

Reversibility of facultative heterochromatin in early embryos and ES cells is mirrored in the capacity of these cell types to reactivate the X chromosome in a somatic cell nucleus in ES cell fusion hybrids (19) or after nuclear transfer (20). Indeed, our results help to understand these findings. First, repression of Xp *Xist* occurs specifically in Nanog-positive cells at the time they are first allocated, suggesting that this is a property inherent to the pluripotent ICM lineage. The same activity in ES cells could result in repression of the somatic Xi *Xist* allele in ES-somatic cell hybrids. This then would lead to X reactivation in the ES nuclear environment, where heritability of X inactivation is strictly *Xist*-dependent. In the case of nuclear transfer, the Xi from the donor somatic cell is also the Xi in TE and PE lineages, but random X inactivation occurs in the embryo proper (20). This would be explained again if repression of *Xist* occurs specifically in ICM cells. TE and PE lineages would inactivate in response to maintained expression of the somatic Xi *Xist* allele, whereas ICM cells would repress *Xist*, establishing the ground state for random X inactivation in the embryo proper.

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21. We thank colleagues for helpful discussions and T. Jenuwein for kindly providing antisera. This work was supported by the Medical Research Council. UK.

www.sciencemag.org/cgi/content/full/303/5658/666/DC1

17 October 2003: accepted 29 December 2003

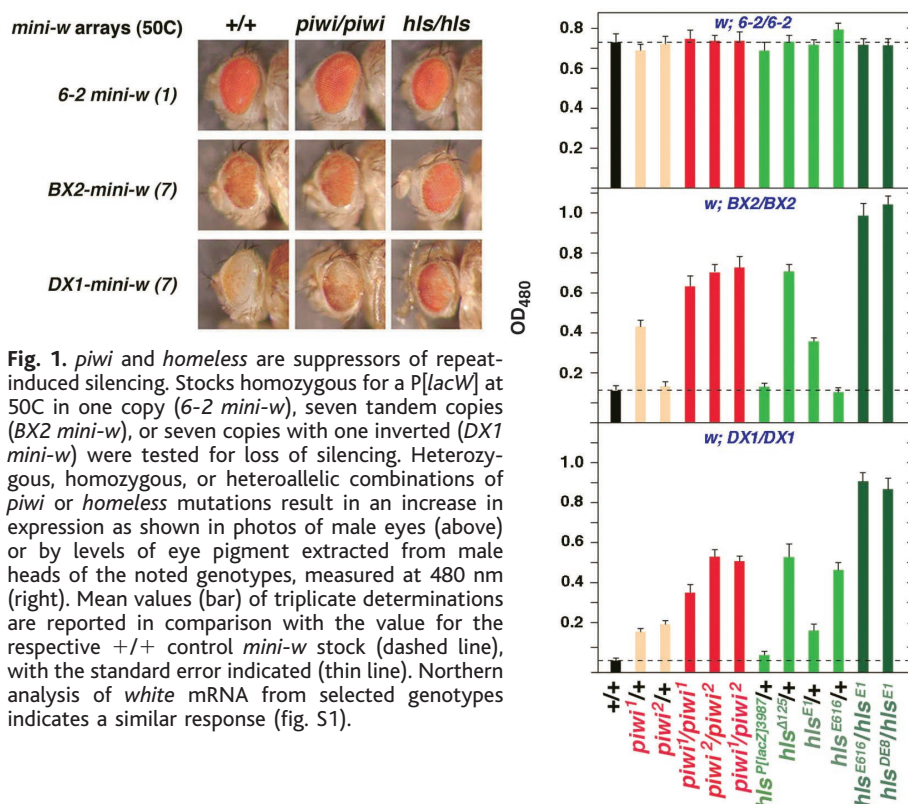
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Genes normally resident in euchromatic domains are silenced when packaged into heterochromatin, as exemplified in *Drosophila melanogaster* by position effect variegation (PEV). Loss-of-function mutations resulting in suppression of PEV have identified critical components of heterochromatin, including proteins HP1, HP2, and histone H3 lysine 9 methyltransferase. Here, we demonstrate that this silencing is dependent on the RNA interference machinery, using tandem *mini-white* arrays and *white* transgenes in heterochromatin to show loss of silencing as a result of mutations in *piwi*, *aubergine*, or *spindle-E* (*homeless*), which encode RNAi components. These mutations result in reduction of H3 Lys<sup>9</sup> methylation and delocalization of HP1 and HP2, most dramatically in *spindle-E* mutants.

Small RNA molecules have been found to play multiple roles in regulating gene expression. These include targeted degradation of mRNAs by small interfering RNAs (siRNAs) (posttranscriptional gene silencing, PTGS) (*1, 2*), developmentally regulated sequence-specific translational repression of mRNA by micro-RNAs (miRNAs) (*3*), and targeted transcriptional gene silencing (TGS) (*4-9*). RNAi activity limits transposon mobilization and provides an antiviral defense (*10*). Recent work demonstrated that RNA interfer-

ence (RNAi) is required to establish silencing at heterochromatic domains in fission yeast (8, 9); appearance of transcripts from centromeric repeats is accompanied by loss of histone H3 Lys<sup>9</sup> methylation (8, 9).

Many components of the RNAi machinery have been identified in *Drosophila melanogaster*, where they have been implicated in PTGS of the tandemly repeated *Stellate* genes, several retrotransposons, and *Alcohol dehydrogenase (Adh)* transgenes (5, 11, 12). Mutations in *aubergine (aub)*, encoding a PAZ domain/



**Fig. 1.** *piwi* and *homeless* are suppressors of repeat-induced silencing. Stocks homozygous for a P[*lacW*] at 50C in one copy (6-2 *mini-w*), seven tandem copies (*BX2 mini-w*), or seven copies with one inverted (*DX1 mini-w*) were tested for loss of silencing. Heterozygous, homozygous, or heteroallelic combinations of *piwi* or *homeless* mutations result in an increase in expression as shown in photos of male eyes (above) or by levels of eye pigment extracted from male heads of the noted genotypes, measured at 480 nm (right). Mean values (bar) of triplicate determinations are reported in comparison with the value for the respective *+/+* control *mini-w* stock (dashed line), with the standard error indicated (thin line). Northern analysis of *white* mRNA from selected genotypes indicates a similar response (fig. S1).

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PIWI domain (PPD) protein, and in *spindle-E* (also known as *homeless*, *hls*), encoding a DEAD-motif RNA helicase, up-regulate *Stellate* expression. *hls* mutations also increase the

expression of some retrotransposons and genomic repeats (11, 12). Mutations in *piwi* (also a member of the PAZ domain family) block PTGS of *Adh* transgenes (5). Embryos with mutations in *aub* and *hls* do not support RNAi in response to injection of double-stranded RNA (13).

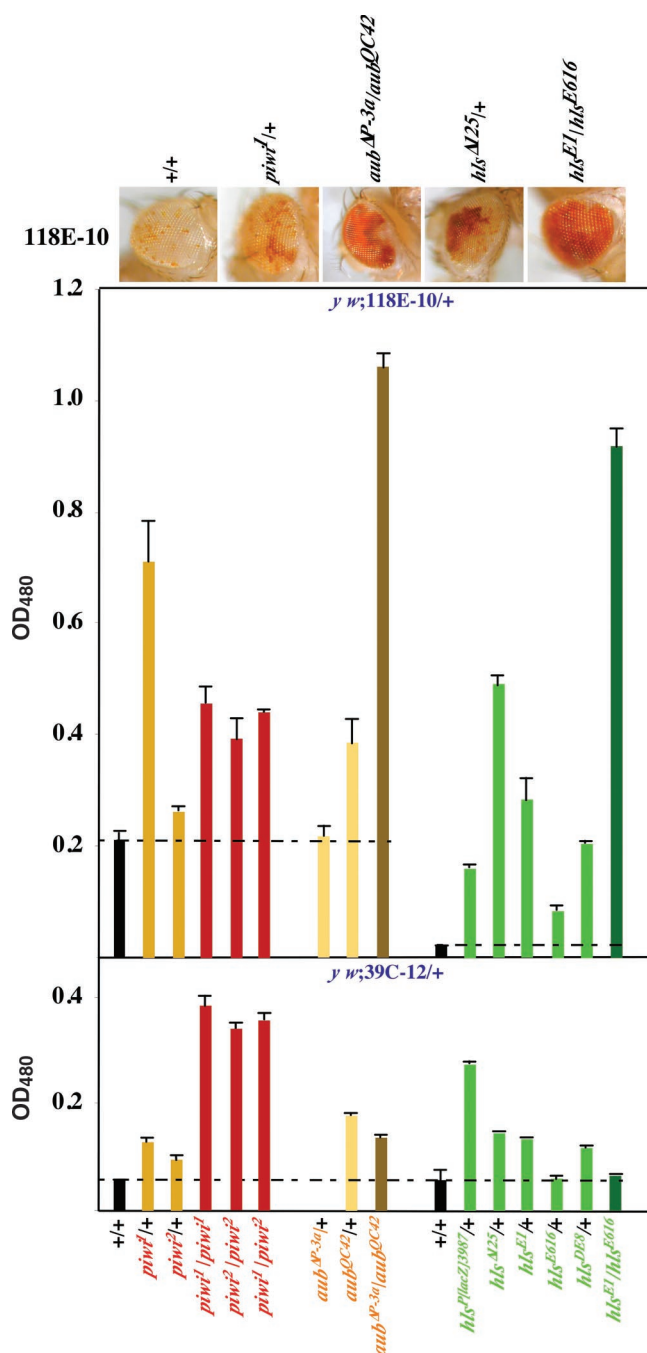
These findings suggest that the RNAi system could also play a role in targeting heterochromatin formation in *Drosophila*. Components of the heterochromatin-silencing complex have been identified by screens for dominant suppressors of position effect variegation (PEV), the silencing that occurs when a normally euchromatic gene is juxtaposed with a heterochromatic domain. The above mutations were originally identified in screens for germline or embryonic abnormalities; we have tested their potential to impact heterochromatic silencing using two systems.

Tandem repeats of a *Drosophila* white transgene *P[lacW]* result in a variegating phenotype (14). Silencing is lost in *Su(var)205/+* mutants [reduction of heterochromatin protein 1 (HP1)] and altered by changes in the number of Y chromosomes, as expected for heterochromatin-induced silencing. We examined *mini-white* lines 6-2 (*mini-w*, one copy), *BX2* (seven tandem copies), and *DX1* (seven copies, one inverted) (15). Mutations in *piwi* and *homeless* do indeed relieve silencing at the repeat loci (Fig. 1). Two alleles of *piwi* (*piwi*<sup>1</sup> and *piwi*<sup>2</sup>) and their heteroallelic combination were tested with similar results. Three tested alleles of *hls* (*E1*, *E616*), as well as the heteroallelic combinations *E1/E616* and *E1/DE8*, cause suppression of variegation at *DX1*, the *mini-w* array showing the strongest silencing. Similar results were obtained for *BX2*. In some instances, these mutations fail to show a dominant phenotype, but loss of silencing is consistently observed when the mutation is homozygous or present in a heteroallelic combination.

Insertion of the *P* element *P[hsp26-pt, hsp70-w]* in a euchromatic domain results in a uniform red eye, whereas insertion in the pericentric heterochromatin or much of the small fourth chromosome results in a variegating phenotype. These variegating lines show loss of silencing on introduction of dominant suppressors of PEV and respond to changes in copy number of the sex chromosomes as anticipated for heterochromatic silencing (16). We have examined the impact of mutant alleles of *piwi*, *aubergine*, and *homeless* on two such lines (15), and we have observed that the functions of all three loci are required for heterochromatic silencing (Fig. 2). Homozygous or heteroallelic mutations in *piwi* result in a twofold increase in *white* expression in line 118E-10. The heteroallelic mutant combination of *aubergine* produces more than a fivefold increase in pigment. Five alleles of *homeless* were tested, and all show a dominant suppression, i.e., cause a loss of silencing. Similar results were obtained with line 39C-12.

The RNAi machinery may function throughout an RNA molecule that directs sequence-specific targeting of heterochromatin formation. Known components of heterochromatin in *Drosophila* include histone H3 specifically modified by methylation at Lys<sup>9</sup> (H3-mK9), HP1, and HP2, a partner of HP1 in *Drosophila* (17). Volpe *et al.* (8) have reported a loss of H3-mK9 and Swi6, the HP1 homolog, at centromeric repeats in *S. pombe* as a result of mutations in the RNAi system. We examined the effects of homozygous mutations in *piwi*, *aubergine*, and *homeless* on HP1 and H3-mK9 by immunofluorescent staining of the

**Fig. 2.** Suppression of PEV by components of the RNAi system. Homozygous or heteroallelic mutations in *piwi* result in an increase in *white* gene expression (loss of silencing) in line 118E-10 (transgene in pericentric heterochromatin). The heteroallelic mutant combination of *aubergine* produces strong suppression. The *homeless* mutations have a dominant phenotype with a several-fold increase in expression, depending on the allele. Males were photographed 3 days after eclosion. Similar, but less dramatic, results were obtained by using stock 39C-12 (transgene in the fourth chromosome). Pigment values are reported relative to the control *y w*<sup>67c23</sup> stock carrying the respective transgene.





polytene chromosomes (15). *homeless* mutations have a dramatic effect on the distribution of HP1, normally concentrated at the pericentric heterochromatin and the fourth chromosome. In *hls/hls*, HP1 is distributed across the whole of the polytene chromosomes (Fig. 3A). At the same time, there is a significant reduction of histone H3-mK9 (Fig. 3B). This reduction was also observed on Western blots of adult fly extracts (Fig. 3C). However, the total amount of extractable HP1 in various *hls* genotypes appears similar (Fig. 3C), which suggests that expression of HP1 is not affected in *hls* mutant lines, but rather the distribution within the nucleus. Thus, the RNAi system must be intact to achieve targeted methylation of histone H3 at Lys<sup>9</sup>, and proper localization of HP1. The changes observed readily account for the loss of PEV.

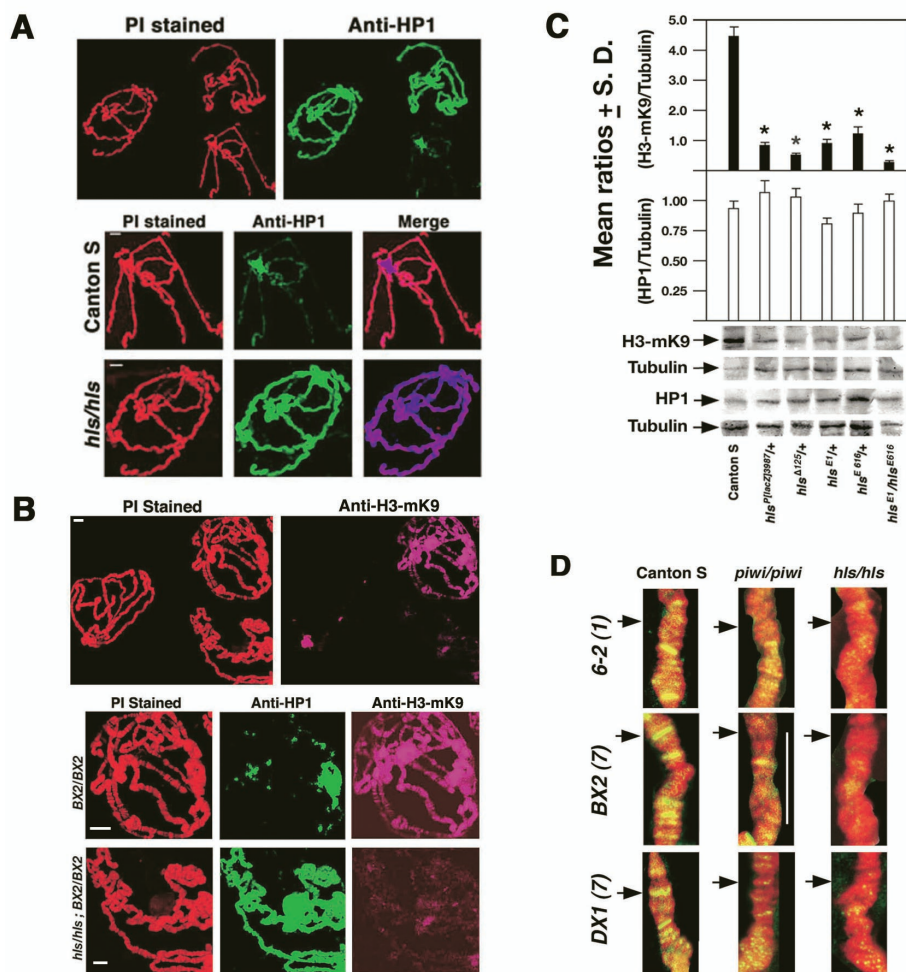
A single copy of the P[*lacW*] transgene at 50C [line 6-2], as well as the *BX2* and *DX1* seven-copy arrays, were examined for the presence of H3-mK9 (15). The location of this heterochromatic array away from the chromocenter allows a determination of whether there is an accumulation of modified H3 correlated with gene silencing. No detectable H3-mK9 above the normal level was found associated with the fully active single copy, but a strong band of labeling was present in the two seven-copy array lines (Fig. 3D). Previous work has shown a strong association of HP1 with the silenced copies (19). In the *piwi* mutant, the strong H3-mK9 labeling is no longer discernible. In the *hls* mutant, there is a general loss of labeling across the nucleus (see also Fig. 3B). Taken together, these results indicate that the *hls* gene product is required for the proper targeting of H3 modification by methylation of Lys<sup>9</sup> at the *mini-white* array.

Mutations in *piwi* and *aubergine* result in partial loss of H3-mK9, most evident at minor sites within the euchromatic arms (Fig. 4; figs. S2 and S3). Mutation of *homeless* has a more pronounced effect, resulting in dramatic loss of H3-mK9 and redistribution of HP1 and HP2 away from the chromocenter, along the euchromatic arms (Fig. 4; fig. S4). Antibodies specific for H3-mK9 and for H3-mK27 (20) were used to confirm that the effect is specific to H3-mK9 (fig. S5). HP1 interacts with SU(VAR)3-9, a major histone H3 methyltransferase, and the normal localization of these proteins to pericentric heterochromatin has been shown to be mutually dependent (21). The general distribution of HP1 along the chromosome arms in the absence of targeted H3-mK9 is not surprising, as HP1 has been shown to bind nonspecifically to nucleosome core particles and naked DNA (22). HP2 interacts with HP1 through the HP1 chromo-shadow domain, and has previously been found to undergo a shift in

distribution in the chromosome upon redistribution of HP1 (17).

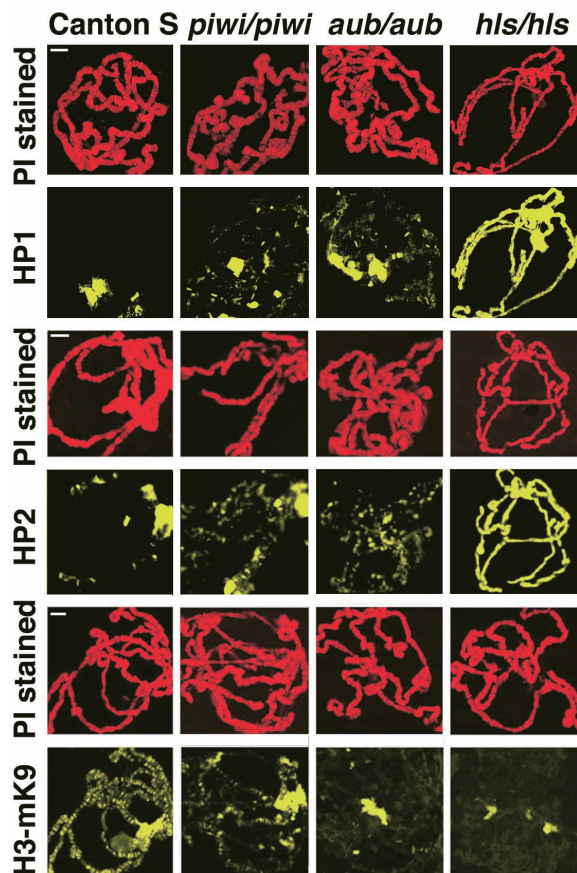
Although the decrease in silencing of the P[*lacW*] array and of the *white* transgenes in pericentric and fourth chromosome heterochromatin is readily detected, it is a partial effect; one does not observe restoration of a uniform red eye phenotype. The fact that HP1 and HP2 retain nearly normal distribution in the presence of *piwi* or *aubergine* mutations,

but not following loss of *homeless* gene product, suggests that *homeless* encodes a more central function than *piwi* and *aubergine* for heterochromatin formation. All three loci appear to be involved in targeting histone H3 methyltransferase activity and localization of HP1 and HP2, demonstrating an important role for the RNAi machinery in establishing this pattern of histone modification and concomitant gene silencing.



**Fig. 3.** Mutations in components of the RNAi system result in a delocalization of HP1 and a strong reduction in H3 methylated at Lys<sup>9</sup>. (A) Salivary glands from wild-type Canton S larvae and *hls/hls* larvae were fixed (3.7% formaldehyde), squashed together on the same slide, treated with mouse monoclonal antibodies specific for HP1 (18) and a secondary Cy5-conjugated goat antibody directed against a mouse antibody, and viewed by confocal microscopy. Simultaneous preparation and treatment of the glands on the same slide permits an assessment of the relative amounts and distribution of the antigen in the two lines. HP1 shows dramatic delocalization in *hls/hls* mutants. Scale bar, 10  $\mu$ m. (B) Salivary glands from wild-type Canton S larvae and *hls/hls*; *BX2/BX2* larvae were squashed together on the same slide and stained using antibodies against histone H3-mK9 (Upstate Biotechnology). Immunostaining was performed in a manner to maximize detection of modified H3. There is a strong reduction of methylated H3 in the mutant line. (C) Western blot analysis showing amounts of HP1 and H3-mK9 in normal and *homeless* mutant flies. The histogram displays the quantification of triplicate blots. HP1 does not vary significantly, but H3-mK9 is reduced, relative to the amount of tubulin in either heterozygous or homozygous *hls* mutants. Means significantly different from Canton S at the 95% level of confidence are marked with an asterisk. (D) Histone H3-mK9 is localized to the 50C *mini-white* arrays that show strong silencing [*BX2*(7), *DX1*(7)] in a wild-type genotype, but is not detectable above the normal level when only a single active transgene [6-2(7)] is present. The strong accumulation on the multiple arrays is lost in lines with heteroallelic mutations in *piwi* or *homeless*. Chromosomes were probed with antibodies specific for H3-mK9. Gray value images were pseudo-colored and merged. Scale bar, 10  $\mu$ m.

**Fig. 4.** Mutations in components of the RNAi system result in a loss of histone H3-mK9, and a delocalization of heterochromatin proteins HP1 and HP2. Polytene chromosomes (prepared as in Fig. 3) were treated with rabbit polyclonal primary antibodies specific to HP1, HP2, or histone H3-mK9, as specified, and with antibodies against the female specific protein, Sex-lethal, used to distinguish mutant from wild-type chromosomes. Antibodies were applied to mixtures of Canton S wild type with *piwi*<sup>1</sup>/*piwi*<sup>2</sup>, *aub*<sup>QC42</sup>/ $\Delta$ P-3a, or *hls*<sup>E1</sup>/*hls*<sup>E616</sup> glands; *piwi*<sup>1</sup>/*piwi*<sup>1</sup>, *hls*<sup>E1</sup>/*hls*<sup>DE8</sup>, and *hls*<sup>E1</sup>/*hls* <sup>$\Delta$ 125</sup> showed similar results. In the supporting online material, adjacent nuclei on the same slide, but of different genotype, are presented for each comparison (figs. S2 to S4). The level of H3 methylated at Lys<sup>9</sup> is progressively reduced, both at heterochromatic and euchromatic sites, in the *piwi*/*piwi*, *aub*/*aub*, and *hls*/*hls* lines, with a progressive delocalization of HP1 and HP2. Scale bar, 10  $\mu$ m.



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23. We thank T. Jenuwein for antibodies specific for modified histones, H. Lin for alleles of *piwi* and *aubergine*, C. Berg for *homeless* alleles, D. Dorer and S. Ronssey for *mini-white* arrays, and members of our research groups for critical review of the manuscript. N. Pavelka, P. Ghana, and C. Craig provided excellent technical assistance. This work was supported by grants from the NIH (HD23844 and GM68388 to S.C.R.E.), the NSF (MCB 0211376 to J.A.B.), the Human Frontier Science Program and a Wellcome Trust Senior Fellowship (RGY20/2003 and Wt2001 to U.B.).

# Supporting Online Material

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Materials and Methods

Figs. S1 to S5

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16 October 2003; accepted 9 December 2003

## RNAi-Mediated Targeting of Heterochromatin by the RITS Complex

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RNA interference (RNAi) is a widespread silencing mechanism that acts at both the posttranscriptional and transcriptional levels. Here, we describe the purification of an RNAi effector complex termed RITS (RNA-induced initiation of transcriptional gene silencing) that is required for heterochromatin assembly in fission yeast. The RITS complex contains Ago1 (the fission yeast *Argonaute* homolog), Chp1 (a heterochromatin-associated chromodomain protein), and Tas3 (a novel protein). In addition, the complex contains small RNAs that require the Dicer ribonuclease for their production. These small RNAs are homologous to centromeric repeats and are required for the localization of RITS to heterochromatic domains. The results suggest a mechanism for the role of the RNAi machinery and small RNAs in targeting of heterochromatin complexes and epigenetic gene silencing at specific chromosomal loci.

The fission yeast *Schizosaccharomyces pombe* contains large stretches of heterochromatin that are associated with telomeres, repetitive DNA

elements surrounding centromeres, and with the silent mating-type loci (*I*). Assembly of heterochromatin at these loci involves an or-

chestrated array of chromatin modifications that lead to the recruitment of two chromodomain histone-binding proteins Swi6, a homolog of the *Drosophila* and mammalian HP1 proteins, and Chp1 (2, 3). The RNAi pathway has also been implicated in regulation at the DNA and chromatin level in *Arabidopsis* (4–6), *Drosophila* (7), and *Tetrahymena* (8), and in heterochromatin assembly in *S. pombe* (9, 10).

RNAi silencing is triggered by double-stranded RNA (dsRNA), which is cleaved by the ribonuclease III (RNase III)-like enzyme Dicer to generate small RNA molecules of ~22 nucleotides (nt) (11–13). These small interfering RNAs (siRNAs), load onto an effector complex called RISC (RNA-induced silencing complex) that contains an Argo-

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