periods [several weeks (1/)], during which TTR becomes modified. Comparisons of guanidinium chloride (GdmCl) and guanidinium thiocyanate (GdmSCN) denaturation curves revealed that WT TTR was more resistant to GdmCl denaturation than was T119M, whereas the opposite was true in GdmSCN, as shown previously (9). These differences in midpoint of denaturation can be attributed to differential anion stabilization, suggesting that the true thermodynamic stabilities of these proteins are very similar, although a quantitative analysis is not possible in these chaotropes (22).

A free-energy landscape diagram consistent with all of the experimental data relevant to T119M trans-suppression is shown in Fig. 4C. T119M trans-suppression is principally mediated by destabilization of the dissociative transition state, consistent with positioning of T119M at the dimer-dimer interface. Increasing the dissociative transition-state energy by 3.1 kcal/mol effectively prevents tetramer dissociation because the activation barrier becomes insurmountable (dissociation half-life $t_{1/2}$ increases from $\sim$42 hours to $>1500$ hours). Small-molecule binding similarly increases the activation barrier associated with tetramer dissociation in a dose-dependent fashion, although this is mediated through tetramer stabilization (Fig. 4A). The extent of stabilization is maximal when the small-molecule dissociation constants $K_{d1}$ and $K_{d2}$ are as low as possible and the concentration of inhibitor is as high as possible. The concentrations used in our experiments for ground-state stabilization are comparable to those observed in plasma for numerous orally available drugs.

Small-molecule binding and trans-suppression increase the activation energy associated with tetramer dissociation, the rate-limiting step of TTR fibril formation. Establishing this analogy is important because it is known that trans-suppression prevents disease in V30M compound heterozygotes (7, 8). Kinetic stabilization of the native state is a particularly attractive strategy, considering the emerging evidence that small misfolded oligomers are neurotoxic (31).

Members of the ARGOXAUTE family are important in diverse posttranscriptional RNA-mediated gene-silencing systems as well as in transcriptional gene silencing in Drosohila and fission yeast, and programmed DNA elimination in Tetrahymena. We cloned ARGOXATE4 (AGO4) from a screen for mutants that suppress silencing of the Arabidopsis SUPERMAN (SUP) gene. The ago4-1 mutant reactivated silent SUP alleles and decreased CnpGp and asymmetric DNA methylation as well as histone H3 lysine-9 methylation. In addition, ago4-1 blocked histone and DNA methylation and the accumulation of 25-nucleotide small interfering RNAs (siRNAs) that correspond to the retroelement AtSN1. These results suggest that AGO4 and long siRNAs direct chromatin modifications, including histone methylation and non-CpG DNA methylation.

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References and Notes

10. Supporting material is available on Science Online.
16. Small-molecule inhibitors 6 and 8 through 10 do not inhibit TTR amyloidosis when a monomeric variant of TTR (M-TTR) (24) is employed for acid-mediated amyloid fibril formation studies (Fig. 2F), demonstrating that the inhibitors mediate amyloid inhibition through tetramer binding (Fig. 2, E and F). Inhibitor 7 slightly inhibits fibril formation from M-TTR because it drives a small fraction of M-TTR into a tetrameric quaternary structure (Fig. 2F).
18. The calculated and observed amplitude changes do not match the populations of T and T-12, displayed in Fig. 3, C and D, exactly in all cases owing to the fact that $K_{d1}$, $K_{d2}$, and the free energy of stabilization resulting from the binding of one (AG1) and two (AG2) ligands were all evaluated under physiological conditions (Fig. 18), and the experiments described here were carried out in 6 M urea.
19. $\Delta G_1 = RT \ln [T + I]/[T] = RT \ln [K_{d1}/K_0]$ and $\Delta G_2 = RT \ln [T - I]/[T] = RT \ln [K_{d2}/K_0]$. $\Delta G_1$.
23. Emerging data from the laboratories of S. Lindquist (25), D. Selkoe (26), C. Dobson (27), P. Lansbury (28), G. Kraft (29), and others reveal that oligomeric aggregates of proteins with a tendency to misfold and misassemble are more toxic to cells than are the insoluble aggregates with high molecular weight (e.g., amyloid fibrils and the scrapie form of the prion protein, PrPSc).
34. We thank NIH (grant DI 6R355), The Skags Institute of Chemical Biology, and the Uta Annabenzen Foundation for financial support; R. A. Lerner and I. Wilson for useful suggestions; and the Wenner-Gren Foundation for a postdoctoral fellowship (P.H.).

Supporting Online Material

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(H3K9) methylation (5, 6). In Tetrahymena, TiWI, a member of the PIWI subfamily of AGOs, is required for programmed DNA elimination, which is associated with 28-nt siRNAs and histone H3K9 methylation (7, 8).

Finally, Drosophila PIWI is required for both posttranscriptional and transcriptional repression of alcohol dehydrogenase transgenes (9).

The clark kent (clk) mutants are epigenetic alleles of the Arabidopsis SUP gene caused by SUP gene silencing and extensive DNA methylation of CpG, CpNpG (where N is either A, C, T, or G), and asymmetric (CpHpH, where H is either A, C, or T) cytosines (10). The clk mutants are recessive and meiotically heritable, suggesting a primarily chromatin-based gene-silencing mechanism. Indeed, the initiation of SUP silencing requires the DRMZ de novo DNA methyltransferase (11). Furthermore, by screening for suppressors of the clk-st allele, we isolated two chromatin modification enzymes required for the maintenance of SUP gene silencing, CHROMOMETHYLASE3 (CMT3) and KRYPTONITE (KYP). CMT3 encodes a DNA methyltransferase, and KYP encodes a histone H3K9-specific protein methyltransferase (12, 13). kyp and cmt3 mutants both cause a loss of CpNpG methylation at SUP and all other loci tested. Here we describe the cloning of a third clk-st suppressor mutation in the AGO4 gene.

We identified one recessive allele of a clk-st suppressor gene that mapped to chromosome II. Other than suppression of SUP silencing, we did not observe morphological defects in the homozygous mutant. By sequencing candidate genes, we identified a mutation in the AGO4 gene, previously named on the basis of its sequence similarity to AGO1 (1). The mutation destroyed a splice acceptor site, causing a deletion and frameshift that terminated translation after 595 amino acids (fig. S1). The AGOs comprise a conserved family of eukaryotic genes (fig. S1) containing two domains of unknown function: an NH2-terminal PAZ domain and a COOH-terminal PIWI domain (14). Because the frameshift deleted almost the entire PIWI domain, the mutation is likely to cause severe loss of AGO4 function. To confirm that the suppressor mutation is within AGO4, we transfected mutant plants with the AGO4 gene and found that the original clk-st phenotype was restored. Thus we named this suppressor mutation ago4-1.

We analyzed the effect of ago4-1 on SUP DNA methylation using bisulfite genomic sequencing (Fig. 1A and table S1). Whereas CpG methylation levels were unchanged, ago4-1 showed a 2.8-fold reduction in CpNpG and a 4.5-fold reduction in asymmetric methylation. This methylation phenotype was similar to that of the mutant but not of the ago4-1 mutant. We previously found that, at all loci tested, cmt3 and kyp showed a reduction of CpNpG methylation but not of CpG methylation (12, 13). Therefore, we used Southern blot analysis with methylation-sensitive restriction enzymes to assay the effect of ago4-1 on both CpG and CpNpG methylation at three additional loci: the 180–base-pair (bp) centromeric repeat (CEN) sequence (Fig. 1B), the Ta3 retrotransposon (Fig. 1C), and the FWA gene (Fig. 1D). The ago4-1 mutation did not affect either CpNpG or CpG methylation levels at these loci. The FWA locus also contains a substantial amount of asymmetric methylation (15), and bisulfite sequencing of FWA showed that the ago4-1 mutation did not reduce this methylation. Thus, the methylation phenotype of ago4-1 is locus-specific and different than that of the cmt3 and kyp mutations.

We found three other loci at which ago4-1 did have an effect on DNA methylation: MEA-ISR, AtSN1, and AtMu1. MEA-ISR is an approximately 183-bp sequence present in seven direct repeats in an intergenic region adjacent to the imprinted MEDEA gene (16). In the wild type, MEA-ISR locus contains 95% CpG, 58% CpNpG, and 26% asymmetric methylation (Fig. 1A and table S1). ago4-1 essentially eliminated the CpNpG and asymmetric methylation but did not affect the CpG methylation (Fig. 1A). We used Southern blot analysis with methylation-sensitive restriction enzymes to confirm these results. We found that CpNpG methylation was eliminated in ago4-1 but that CpG methylation was unaffected (Fig. 1E). AtSN1 is a retrotransposon sequence previously shown to be methylated (17). We found that the wild-type AtSN1 locus contains 75% CpG, 70% CpNpG, and 24% asymmetric methylation (Fig. 1A and table S1). ago4-1 greatly reduced
the non-CpG methylation to 14% CpNpG and 0.8% asymmetric methylation. The AtMu1 sequence is the 3′-terminal inverted repeat of the Arabidopsis DNA transposon Mu1 (18). We found that wild-type AtMu1 shows 58% CpG, 35% CpNpG, and 11% asymmetric methylation. The ago4-1 mutation did not affect the CpG methylation but reduced the CpNpG methylation to 19% and the asymmetric methylation. One explanation for AGO4-independent non-CpG methylation is that another AGO gene (nine of which are present in the Arabidopsis genome) could act redundantly with AGO4. Alternatively, pathways that do not involve AGO genes could function at some loci. For instance, if AGO4’s primary role is to establish methylation, effects will only be visible at loci that require frequent establishment.

A comparison of the methylation phenotype of ago4-1 with those of mutants of CMT3 and DRM, the two types of DNA methyltransferase genes known to control non-CpG methylation, did not show a simple relationship (Fig. 1A). In particular, ago4-1 mimicked the dnm1 dnm2 double mutant at MEA-ISR (16). However, at both SUP and AtSN1, ago4-1 showed a reduction in CpNpG methylation that was intermediate between the effects of the cmt3-7 and dnm1 dnm2 mutants and a reduction of asymmetric methylation that was stronger than the effect of either (Fig. 1A). These results suggest that both CMT3 and DRM are involved in AGO4-dependent methylation.

To determine the relationship between AGO4, KYP, and CMT3, we performed chromatin immunoprecipitation (ChIP) experiments to examine histone H3K9 methylation levels at SUP. We previously found that kyp, but not cmt3, reduced H3K9 methylation at SUP (19), suggesting that CMT3 acts downstream of KYP, because of targeting of CMT3 to methylated histones (13). Figure 2 shows that ago4-1 reduced H3K9 methylation of SUP relative to the wild-type strain clk-st. The simplest interpretation of these results is that AGO4 acts upstream of KYP to target H3K9 methylation. We also found that ago4-1 reduced H3K9 methylation at AtSN1, a locus where ago4-1 also reduced DNA methylation (Fig. 2). However, ago4-1 did not reduce H3K9 methylation of Ta3 (Fig. 2) or of the CEN repeats, where ago4-1 showed no DNA methylation effect. Thus, the effects of ago4-1 on H3K9 methylation are locus-specific and correlate with effects on DNA methylation.

We tested whether AGO4 function is associated with siRNAs by probing Northern blots of RNA preparations that had been enriched for small RNAs. AtSN1 was recently shown to be associated with a newly discovered class of long (approximately 25-nt) siRNAs (17). We could easily detect AtSN1 siRNAs in the wild-type Ler or clk-st strains and in the cmt3 or kyp mutant strains (Fig. 3A). However, these siRNAs were reduced to below the level of detection in ago4-1. We did not detect siRNAs specific for the SUP or AtMu1 sequences. This may be due to the limited sensitivity of Northern blot analysis, because siRNAs to AtSN1 (present in approximately 70 copies per genome) are probably easier to detect than siRNAs to low-copy-number genes such as SUP and AtMu1 (17).

It has been shown that long siRNAs of tobacco TS SINE retroelements did not mediate resistance to a virus carrying TS SINE sequences, suggesting that, unlike the 21- to 22-nt siRNAs, long siRNAs do not participate in PTGS (17). In addition, mutants that affect RNA silencing were used to show a correlation of long siRNAs with DNA methylation. In particular, mutants in SDE1/SGS2 (an RNA-dependent RNA polymerase), SDE3 (an RNA helicase), and SGS3 (a novel gene) did not suppress the accumulation of long siRNAs or affect DNA methylation of AtSN1, but the sde4 mutant (not yet cloned) suppressed both long siRNAs and DNA methylation (17). ago4 and sde4 map to different chromosomes and are therefore not allelic (20).

Thus, AGO4 and SDE4 likely encode components of a silencing system that generates long siRNAs specialized for chromatin level gene silencing (Fig. 3B). Presumably, a Dicer-like enzyme (21) and possibly an RNA-dependent RNA polymerase are also involved in siRNA production. Once generated, the long siRNAs guide KYP-dependent histone methylation and CMT3- and DRM-dependent DNA methylation to specific regions of chromatin. The targeting of this system to transposable elements likely contributes to suppression of transposon proliferation and to genome stability.

**Note added in proof:** The long and short classes of small RNAs have been shown to be bona fide siRNAs and are likely made by distinct Dicer-like enzymes (22).

**References and Notes**


**Fig. 2.** ChIP analysis of H3K9 methylation, showing multiplex polymerase chain reaction analyses of SUP, AtSN1, and Ta3 together with ACTIN, a locus with a low level of H3K9 methylation (19). The input is clk-st chromatin before immunoprecipitation. "No AB" lanes are control immunoprecipitations with no antibody. The fold enrichment of the SUP, AtSN1, and Ta3 signal over the ACTIN signal is shown.

**Fig. 3.** Effect of ago4-1 on AtSN1 siRNAs. (A) Northern blot of small RNAs hybridized with a sense AtSN1 RNA probe. —25-nt siRNAs are found in all genetic backgrounds shown except for the ago4-1 mutant strain. Positions of 20- and 30-nt RNA markers are indicated. (B) Model for the function of AGO4 and long siRNAs in the control of histone and DNA methylation.
Modulation of Heterochromatin Protein 1 Dynamics in Primary Mammalian Cells

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Heterochromatin protein 1 (HP1), a key component of condensed DNA, is strongly implicated in gene silencing and centromeric cohesion. Heterochromatin has been considered a static structure, stabilizing crucial aspects of nuclear organization and prohibiting access to transcription factors. We demonstrate here, by fluorescence recovery after photobleaching, that a green fluorescent protein–HP1 fusion protein is highly mobile within both the euchromatin and heterochromatin of ex vivo resting murine T cells. Moreover, T cell activation greatly increased this mobility, indicating that such a process may facilitate (hetero)chromatin remodeling and permit access of epigenetic modifiers and transcription factors to the many genes that are consequently derepressed.

The role of HP1 (1, 2) in heterochromatin formation and function was revealed by studies of position effect variegation (PEV) in which a gene juxtaposed to a heterochromatic region is stochastically silenced in a proportion of the cells that normally would express it (3–5). The probability of silencing in PEV is regulated by the concentration of heterochromatin components such as HP1 (6, 7) or SU(VAR)3-9, a methyltransferase enzyme (8, 9) that methylates the Lys4 on the histone H3 tail (10, 11), creating an HP1 binding site (12, 13). Silencing in PEV correlates with decreased accessibility of the affected gene to nucleases (5, 7, 14). Because HP1 is an integral component of H3 Lys4-methylated chromatin and therefore is thought to restrict access to factors required for crucial processes (for example, transcription, replication, repair), it is essential to know (i) whether the binding of HP1 to chromatin is static or dynamic and (ii) whether this binding can be modulated physiologically. To address these questions, we established an in vivo mammalian system to measure HP1 mobility within heterochromatin and euchromatin in resting and activated primary murine T cells.

To use fluorescence recovery after photobleaching (FRAP) as a measure of the mobility of HP1 in living cells, we generated transgenic mice that express a green fluorescent protein (GFP)–HP1 chimeric protein in T cells by using a fusion cDNA construct (15) under the control of the human CD2 locus control region (16, 17). We established lines expressing GFP-HP1 at <10% the level of endogenous HP1 protein (fig. S1). Flow cytometry confirmed T cell–specific transgene expression and normal T cell development (that is, normal proportions of T cells in the subpopulations identified by CD4 and CD8 staining). Microscopy (18) of ex vivo T cells showed GFP signal concentrated in several discrete regions in the nucleus that colocalized with 4′,6-diamidino-2-phenylindole (DAPI)–dense staining (fig. 1). Such DAPI–dense regions have been found to colocalize with heterochromatic centromeric clusters that bind endogenous HP1 in murine lymphocytes (19). HP1α staining of transgenic T cells revealed similar colocalization of DAPI- and GFP–dense regions (fig. S2). A smaller amount of green fluorescence was seen throughout the rest of the T cell nuclei. These euchromatic regions, which stained densely with antibody against acetylated histone H3 (fig. S3), are likely to contain both chromatin-bound and free HP1.

Fig. 1. GFP-HP1 protein is concentrated in heterochromatic foci in transgenic T cells, as shown by (A) DAPI staining (blue) and (B) GFP fluorescence (green). This confirms that the GFP-HP1 protein is localized to heterochromatin foci, as is the case for endogenous HP1. In addition, the regions between these foci contain lower levels of green fluorescence, indicating that GFP-HP1 is also present in “euchromatic” areas. Non-T cells have very little green fluorescence, indicating that the background levels are low.

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