after release of the TetR-Clr4-I initiator. (**E**) Replacement of *clr4*$^+$ with *clr4ΔCD*, encoding Clr4 lacking the chromodomain, abolishes initiator-independent silencing.

di- and trimethylated H3K9, suggesting a direct read-write mechanism for this mode of epigenetic inheritance.

Several recent studies have described the transgenerational inheritance of environmentally induced changes in gene expression from parent to offspring (*41*). The mechanism of this transgenerational inheritance has not been fully defined but appears to occur via both sRNA-dependent and -independent pathways. In *Caenorhabditis elegans*, plants, fission yeast, and possibly other systems, the transmission of histone modification patterns is often coupled to sRNA generation and/or CpG DNA methylation (*42*–*44*). The latter pathways can form positive-feedback loops that help maintain histone modification patterns (*2*, *45*). This coupling of positive-feedback loops would increase the rate of reestablishment of silent domains and thus counteract the erasure activity of enzymes such as Epe1 or mechanisms that increase the rate of histone turnover.

A previous study used a small-molecule dimerization strategy to show that ectopically induced domains of H3K9me at the Oct4 locus in murine fibroblasts can be maintained after the removal of the small-molecule inducer by a mechanism that is reinforced by CpG DNA methylation (*46*). However, unlike the experiments presented here, the use of the Oct4 locus, which is normally packaged into heterochromatin in fibroblasts (*46*), precludes any conclusions about sequence-independent inheritance, as contributions from locus-specific sequence elements that normally silence Oct4 in differentiated cells cannot be ruled out. It therefore remains to be determined whether H3K9me can be inherited independently of specific DNA sequences or modulation of H3K9 demethylase activity in mammalian cells. Also, in fission yeast, a previous study reported that ectopic heterochromatin-dependent silencing of an *ade6*$^+$ allele induced by a centromeric DNA fragment is maintained in an RNAi-dependent manner after excision of the centromeric DNA fragment (*47*), suggesting that a sRNA amplification loop may somehow be established at the *ade6*$^+$ locus, which helps to maintain the silent state. However, these findings contradict other studies, which have demonstrated that silencing of *ura4*$^+$ alleles by the generation of sRNA from a hairpin could not be maintained in the absence of the inducing hairpin (*48*, *49*). Our findings indicate that even in the absence of coupling to other positive-feedback loops, or in the absence of sequence-dependent initiation signals, H3K9me defines a silent state that can be epigenetically inherited (fig. S7). Maintenance of the OFF state is probably determined by the balance between the rate of H3K9me by the Clr4 reader-writer module and the loss rate due to demethylation by an Epe1-dependent mechanism, transcription-coupled nucleosome exchange, and dilution of histones during DNA replication.

Although demethylase activity for the *S. pombe* Epe1 protein has not yet been demonstrated in vitro, key residues required for demethylase activity in other Jumonji domain proteins are conserved in Epe1 and required for its in vivo effects on silencing (*30*, *50*) and its effect on initiator-independent inheritance of heterochromatin (this study, Fig. 4C). In addition, a recent study showed that the activity of human PHF2, another member of the Jumonji domain protein family, is regulated by phosphorylation (*51*), raising the possibility that a posttranslational modification or cofactor may be required for reconstitution of Epe1 demethylase activity. Regulation of histone demethylation activity may play a broad role in determining the reversibility of epigenetic states. Although it is unknown whether Epe1 activity or levels regulate epigenetic transitions in *S. pombe*, regulation of histone demethylase activity has been implicated in control of developmental transitions in multicellular eukaryotes. In mouse embryonic stem cells, the pluripotency transcription factor Oct4 activates the expression of Jumonji domain Jmjd1a and Jmjd2c H3K9 demethylases, and this activation appears to be important for stem cell self-renewal (*52*). An attractive possibility is that as epigenetic states become established during transition from pluripotency to the differentiated state, reduction in the expression of H3K9 demethylases helps to stabilize the differentiated state (*52*). In another example, down-regulation of the amine oxidase family histone demethylase LSD1 during activation of individual olfactory receptor (OR) genes in the mammalian nose has been suggested to create an epigenetic "trap" that prevents the activation of additional OR genes (*53*). More generally, H3K9 demethylases may act as surveillance enzymes that prevent the formation of spurious H3K9 methylated domains, which may lead to epigenetic mutations and gene inactivation.

Materials and methods

Plasmids

Plasmids containing *10XtetO* binding sites upstream of *ade6*$^+$ and *ura4-GFP* reporter genes were constructed by first synthesizing a plasmid

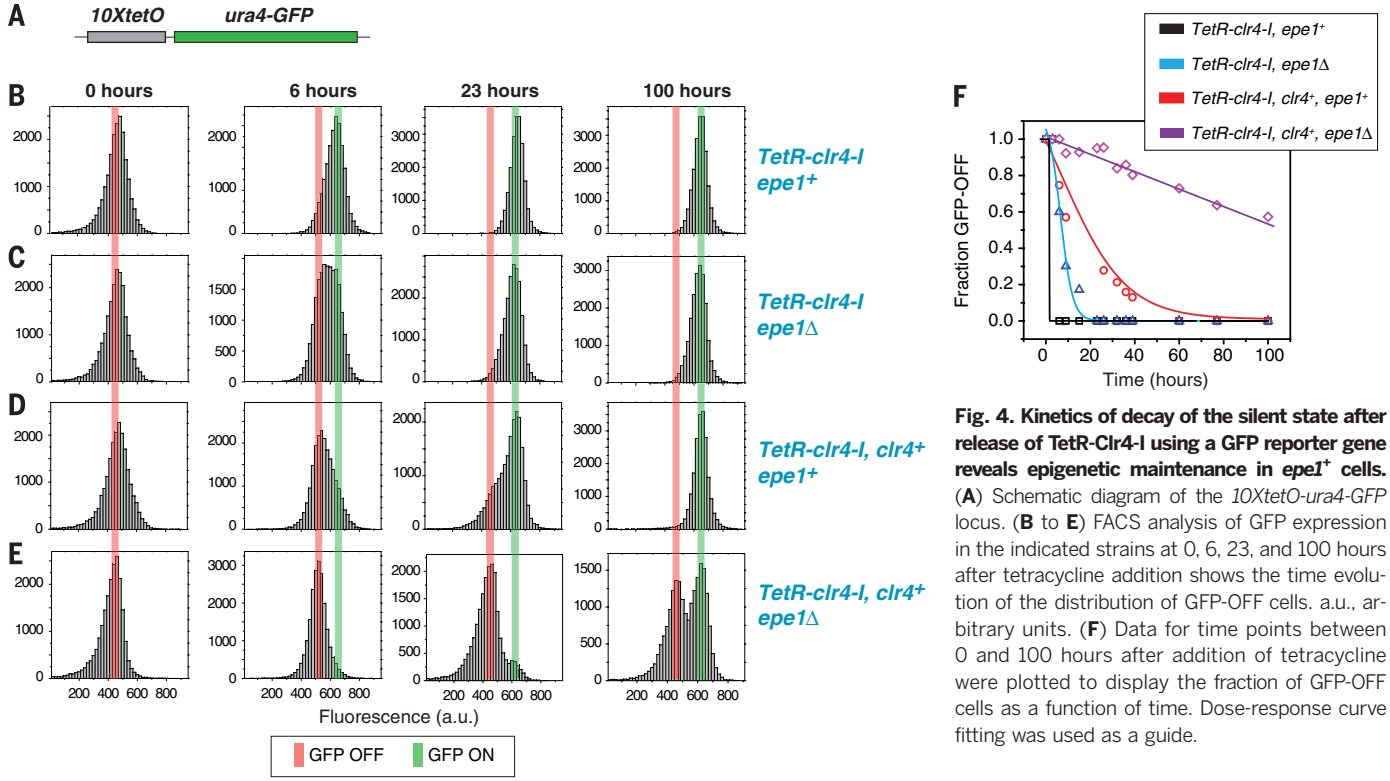

Fig. 4. Kinetics of decay of the silent state after release of TetR-Clr4-I using a GFP reporter gene reveals epigenetic maintenance in *epe1*$^+$ cells. (**A**) Schematic diagram of the *10XtetO-ura4-GFP* locus. (**B** to **E**) FACS analysis of GFP expression in the indicated strains at 0, 6, 23, and 100 hours after tetracycline addition shows the time evolution of the distribution of GFP-OFF cells. a.u., arbitrary units. (**F**) Data for time points between 0 and 100 hours after addition of tetracycline were plotted to display the fraction of GFP-OFF cells as a function of time. Dose-response curve fitting was used as a guide.

containing 10 *tetO* sites flanked by 200-bp homology sequences to facilitate reporter insertion at the *ura4* locus. The reporter genes were cloned downstream of the *tetO* binding sites using *PacI* and *AscI* restriction sites that were incorporated during the initial synthesis of the plasmid. The *ade6*$^+$ reporter construct consists of the full-length WT *ade6*$^+$ gene with endogenous upstream promoter and downstream terminator sequences. The *ura4-GFP* reporter consists of the full-length *ura4*$^+$ gene fused at the C terminus with a monomeric yeast codon–optimized GFP using Gibson assembly (*54*). This construct was subsequently cloned downstream of the 10 *tetO* sites and appended with a 114-bp *ura4* promoter element and the corresponding endogenous *ura4* downstream terminator sequence. The plasmid containing *TetR-clr4-I* was constructed by modifying a pFA6a-natMX6-P$_{nmt1}$ plasmid. The promoter elements in the original plasmid were replaced with the endogenous *clr4*$^+$ promoter (using *BglII* and *PacI* restriction sites). The TetR construct consists of an N-terminal SV40 nuclear localization sequence followed immediately by a 2X-FLAG tag. The *clr4*$^+$ chromodomain deletion construct consists of a *clr4* allele lacking amino acids 7 to 59. The synthesis of the *TetR-clr4-I* fusion with the upstream endogenous clr4 promoter elements was achieved by Gibson assembly. The deletion of the TetR DNA binding element was achieved after modifying a pFA6a-hphMX6-P$_{nmt1}$ plasmid by insertion of the endogenous *clr4*$^+$ promoter and a *clr4* allele lacking the chromodomain.

Strains

A strain containing the 10 *tetO* sites was first made by insertion of the reporter gene at the *ura4*$^+$ locus. The subsequent introduction of the TetR-Clr4-I fusion protein was achieved with the use of a PCR-based gene-targeting approach (*55*). Strains with the designation TetR-Clr4-I are those in which the endogenous copy of *clr4* is replaced with the TetR-Clr4-I fusion, making it the only source of Clr4 expression in the cells. In strains where the WT copy of *clr4*$^+$ is intact (i.e., *TetR-clr4-I,clr4*$^+$), the fusion protein is inserted at the *trp1*$^+$ locus. The deletions of the various RNAi and chromatin components were achieved either by PCR-based gene-targeting approaches or by a cross followed by random spore analysis and PCR-based screening to select for colonies that harbored the reporter gene, the TetR fusion protein, and the appropriate deletion. Strains containing deletions of the TetR DNA binding domain (*clr4-I*Δ) were constructed by both PCR-based targeting approaches and crosses followed by random spore analysis. The resulting colonies were tested using allele specific primers. To isolate red colonies that harbor a deletion of the TetR DNA binding domain, sectored colonies, which tested positive for the deletion in the allele-specific PCR screen, were replated to isolate single red colonies on plates containing limiting adenine. All strains used in this study are listed in table S1.

Crosses were performed between red isolates of haploid cells of opposite mating type that harbored a deletion of the TetR DNA binding

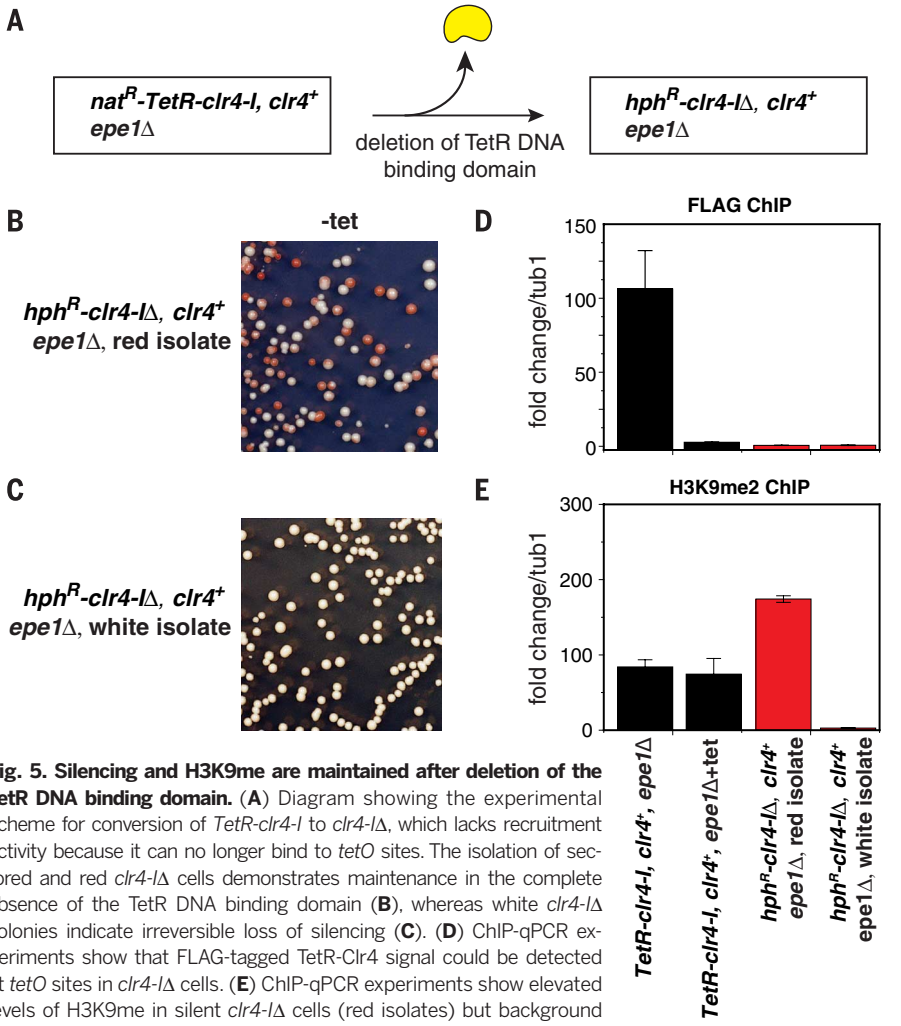

Fig. 5. Silencing and H3K9me are maintained after deletion of the TetR DNA binding domain. (**A**) Diagram showing the experimental scheme for conversion of *TetR-clr4-I* to *clr4-I*Δ, which lacks recruitment activity because it can no longer bind to *tetO* sites. The isolation of sectored and red *clr4-I*Δ cells demonstrates maintenance in the complete absence of the TetR DNA binding domain (**B**), whereas white *clr4-I*Δ colonies indicate irreversible loss of silencing (**C**). (**D**) ChIP-qPCR experiments show that FLAG-tagged TetR-Clr4 signal could be detected at *tetO* sites in *clr4-I*Δ cells. (**E**) ChIP-qPCR experiments show elevated levels of H3K9me in silent *clr4-I*Δ cells (red isolates) but background levels of methylation in ade6$^+$-expressing (white) *clr4-I*Δ cells. Error bars in (D) and (E) represent SD.

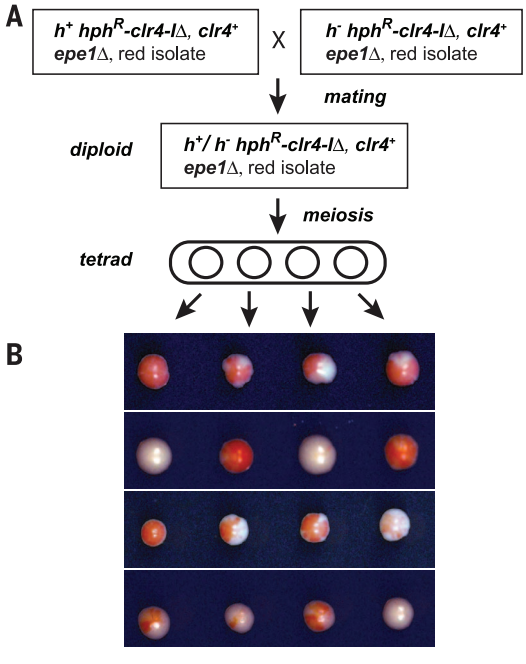

Fig. 6. Inheritance of initiator-independent silencing through meiotic cell divisions. (**A**) Scheme for mating of silent (red) *ade6*$^+$ haploid cells of the indicated genotypes in which the TetR-Clr4-I was deleted. After sporulation of the resulting diploid cells, tetrads were dissected, and the haploid meiotic progeny were plated on low-adenine medium (**B**). Results of four tetrad dissections are presented and show inheritance and variegation of the silent state.

module. The resulting diploid, which lacks any sequence-specific establishment factors, was then allowed to sporulate. After tetrad dissection, spores were plated on low-adenine medium and allowed to grow at 32°C for 3 days.

Chromatin immunoprecipitation

Cells were grown to a density of 2.5×10^7 cells/ml at 32°C in yeast extract supplemented with adenine (YEA) or YEA containing tetracycline (2.5 μg/ml). Cells were cross-linked with 1% formaldehyde for 30 min at room temperature before quenching with 125 mM glycine for 5 min. The subsequent steps for sample processing were performed as previously described (*56*). Immunoprecipitation was performed using the following antibodies: 2.5 μl α-H3K9me2 (ab1220, Abcam) for quantifying H3K9me2 levels, 2 μg α-H3K9me3 (*57*) for quantifying H3K9me3 levels, and 2.5 μl α-FLAG (M2, Sigma) for quantifying TetR-Clr4-I occupancy at the ectopic locus before and after addition of tetracycline. DNA purified from the ChIP experiments was analyzed by quantitative PCR using an Applied Biosystems 7900HT Fast Real-Time PCR system. See table S2 for primer sequences. ChIP-seq libraries were constructed, sequenced using an Illumina HiSeq platform, and processed as described previously (*49*).

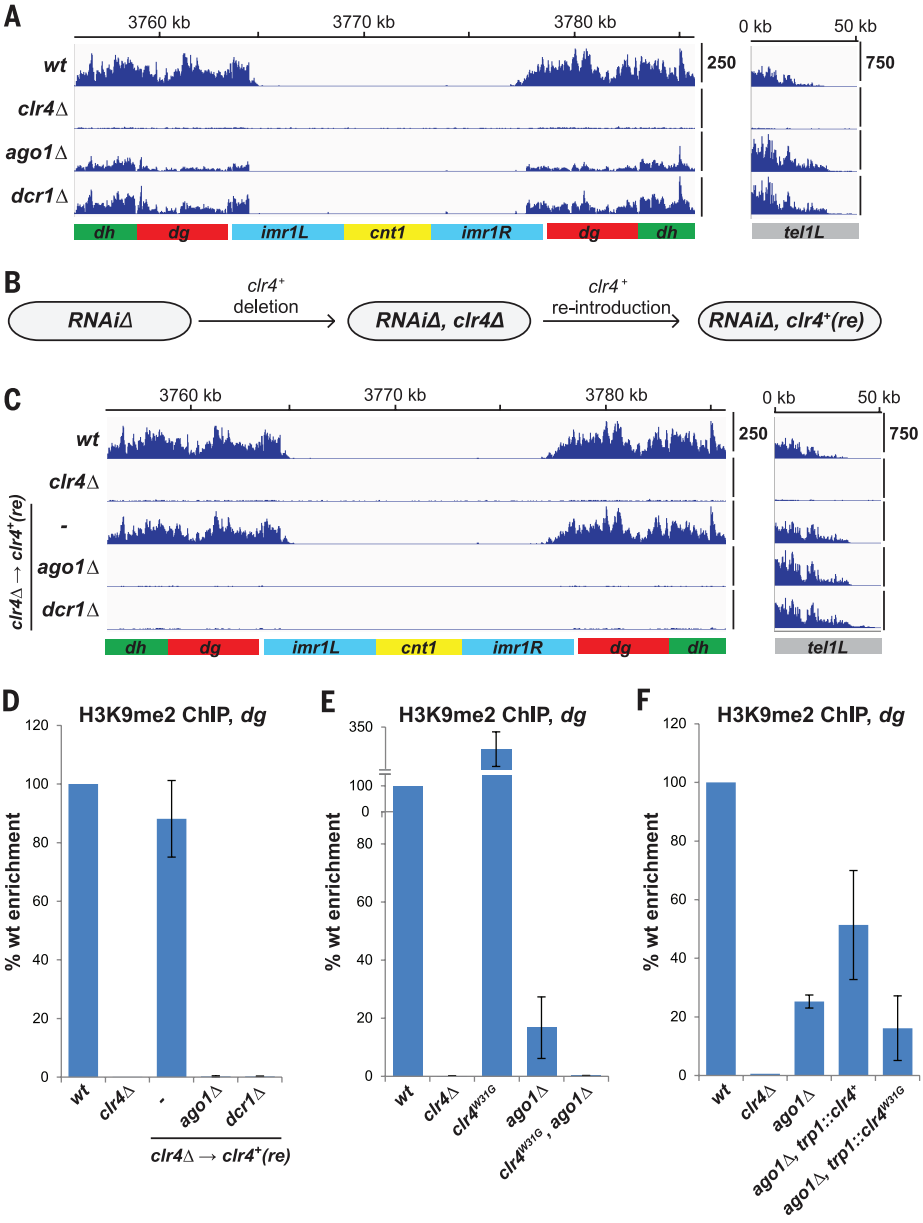

Fig. 7. RNAi-independent H3K9me at pericentromeric repeats is epigenetically inherited. (**A**) ChIP-seq experiments showing the persistence of residual histone H3K9me2 at the pericentromeric *dg* and *dh* repeats of chromosome 1 in *ago1*Δ and *dcr1*Δ cells. Libraries were sequenced on the Illumina HiSeq2500 platform and normalized to reads per million (*y* axis). Chromosome coordinates are indicated above the plots. (**B**) Scheme for the reintroduction of *clr4*$^+$ into *RNAi*Δ, *clr4*Δ cells to test the requirement for RNAi in H3K9me establishment. *clr4*$^+$ was reintroduced to the native locus to avoid overexpression. (**C**) ChIP-seq experiments showing that the reintroduction of *clr4*$^+$ into *clr4*Δ cells, but not *clr4*Δ *ago1*Δ or *clr4*Δ *dcr1*Δ cells, restores H3K9me2 at the pericentromeric repeats of chromosome 1 (left). Reads for H3K9me2 at the telomeres of chromosome 1 (*telL1*) on the right side show that, unlike the centromeres, establishment of telomeric H3K9me does not require RNAi. (**D**) ChIP-qPCR experiments verify that RNAi is required for the reestablishment of H3K9me2 at the pericentromeric *dg* repeats. (**E**) ChIP-qPCR experiments show that a mutation in the chromodomain of Clr4 (*clr4W31G*) abolishes the maintenance of H3K9me2 at *dg* repeats. (**F**) ChIP-qPCR experiments show that an additional copy of WT *clr4*$^+$, but not *clr4W31G*, boosts residual H3K9me2 levels at *dg*. Error bars in (D) to (F) represent SD.

Silencing assays

Strains containing the *ade6*$^+$ reporter construct were grown overnight, after which fivefold dilutions of each culture were spotted on plates containing only yeast extract and glucose without any additional adenine supplements with (+tet) or without (–tet) tetracycline (2.5 μg/ml). Each silencing assay also included centromeric silencing reporter strains that are unresponsive to tetracycline (*otr1R:ade6*$^+$ and *ura4::10XtetO- ade6*$^+$) as controls to ensure that the addition of tetracycline does not induce any changes in reporter gene expression.

FACS analysis

Cells containing *TetR-clr4-I* and *10XtetO- ura4-GFP* reporter were maintained in log phase (~2.5×10^7 cells/ml) through the course of sample preparation at various time points after addition of tetracycline (2.5μg/ml). Approximately 2.5×10^7 cells were harvested and fixed by addition of 70% ethanol for 20 min. The cells were then washed twice with 1X tris-buffered saline (TBS) (200 mM Tris pH 7.5, 150 mM NaCl) and resuspended in 1 ml of 1X TBS in a FACS tube (BD Falcon). GFP fluorescence was then measured using a FACScalibur instrument (Becton Dickinson), and excitation was achieved by using an argon laser emission of 488 nm. Data collection was performed using Cellquest software (Becton Dickinson), and a primary gate based on physical parameters (forward and side light scatter) was set to exclude dead cells or debris. Typically, 20,000 cells were analyzed for each sample and time point. The resulting GFP fluorescence profiles were fit using Gaussian curves (Origin 8.0), assuming a model in which cells exhibit two expression states: either GFP-ON or GFP-OFF. The fraction of cells in each state was calculated by measuring the area under the curve for each Gaussian fit.

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SUPPLEMENTARY MATERIALS

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