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 normal module. Furthermore, the chromodomain of Clr4, which is involved in recognition of the H3K9me mark, was dispensable 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.

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.

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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.
**Research Article**

**Epigenetic inheritance uncoupled from sequence-specific recruitment**

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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 methyltransferase, Clr4/Suv39h, is associated 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 Crl4 methyltransferase catalytic domain to the bacterial tetracycline repressor (TetR) protein. This allowed us to establish an extended heterochromatin domain and study its initiator-independent maintenance either by tetracycline-mediated release of TetR-Crl4 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 maintenance of H3K9me and the chromodomain of the Crl4 methyltransferase in its maintenance.

**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 Crl4 protein lacking its N-terminal chromodomains (required for binding to methylated histone H3K9) while retaining its enzymatic methyltransferase activity to the bacterial TetR protein (designated “TetR-Crl4-I” for TetR-Crl4 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 (TetRrep system) (27). We generated cells in which TetR-Crl4-I replaced the wild-type (WT) Crl4 (TetR-clr4-I) or in which WT Crl4 was intact and TetR-Crl4-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 Crl4 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-Crl4-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 Crl4 was not required for de novo heterochromatin establishment (Fig. 1B)—but depended on HP1 proteins (Swi6 and Chp2) and histone deacetylases (Cir3 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-Crl4-I from DNA. Tetracycline-dependent release of TetR-Crl4-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-Crl4-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-Crl4-I (Fig. 1C). Furthermore, ChIP combined with high-throughput sequencing (ChIP-seq) and ChIP
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-CI is (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 heterochromatric domains for a limiting pool of proteins that play critical roles in maintenance.

To test these different scenarios, we constructed TetR-CI, 10XtetO-ade6+ cells (with or without CI) that carried deletions for genes involved in various chromatin-maintenance pathways (28–30). The deletion of epi, 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-CI, epi and TetR-CI, CI (Fig. 2A, left side). In contrast to epi, cells, epi 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 CI (Fig. 2A, right side; compare last two rows with first two rows). ChIP experiments verified that tetracycline addition promoted the release of TetR-CI from tetO sites (Fig. 2B). Consistent with the colony color silencing assays, ChIP-qPCR experiments also indicated that the large domain of H3K9 di- and trimethylation surrounding the 10XtetO-ade6+ locus was lost in TetR-CI, CI, epi cells but was maintained in TetR-CI, CI, epi cells (Fig. 2C and fig. S2, A to C) 24 hours after the addition of tetracycline. Within this domain, the expression of other transcriptional units remained on -tet medium, and this silencing was maintained for several hours after tetracycline addition (fig. S3).

Maintenance of the silent state in epi 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 epi with alleles containing active-site mutations (epi-34 and epi-H297A) (30, 31) displayed a maintenance phenotype similar to its deletion (epiΔ) (Fig. 3D). Furthermore, consistent with a requirement for the chromodomain of CI in maintenance (Fig. 2A), the replacement of CI with a CI allele (which lacks the chromodomain) resulted in the formation of only white colonies on -tet medium (Fig. 3E). Therefore, rather than differences in CI dosage, the appearance of red or sectored colonies depends on the presence of CI 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 set2, 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 B).
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 (GFP ON cells). H3K9me2 is maintained in both deletion backgrounds (Fig. S5) (for RNAi-dependent heterochromatin formation at centromeres (27), or deletion of tetracycline (fig. S4, B and C). We then tested whether destabilizing endogenous heterochromatic regions could release factors that affect maintenance by deleting 70xΔ, a DNA binding protein required for heterochromatin formation at telomeres (36), or dcr3+, which is required for RNAi-dependent heterochromatin formation at centromeres (35) (fig. S5). In these mutants, effects on colony color were absent (dcr1Δ, fig. S5A) or subtle (pozlΔ, 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.
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-I, 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+, msu2+, and poz1+ 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
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 Ctr4 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 demethylation 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

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**Fig. 4. Kinetics of decay of the silent state after release of TetR-Ctr4-I using a GFP reporter gene reveals epigenetic maintenance in epe1Δ 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 Pau1 and Ascl 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-Pnmt 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-Pnmt 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 trpl locus. The deletions of the various RNAs 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) 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...
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 × 10⁷ 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 (36). 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 pericentromeric repeats. 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).

**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 × 10⁷ cells/ml) through the course of sample preparation at various time points after addition of tetracycline (2.5 µg/ml). Approximately 2.5 × 10⁷ 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.

**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 repeats of chromosome 1 in ago1Δ and dcr1Δ cells. Libraries were sequenced on the Illumina HiSeq platform, and normalized to reads per million (y axis). Chromosome coordinates are indicated above the plots. (B) Scheme for the reintroduction of clr4+ into RNAiΔ, clr4Δ 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 clr4Δ cells, but not clr4Δ ago1Δ or clr4Δ dcr1Δ 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 H3K9me2 does not require RNAi. (D) ChIP-qPCR experiments verify that RNAi is required for the establishment of H3K9me2 at the pericentromeric repeats. (E) ChIP-qPCR experiments show that a mutation in the chromodomain of Cir4 (clr4W31G) abolishes the maintenance of H3K9me2 at clr4Δ repeats. (F) ChIP-qPCR experiments show that an additional copy of WT clr4+ but not clr4W31G, boosts residual H3K9me2 levels at clr4Δ. Error bars in (D) to (F) represent SD.

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