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#### Supporting Online Material

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# Hairpin RNAs and Retrotransposon LTRs Effect RNAi and Chromatin-Based Gene Silencing

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The expression of short hairpin RNAs in several organisms silences gene expression by targeted mRNA degradation. This RNA interference (RNAi) pathway can also affect the genome, as DNA methylation arises at loci homologous to the target RNA in plants. We demonstrate in fission yeast that expression of a synthetic hairpin RNA is sufficient to silence the homologous locus in trans and causes the assembly of a patch of silent Swi6 chromatin with cohesin. This requires components of the RNAi machinery and Clr4 histone methyltransferase for small interfering RNA generation. A similar process represses several meiotic genes through nearby retrotransposon long terminal repeats (LTRs). These analyses directly implicate interspersed LTRs in regulating gene expression during cellular differentiation.

Eukaryotic genomes invariably contain repetitive DNA sequences of uncertain functional significance: large expanses of heterochromatin are associated with centromere regions and flank the kinetochores at centromeres. This centromeric heterochromatin is frequently made up of tandem arrays of simple satellites interspersed with transposable elements (1–5). Moreover, recent studies support a direct role for such tandem repeats in centromere function, because arrays of human centromeric  $\alpha$ -satellite allow de novo kinetochore assembly (1). In addition, the heterochromatin that coats centromeric repeats in fission yeast (*Schizosaccharomyces pombe*) plays an important role in sister-centromere cohesion (6, 7).

Other repetitive elements (e.g., retrotransposons) are found scattered throughout eukaryotic genomes (8). It is becoming apparent that interspersed repeats can influence the expression of nearby genes (9, 10). McClintock first recognized this potential role for transposons in maize and referred to them as controlling elements (11). Consistent with this, mutations that reduce DNA methylation in plants result in activation and mobilization of transposons and lead to epimuta-

tions (12, 13). Although transposable elements can alter the expression of neighboring genes in plant and animal cells (8–10), it remains unclear whether these elements play a direct role in endogenous gene regulation. Nevertheless, early models proposed that families of interspersed repetitive sequences might regulate networks of genes and lead to cellular differentiation in response to a signal (14).

The heterochromatic nature of centromeres and the abundance of transposable elements at pericentromeric regions in some organisms suggests links between transposon silencing and centromeric heterochromatin formation. In mammalian cells, both centromeric and retrotransposon repeats have a similar repertoire of both DNA and chromatin modifications that are usually found associated with silent chromatin and also attract cohesin (15–18).

Fission yeast centromeres are composed of outer repeats that flank the central kinetochore domain. Marker genes placed within these repeats are silenced by a process requiring Clr4, which methylates histone H3 on lysine 9 (MeK9-H3), which allows binding of Swi6 (the ortholog of metazoan HP1) and Chp1 to these outer repeats (19–24). Overlapping noncoding transcripts are produced from these repeats, and analyses suggest that their processing by the RNA interference (RNAi) machinery results in the generation of small interfering RNAs (siRNAs), which are continually required for the

formation of silent chromatin over these centromere repeats (25, 26) and for centromere function (27–29). Proteins related to CENP-B and the mariner class of transposases also bind the outer repeat sequences at fission yeast centromeres and contribute to the formation of heterochromatin (30). In addition to centromeric repeats, the standard fission yeast genome contains 13 intact Tf2 retrotransposable elements plus 249 Tf1 and Tf2 long terminal repeats (LTRs), or related LTRs, and LTR fragments dispersed throughout the genome (31).

In many organisms, the process of RNAi has been exploited to nullify gene expression (32). Silencing can occur in two ways: posttranscriptional gene silencing (PTGS) involves degradation of the targeted RNA; studies in plants indicate that expression of double-stranded RNAs (dsRNAs) can result in DNA methylation and transcriptional gene silencing (TGS) at the target locus (33–35). Moreover, the RNAi pathway is required for the generation of siRNAs homologous to, and DNA methylation of, retrotransposons in plants (36, 37). Here we test whether, in fission yeast, the RNAi machinery is preserved to specifically process transcripts generated from centromeric repeat sequences or if it can act to silence noncentromeric transcripts in trans and trigger heterochromatin formation. We also demonstrate that endogenous, developmentally regulated, lineage-restricted genes are subject to control by this same process and that adjacent retrotransposon LTRs effect this regulation.

**Short hairpin RNA (shRNA) can silence a normally expressed *ura4<sup>+</sup>* gene.** To determine whether the RNAi machinery of fission yeast can act to process noncentromeric repeat transcripts, we cloned an internal region [280 base pairs (bp) of *ura4<sup>+</sup>*, Stu I–Eco RV (SE)] from the *ura4* open reading frame (ORF) in an inverted orientation around a 355-bp spacer under the control of the *nmt41* promoter (*shuraSE*; Fig. 1A). To assess whether expression of *shuraSE* could induce silencing of the *ura4<sup>+</sup>* gene, we transformed the construct and empty vector into two strains; one in which the endogenous *ura4<sup>+</sup>* locus remained intact and the other in which the 280-bp Stu I–Eco RV region was deleted from the *ura4* gene (*ura4-DS/E*, which lacks homology to *shuraSE*) and a wild-type copy of *ura4<sup>+</sup>* had been inserted at another location (*Rint.ura4<sup>+</sup>*). Previous analyses demon-

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strate that *Rint:ura4<sup>+</sup>* is expressed at the same levels as the truncated, nonfunctional transcript arising from *ura4-DS/E* in the same cells and is never silenced (38). Cells expressing *ura4<sup>+</sup>* grow on medium lacking uracil (-URA) but are unable to grow on counter-selective plates containing 5-fluoro-uracil (FOA). Conversely, silencing of *ura4<sup>+</sup>* inhibits growth on medium lacking uracil but allows growth on FOA. Expression of *shuraSE* blocked growth of both strains *Rint:ura4<sup>+</sup>* and the normal *ura4<sup>+</sup>* locus; also *ura4<sup>+</sup>* inserted at eight other distinct locations (39) on medium lacking uracil and allowed colony formation on FOA plates (Fig. 1B). The level of *Rint:ura4<sup>+</sup>* transcription was significantly reduced relative to the *ura4-DS/E* internal control (Fig. 1C). This demonstrates that only sequences homologous to the shRNA (*ura4<sup>+</sup>* not *ura4-DS/E*) are targeted for silencing.

**shRNA silencing of *ura4<sup>+</sup>* requires RNAi and heterochromatin components.** Argonaute, Dicer, and RNA-directed RNA polymerase act to mediate RNAi in many organisms (32). The *S. pombe* homologs Ago1, Dcr1, and Rdp1 are required for both silencing and the assembly of silent chromatin over outer centromere repeats (26). Expression of *shuraSE* in strains with deletions of *ago1<sup>+</sup>*, *dcr1<sup>+</sup>*, or *rdp1<sup>+</sup>* (*ago1Δ*, *dcr1Δ*, or *rdp1Δ*) did not induce silencing of *Rint:ura4<sup>+</sup>* (Fig. 1, B and C). Thus, silencing of *Rint:ura4<sup>+</sup>* by *shuraSE* expression is dependent on RNAi components, consistent with silencing by PTGS.

To determine whether known factors required for chromatin-based transcriptional silencing affect *shuraSE* mediated silencing, we also introduced the constructs into cells containing *Rint:ura4<sup>+</sup>*, which lack Clr4 K9-H3 methyltransferase function or the HP1 ortholog, Swi6. Because these are chromatin-based silencing factors these lesions are not expected to affect PTGS. Surprisingly, phenotypic silencing of *ura4<sup>+</sup>* was completely abolished in *clr4-s5* (no growth on FOA) but persisted in *swi6Δ* cells (Fig. 1, B and C). *ura4<sup>+</sup>* transcript levels remained relatively low in *swi6Δ* cells as compared to *clr4-s5* and *dcr1Δ* cells expressing *shuraSE*. The fact that *clr4-s5* lacks silencing of *ura4<sup>+</sup>* indicates that RNAi is defective and suggests that, apart from methylating histone H3, Clr4 acts in the PTGS pathway. Consistent with this siRNAs homologous to *shuraSE* are not detected in *clr4-s5*, in addition to *ago1Δ*, *dcr1Δ*, or *rdp1Δ* cells, but are generated in *swi6Δ* cells (Fig. 1D). Thus, the RNase III endonuclease Dcr1 requires Ago1, Rdp1, and Clr4 to attack dsRNA and to generate siRNAs that target endogenous *ura4<sup>+</sup>* transcripts for degradation.

***shura4* expression recruits silent chromatin modifications and proteins to *ura4<sup>+</sup>*.** In wild-type fission yeast, the RNAi pathway generates siRNAs homologous to centromeric repeats that then induce the degradation of the corresponding transcripts. Modification of chromatin [dimethyl-K9-H3 (diMeK9-H3) and Swi6

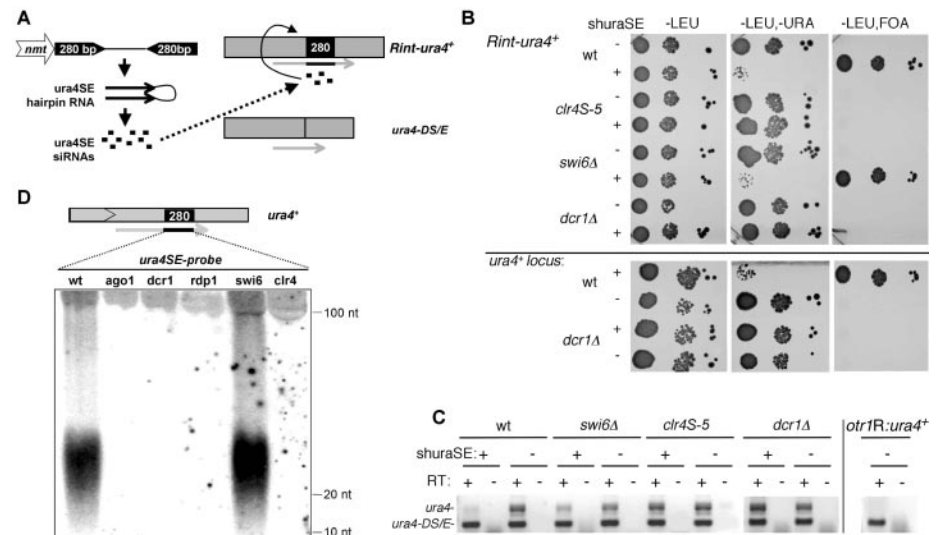
binding] over the centromeric repeats appears to be coupled to this process (25, 26). The requirement of Clr4 for *shuraSE*-mediated silencing suggested that chromatin modifications might be transmitted to the target locus.

To test whether expression of *shuraSE* leads to chromatin modifications at the *ura4* locus, we used chromatin immunoprecipitation (ChIP) to assess the methylation status of histone H3 (diMeK9-H3 and diMeK4-H3) and Swi6 association at the *Rint:ura4<sup>+</sup>* locus compared with the *ura4-DS/E* internal control in wild-type, *ago1Δ*, *dcr1Δ*, *rdp1Δ*, *clr4-s5*, and *swi6Δ* cells (Fig. 2A). In wild-type cells, expression of *shuraSE* resulted in accumulation of diMeK9-H3 and recruitment of Swi6 at the *Rint:ura4<sup>+</sup>* locus but not at the *ura4-DS/E* locus, which lacks homology with *shuraSE*. Dimethyl-K9-H3 and Swi6 association was not detectable at *Rint:ura4<sup>+</sup>* in *ago1Δ*, *dcr1Δ*, *rdp1Δ*, or *clr4-s5* cells compared with wild-type cells; instead, the diMeK4-H3 mark, normally associated with active chromatin, remained comparable to that detected in wild-type cells not expressing *shuraSE*. In contrast, in *swi6Δ* cells, diMeK4-H3 persisted even though diMeK9-H3 is still induced by *shuraSE* at *Rint:ura4<sup>+</sup>* (Fig. 2A). This pattern of modifications is identical to that seen at centromeric repeats in *swi6Δ* cells (22, 24, 29). Further analyses dem-

onstrate that diMeK9-H3 is also detectable at the *ura4<sup>+</sup>* promoter and between the promoter and the target *uraSE* region of the silenced *Rint:ura4<sup>+</sup>* gene (Fig. 2B). Thus, silent chromatin can spread outward from the targeted *uraSE* region (see last figure). In the absence of Swi6, diMeK9-H3 is no longer detected at the promoter. Thus, as at the silent mating type locus (40), Swi6 is required to spread silent chromatin outward from the nucleation site, which, in this case, is the 280-bp *uraSE* region homologous with the shRNA.

Cohesin is also known to bind Swi6-containing chromatin (6, 7). ChIP was performed on a strain expressing hemagglutinin (HA)-tagged Rad21 and *shuraSE*. Rad21 cohesin is also associated with shRNA-induced heterochromatin over the *Rint:ura4<sup>+</sup>* gene (Fig. 2C). Thus, the expression of an shRNA is sufficient to induce the assembly of silent chromatin at a normally euchromatic locus, and this silent chromatin has all the hallmarks and features of bona fide fission yeast centromeric heterochromatin.

**Meiotic genes are up-regulated in cells lacking RNAi or heterochromatin.** Because *shuraSE* RNA expression results in repression of *ura4<sup>+</sup>* by coupled posttranscriptional events and chromatin modifications, it seemed likely that endogenous genes



**Fig. 1.** An shRNA homologous to the normally expressed *ura4<sup>+</sup>* gene induces silencing of *ura4<sup>+</sup>*. (A) Expression of *uraSE* shRNA should generate siRNAs homologous to the 280-bp *uraSE* region of *Rint:ura4<sup>+</sup>* gene but not the *ura4-DS/E* minigene. *Rint:ura4<sup>+</sup>* but not *ura4-DS/E* mRNA may be targeted for degradation (dotted line) perhaps resulting in chromatin modifications (curved line). *shuraSE* is expressed from *nmt41* on pREP41 (54). (B) Comparative growth assay of serially diluted strains transformed with pREP41-*shuraSE* (+) or empty pREP41 (-) were plated on selective media (-LEU), lacking uracil (-LEU, -URA) or containing 5-FOA (-LEU, FOA) (54). (Top) Serial dilution of the wild-type (wt), *clr4-s5*, *swi6Δ*, and *dcr1Δ*, strains containing *Rint:ura4<sup>+</sup>* and *ura4-DS/E*. (Bottom) Serial dilution of wild-type and *dcr1Δ* strains with an intact *ura4<sup>+</sup>* locus (39). (C) RT-PCR analysis of *ura4<sup>+</sup>* transcripts was performed on oligo(dT)-primed complementary (cDNA) from RNA samples of the indicated strains in the presence (+) or absence (-) of reverse transcriptase (RT). Expression of *Rint:ura4<sup>+</sup>* relative to the *ura4-DS/E* minigene compared with (+) or without (-) *shuraSE*. A strain with silenced *ura4<sup>+</sup>* at *cen1* (*otr1R:ura4<sup>+</sup>*) is included for comparison (19). *ago1Δ* and *rdp1Δ* have the same effect as *dcr1Δ* and *clr4-s5* (not shown). (D) Detection of *shuraSE* homologous siRNAs. Small RNAs were enriched from the indicated strains separated and transferred to filters. Labeled *uraSE* riboprobe was hybridized, and filters were washed and exposed to obtain this phosphorimage. Relative size was determined from markers in neighboring tracks.

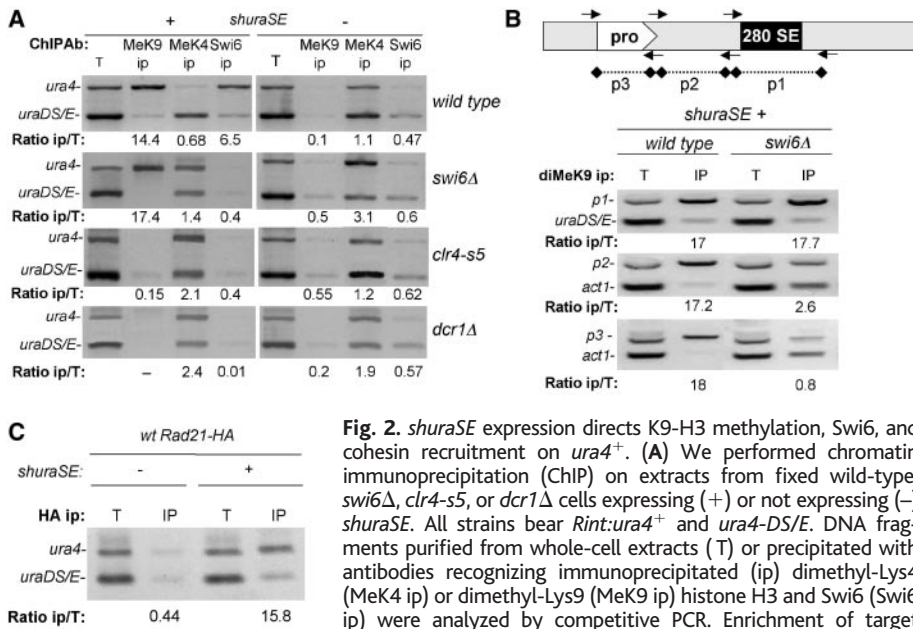
might also be regulated by RNAi-mediated chromatin modifications.

In rich growth media, mitotically dividing fission yeast cells repress a battery of genes that

are induced after nitrogen starvation, which sets off sexual differentiation, resulting in conjugation, zygote formation, and meiosis (41, 42). One study identified 31 cDNAs that were con-

firmed to be induced by nitrogen starvation (42). We noticed that 14 of these induced genes reside in close proximity (within 10 kb) of a retrotransposon LTR (see Fig. 3A). Microarray analyses demonstrated that 700 genes are induced at levels at least 5 times higher after nitrogen starvation, meiosis, and sporulation, and some of these reside close to LTRs (41). Because plant retroelements have been shown to be targets for RNAi and are associated with MeK9-H3 (36, 43), we suspected that LTRs might play a role in repression of nearby genes during vegetative growth. We therefore used reverse transcription-polymerase chain reaction (RT-PCR) to test whether meiotically induced genes remain repressed in rich medium (R) in wild-type, *ago1Δ*, *dcr1Δ*, *rdp1Δ*, *clr4-s5*, and *swi6Δ* cells. We examined expression of four *meu* genes (*meu6*, *meu19*, *meu28*, and *meu30*) and seven genes (*ste7*, *SPAC30D11.02c*, *SPAC26H5.11*, *SPAC56F8.14c*, *SPAC56F8.17c*, *mfr1*, *fbp1*) shown by microarray analyses to be considerably induced during sexual differentiation. Each gene and its position relative to LTRs is depicted in Fig. 3A.

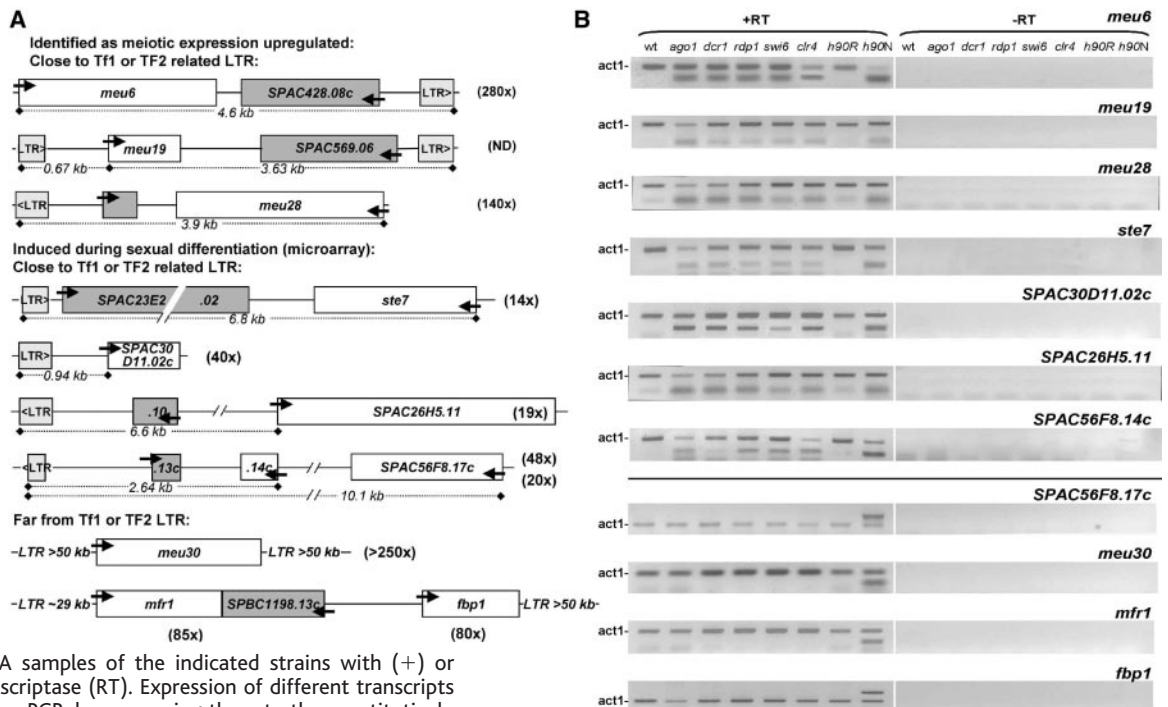
Analyses by RT-PCR confirmed that these eleven genes are induced after nitrogen starvation (-N) of a homothallic [self-mating (*h<sup>90</sup>*)] wild-type strain (Fig. 3B). Further analyses show that the *meu6*, *meu19*, *meu28*, *ste7*, *SPAC30D11.02c* (*D11.02*), *SPAC26H5.11* (*H5.11*), and *SPAC56F8.14c* (*F8.14*) genes, whose promoters reside within 7 kb of an LTR, are depressed in *ago1Δ*, *dcr1Δ*, *rdp1Δ*, *clr4-s5*, and *swi6Δ* backgrounds of a heterothallic [not self-mating (*h<sup>-</sup>*)]



**Fig. 2.** *shuraSE* expression directs K9-H3 methylation, Swi6, and cohesin recruitment on *ura4<sup>+</sup>*. (A) We performed chromatin immunoprecipitation (ChIP) on extracts from fixed wild-type, *swi6Δ*, *clr4-s5*, or *dcr1Δ* cells expressing (+) or not expressing (-) *shuraSE*. All strains bear *Rint:ura4<sup>+</sup>* and *ura4-DS/E*. DNA fragments purified from whole-cell extracts (T) or precipitated with antibodies recognizing immunoprecipitated (ip) dimethyl-Lys4 (MeK4 ip) or dimethyl-Lys9 (MeK9 ip) histone H3 and Swi6 (Swi6 ip) were analyzed by competitive PCR. Enrichment of target *ura4<sup>+</sup>* is compared with the internal control *ura4-DS/E*. Similar

data were obtained in three independent experiments. (B) ChIP samples prepared from wild-type and *swi6Δ* cells expressing *shuraSE* were used to detect diMeK9-H3 over the *ura4SE* region (400-bp PCR product, p1); the 5' adjacent region (300-bp product, p2); and the *ura4<sup>+</sup>* promoter (pro; 250-bp product, p3). The p1 primers also amplify a smaller product from the *ura4-DS/E* allele as an internal control. Primers that amplify 200 bp from *act1<sup>+</sup>* were used as an internal control with p2 and p3 primers. (C) HA-specific antibodies were used to immunoprecipitate chromatin from wild-type cells bearing 3xHA epitope-tagged *rad21<sup>+</sup>* at the endogenous locus, the euchromatic *Rint:ura4<sup>+</sup>* insertion, and the *ura4-DS/E* allele. Cells expressing (+) or not expressing (-) *shuraSE* were examined. Enrichment of *ura4* relative to *ura4-DS/E* was reproducible.

**Fig. 3.** Meiotic genes residing in close proximity to LTRs are depressed in cells lacking RNAi, *clr4<sup>+</sup>*, or *swi6<sup>+</sup>*. (A) Schematic representation of different meiotic genes (white rectangle) and their position relative to LTRs (light gray rectangle). Black arrowheads indicate the direction of gene transcription. The induction level determined in *pat1* synchronous meiosis by microarray analysis is shown in brackets (41). Distance (in kilobases) of the LTRs from the 5' end of each ORF examined is indicated. (B) RT-PCR analysis was performed on oligo(dT)-primed cDNA from RNA samples of the indicated strains with (+) or without (-) reverse transcriptase (RT). Expression of different transcripts was assessed by multiplex PCR, by comparing them to the constitutively expressed *act1<sup>+</sup>* gene. All meiotic genes are induced in a homothallic (*h<sup>90</sup>*) wild-type strain after nitrogen starvation (*h<sup>90</sup>N*) but not when grown in rich medium (*h<sup>90</sup>R*). *SPAC56F8.17c* and *fbp1* PCR products migrate above, whereas all others migrate below, the *act1<sup>+</sup>* product.



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strain (Fig. 3B). In contrast, repression of *meu30*, *mfr1*, and *fbp1*, which are at least 29 kb away from an LTR, was unaffected in cells lacking Ago1, Dcr1, Rdp1, Clr4, or Swi6. In all cells and conditions, actin (*act1*<sup>+</sup>) transcripts were clearly detected. Repression of SPAC56F8.17c (*F8.17*), the promoter of which lies 7.5 kb distal to that of the responding *F8.14* gene and 10.1 kb from the same LTR, was unaffected by loss of Ago1, Dcr1, Rdp1, Clr4, or Swi6 function.

Although not comprehensive analyses, these data show a compelling correlation between the proximity of an LTR and RNAi/Swi6-dependent regulation of a gene. Surveying the *S. pombe* gene database (44) indicates that not all meiotically induced genes reside close to an LTR and that not all LTRs are found close to genes induced during sexual differentiation. However, our data suggest that, in some cases, retrotransposon LTRs may be required to maintain repression of nearby meiotically in-

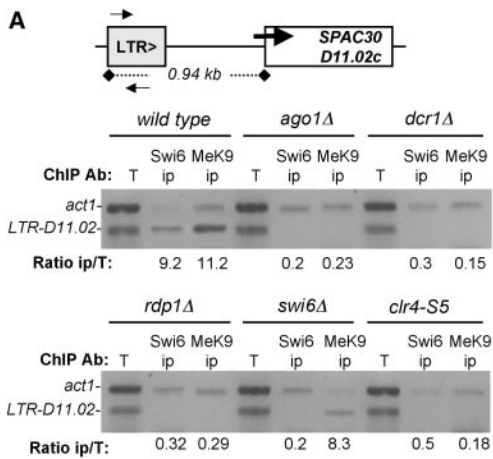
duced genes during vegetative growth. Other LTRs may act to enforce repression of genes normally induced under other conditions.

**LTRs are coated with silent chromatin.**

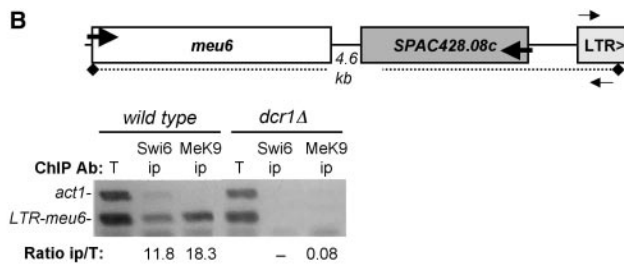
The above analyses implicate Tfl1- and Tf2-related LTRs in the repression of nearby genes by RNAi-dependent chromatin-based silencing. To further assess this, we examined two LTRs to determine whether they are packaged in chromatin methylated on lysine 9 of histone H3 and bound by Swi6. ChIP assays indicated that, in wild-type cells, both LTRs were found to be coated by diMeK9-H3 chromatin and associated with Swi6 (Fig. 4). This modification and association were lost in the absence of Ago1, Dcr1, Rdp1, or Clr4, whereas *swi6*Δ retained diMeK9-H3. (We found similar results for LTR-*meu6*; only wild type and *dcr1*Δ are shown.) This suggests that the effects on *D11.02* and *meu6* gene expression are mediated by RNAi-dependent modifications at these LTRs.

**LTRs are transcribed and required to mediate gene repression.**

Intact LTRs contain the promoter that in a complete retrotransposon drives transcription of the transposon ORFs (45). Solo LTRs may interfere with expression of nearby genes by driving expression of non-coding RNAs, which triggers RNAi-dependent silent chromatin assembly. To test this, we used primers to detect RNA derived from both strands across the *meu6*<sup>+</sup> and *D11.02* LTRs by RT-PCR (Fig. 5A). No transcript could be detected in wild-type cells; however, in *ago1*Δ, *dcr1*Δ, *rdp1*Δ, and *clr4-s5* cells, transcripts from both the top and bottom strands accumulated, whereas the bottom transcript was predominantly detected in *swi6*Δ cells. We surmise that, as with fission yeast centromere transcripts and *shuraSE*, these LTR transcripts are usually processed by the RNAi pathway and form siRNAs and that this process results in the modification of chromatin at the LTR which

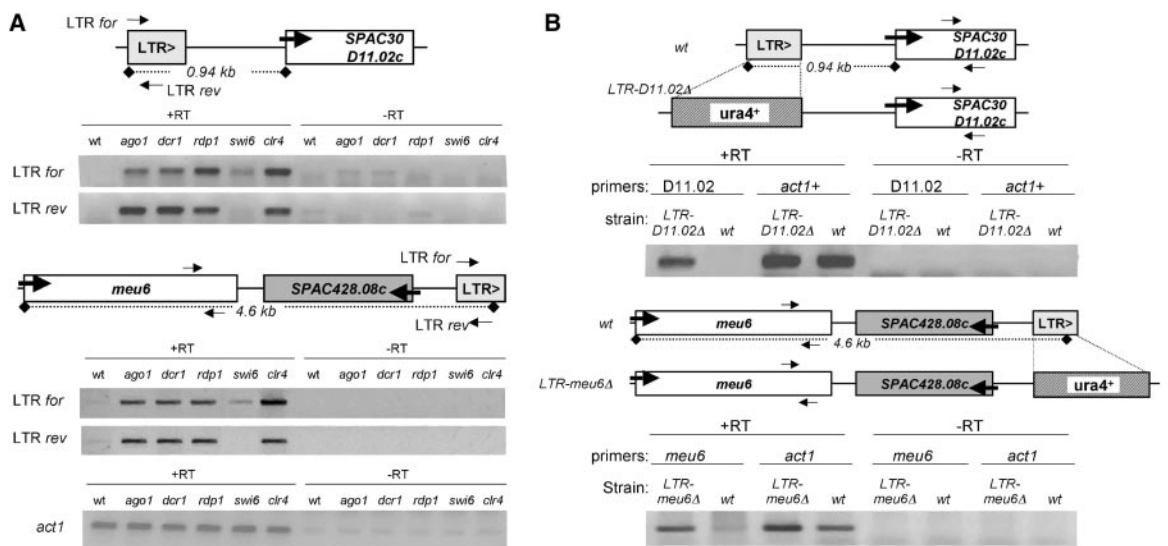


**Fig. 5.** LTRs are transcribed, and they enforce repression of nearby meiotically induced genes. (A) Strand-specific transcript analyses at *D11.02* and *meu6* LTRs. Diagrams show the position of the LTRs relative to the meiotically up-regulated genes. Transcription direction of meiotic genes is indicated by large black arrows. The top (LTR for) or bottom strand (LTR rev) primers (small black arrows) were used to initiate cDNA synthesis; subsequently, the other primer was added to PCR amplify the product. RT-PCR analysis of LTR-specific transcripts from LTRs close to the *D11.02* and *meu6* genes was performed on LTR for or LTR rev primed cDNA from RNA samples of the indicated strains with (+) or without (-) reverse transcriptase (RT). Using PCR, 150 bp was amplified with LTR for and LTR rev primers specific for the *D11.02* and *meu6* LTRs. Amplification of 200 bp of *act1* from the same RNA samples was included as a control. (B) Replacement of the *D11.02* and



We assessed enrichment of dimethyl-K9-H3 and Swi6 on the LTR region by multiplex PCR using primers (indicated by small black arrows) specific for the LTRs adjacent to *D11.02* (A) and *meu6* (B), respectively (lower bands). Primers that amplify 200 bp from *act1*<sup>+</sup> were included as an internal control. LTR-*meu6* primers gave similar results to those of LTR-*D11.02* in *ago1*Δ, *rdp1*Δ, *clr4-s5* and *swi6*Δ (not shown).

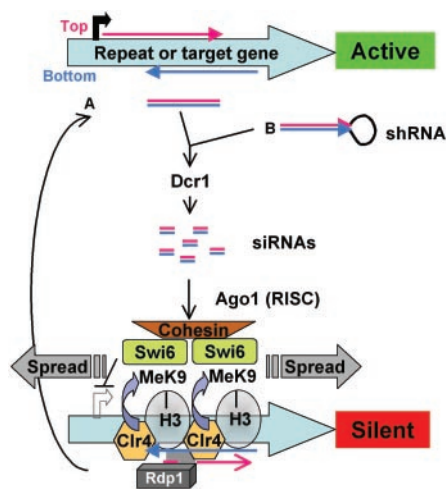
**Fig. 4.** LTR chromatin containing diMeK9-H3 is associated with Swi6, and this requires RNAi, *clr4*<sup>+</sup>, and *swi6*<sup>+</sup>. ChIPs with antibodies recognizing dimethyl-K9-H3 and Swi6 were performed as described in Fig. 2A on wild-type or mutant cells.



*meu6* LTRs by the *ura4*<sup>+</sup>. Diagrams show the generation of the *LTR-D11.02*Δ and *LTR-meu6*Δ strains. The position of primers in *D11.02* and *meu6* are indicated (small black arrows). RT-PCR analysis of the *D11.02* and *meu6* transcripts was carried out in the indicated strains as described in Fig. 3B. Primers specific for *act1*<sup>+</sup> were used as positive controls.

can silence nearby genes. Loss of Ago1, Dcr1, Rdp1, Clr4, or Swi6 allows accumulation of the LTR transcripts and expression of the neighboring genes. Curiously, the bottom transcript preferentially accumulates in cells lacking Swi6, and this is reminiscent of the pattern of centromere transcript accumulation (26, 29).

To determine whether these LTRs are directly required for repression of nearby meiotically induced genes, we replaced the LTRs lying 0.94 kb upstream and 4.6 kb downstream of the *D11.02* and *meu6* gene promoters, respectively, with the *ura4<sup>+</sup>* gene by homologous recombination by sequences immediately flanking the LTRs to generate *LTR-D11.02Δ* and *LTR-meu6Δ*. During vegetative growth conditions,



**Fig. 6.** Model for the nucleation and spreading of silent chromatin from centromeric repeats, LTRs, or an shRNA target. Top and bottom strand noncoding transcripts from retrotransposon LTR or centromeric repeat DNA overlap, hybridize, and form dsRNA (A). Expression of synthetic shRNAs also allows formation of dsRNA homologous to a target gene (B). dsRNAs are cleaved to siRNAs by Dicer (Dcr1); a putative complex containing Ago1 (RISC) binds these siRNA and uses them to target homologous nascent transcripts at the target locus. Rdp1 (RNA-directed RNA polymerase) may use siRNAs to back-transcribe this transcript, and in the process, Clr4 methylates lysine 9 of histone H3 on the surrounding chromatin, which allows binding of Swi6, the formation of silent chromatin, and recruitment of cohesin (26, 55). Transcription from the top strand of centromere repeats or the shRNA target gene is repressed. Transcription and RNAi-mediated degradation of the bottom strand of centromeric repeats and LTR transcripts continues even after assembly of Swi6 chromatin (26). Rdp1 activity ensures that homologous dsRNA and siRNAs are continually generated, and thus, silent chromatin is maintained over these repeats. Direct recruitment of Clr4 by assembled Swi6 chromatin may allow this to spread along the chromatin fiber and may mediate silencing of genes adjacent to LTRs. Alternatively, noncoding transcripts originating in the LTR that extend into adjacent genes may trigger the formation of siRNAs homologous to that gene, which results in its silencing.

the *D11.02* and *meu6* RNAs were constitutively expressed in *LTR-D11.02Δ* and *LTR-meu6Δ* cells (Fig. 5B). This indicates that the LTR lying adjacent to the *D11.02* gene and the LTR separated by another ORF 3' to the *meu6* gene play a direct role in the repression of these genes during vegetative growth in rich medium. We conclude that LTRs can play a critical role in restricting the expression of a gene to a distinct differentiation pathway and that components of the RNAi pathway and silent chromatin are required to effect this repression.

**Discussion.** Our data indicate that expression of a synthetic shRNA can silence expression of a euchromatic gene in trans and this is coupled to chromatin modification and recruitment of heterochromatin proteins and cohesin to the target locus. Thus, expression of a shRNA can, alone, trigger the assembly of a patch of silent chromatin, which mimics that associated with the centromeric outer repeats.

The synthetic *shuraSE* hairpin RNA trigger requires Argonaute, Dicer, and RNA-directed RNA polymerase, the known components of the RNAi machinery, to mediate silencing. Thus, the RNAi machinery of fission yeast is not just designed to process unusual centromeric transcripts specifically, but, as in other organisms, it appears to degrade any extensive dsRNA. A surprising finding was that expression of *shuraSE* RNA also requires functional Clr4 to produce *shuraSE* siRNAs and to reduce *ura4<sup>+</sup>* expression; however, phenotypic silencing of *ura4* (FOA resistance), siRNA generation, and diMeK9-H3 over the *uraSE* region all persist in the absence of Swi6, even though *ura4* RNA levels appear elevated. This suggests that Swi6 operates downstream of the PTGS (RNAi) pathway to mediate transcriptional silencing at the level of chromatin. Because gene silencing is completely ineffective in *clr4-s5*, this histone methyltransferase is required both for efficient PTGS and the downstream modification of histone H3 on lysine 9 to mediate TGS. It seems likely that Clr4 acts in concert with Dicer, Ago1, and Rdp1 and that, in its absence, complexes [for example, RISC; see (41)] that are required for RNA interference are disrupted (see Fig. 6).

In wild-type cells expressing *shuraSE*, diMeK9-H3 is clearly detected on the *uraSE* target, the 300-bp region upstream and the *ura4* promoter itself. In the absence of Swi6 diMeK9-H3 persists at *uraSE* but is lost from the adjacent region and the promoter (Fig. 2B). Thus, as at the mating-type locus (40), Swi6 is required to spread silent chromatin formation outward from the 280-bp *uraSE* “nucleation center.” Because RNAi and/or PTGS clearly remains operative in *swi6Δ* cells, the presence of homologous siRNAs must not be sufficient to mediate spreading. The most parsimonious explanation is that *uraSE* siRNAs target nascent *ura4* transcripts and, in the process, recruit Clr4 histone methyltransferase, which methylates K9-H3 on local chromatin and thus allows binding of Swi6, which in turn re-

cruits Clr4 independently of RNAi to methylate and spread over adjacent nucleosomes (Fig. 6).

The RNAi pathway is involved in the regulation of differentially expressed genes in a number of systems by means of short temporal or micro-RNAs (46–48). Our analyses also demonstrate that RNAi-dependent chromatin silencing acts to repress some genes that lie in close proximity to some retrotransposon LTRs and that are normally induced during sexual differentiation. Together these data are consistent with a scenario where transcripts, homologous to both strands of the LTR, are acted on by the RNAi pathway to generate siRNAs, which cause the nucleation of a patch of diMeK9-H3, Swi6-bound silent chromatin, which can spread outward to silence adjacent genes (Fig. 6). The generation of siRNA homologous to *shuraSE* depends on Ago1, Dcr1, Rdp1, and Clr4. Although we have not detected siRNA homologous to Tf1 or Tf2, their generation is clearly implied by this silent chromatin’s dependence on these components. More complex mechanisms may account for silencing of RNAi-responding genes located several kilobases from an LTR; for example, long, noncoding transcripts originating in the LTR may interfere with the accumulation of transcripts from these genes and may mediate chromatin-based silencing. Alternatively, the effect may be indirect, owing to activation of some key meiotic transcription factor induced in the absence of RNAi, Clr4, or Swi6. Further detailed investigation will be required to distinguish between these and other possibilities. Nevertheless, the fact that some LTRs elicit a direct effect on neighboring genes is indisputable; RNAi is required for chromatin modification over the LTR adjacent to the *D11.02* and *meu6* genes and deletion of these LTRs leads to their constitutive expression. Thus, a similar mechanism of RNAi-dependent chromatin silencing appears to mediate the formation of silent chromatin at fission yeast centromeres and the repression of some genes that are induced during sexual differentiation, and this machinery can recognize a synthetic dsRNA and mediate silent chromatin formation on homologous DNA sequences (Fig. 6).

Britten and Davidson proposed that subfamilies of related repeats might regulate the differential expression of distinct networks of genes (14). Here we have demonstrated that full repression of at least two meiotically induced genes requires nearby LTRs, and our data are consistent with other LTRs playing a similar role in regulating other meiotic genes. A recent study has subdivided fission yeast LTRs into nine clades (31); we speculate that these distinct but related LTR families might be coregulated to mediate repression of different gene sets. Coregulation of nonoverlapping gene sets could be achieved with a minimal degree of homology being required to trigger RNAi-dependent chromatin silencing by an siRNA at a particular LTR. Indeed, siRNAs need to be highly homologous to recognize and destroy their RNA target (49); therefore, siRNAs

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identical in sequence to one LTR family would only bring about silent chromatin assembly at other member LTRs and not at nonmember LTRs where the sequences have drifted substantially to form a distinct family. Indeed some Tfl LTRs are less than 60% identical, with the longest stretches of identity being less than 20 nucleotides (31).

Analyses in other systems indicate that RNA and protein levels are regulated by endogenous micro-RNAs (46–48). It is possible that some of these also trigger chromatin modifications that allow stable propagation of the silent state through subsequent divisions. Related to this, many studies in metazoans use RNAi for functional analyses of a gene of interest (32). It is possible that not only is the target RNA degraded but that unanticipated chromatin modifications are induced at that genes locus that influence the outcome and interpretation of the result.

Our analyses shows that the mechanism of heterochromatin formation at centromeres and transposable elements are closely related and adds further weight to the idea that ancient transposable elements have been co-opted into centromere structure and function (50). In mammalian cells, there are also similarities between gene-silencing mechanisms, retrotransposon silencing, and centromeric satellite-repeat heterochromatin (9, 15–17, 51–53). Whether the RNAi pathway affects these processes in a manner similar to that described here remains to be determined.

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### Supporting Online Material

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# REPORTS

## Coherent Spin Transfer Between Molecularly Bridged Quantum Dots

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Femtosecond time-resolved Faraday rotation spectroscopy reveals the instantaneous transfer of spin coherence through conjugated molecular bridges spanning quantum dots of different size over a broad range of temperature. The room-temperature spin-transfer efficiency is ~20%, showing that conjugated molecules can be used not only as interconnections for the hierarchical assembly of functional networks but also as efficient spin channels. The results suggest that this class of structures may be useful as two-spin quantum devices operating at ambient temperatures and may offer promising opportunities for future versatile molecule-based spintronic technologies.

Semiconductor quantum dots (QDs) are attractive candidates for scalable solid-state implementations of quantum information processing based on electron spin states (1). Confined car-

riers in QDs are relatively decoupled from their dissipative environment as compared with systems of higher dimensionality, such as quantum wells and bulk semiconductors, which are ex-

pected to inhibit spin relaxation processes and result in extended spin coherence times. Recently, coherence times of isolated spins in an ensemble of chemically synthesized QDs were found to exceed several nanoseconds, limited by inhomogeneous broadening, up to room temperature (2). In this context, a variety of QD-based quantum computation schemes have been suggested in which efficient and tunable spin coupling among QDs is a crucial implementation requirement (3–8). Although scalability and integration of the proposed devices based on conventional top-down fabrication methods remain challenging, colloidal chemistry techniques permit the creation of semiconductor QDs with controllable size and shape (9). These techniques also allow for chemical reactivity to direct the assembly of the nanometer-scale building blocks into ordered arrays by the judicious choice of the coordinating-ligand functionality.