RNA interference (RNAi) is a fundamental biological process that plays a crucial role in regulation of gene expression in many organisms. Transcriptional gene silencing (TGS) is one of the important nuclear roles of RNAi. Our previous data show that Entamoeba histolytica has a robust RNAi pathway that links to transcriptional gene silencing (TGS) or through post-translational gene silencing (TGS). An RNAi-silencing trigger can induce H3K27Me2 deposits at both episomal and chromosomal loci, mediating gene silencing. Our data support two phases of RNAi-mediated TGS: an active silencing phase where the RNAi trigger is present and both H3K27Me2 and Ago2-2 concurrently enrich at chromosomal loci; and an established silencing phase in which the RNAi trigger is removed, but gene silencing with H3K27Me2 enrichment persist independently of Ago2-2 deposition. Importantly, some genes display resistance to chromosomal silencing despite induction of functional small RNAs. In those situations, the RNAi-triggering plasmid that is maintained episomally gets induced and becomes transcriptionally active Argonaute (Ago) proteins. Other important proteins for RNAi include Dicer (an RNAse III enzyme that generates small RNAs) and RNA-dependent RNA polymerase (RdRP), which amplifies small RNAs in some systems (23). The RNAi pathway can mediate gene silencing through transcriptional gene silencing (TGS) or through post-translational gene silencing in diverse situations including worm development and germline X chromosome silencing (10–16). Moreover, TGS mediated by DNA methylation has been noted in plants and the fruit fly (17, 18).

RNAi is a means of regulating gene expression in many biological systems ranging from protozoans to humans (19–22). The initiation of RNAi is via small RNA molecules whose sequence information identifies genes to be targeted for silencing. The machinery that ultimately mediates gene silencing is the RNA-induced silencing complex, containing the catalytically active Argonaute (Ago) proteins. Other important proteins for RNAi include Dicer (an RNAse III enzyme that generates small RNAs) and RNA-dependent RNA polymerase (RdRP), which amplifies small RNAs in some systems (23). The RNAi pathway can mediate gene silencing through transcriptional gene silencing (TGS) or through post-translational gene silencing in diverse situations including worm development and germine X chromosome silencing (10–16). Moreover, TGS mediated by DNA methylation has been noted in plants and the fruit fly (17, 18).

**Background:** Entamoeba histolytica has an RNAi-mediated TGS pathway but the epigenetic marks are not known.

**Results:** H3K27Me2 is an inducible repressive histone modification enriched in loci silenced by small RNAs.

**Conclusion:** Transcriptional gene silencing and chromatin modification are coupled to maintain prolonged gene silencing.

**Significance:** This work identifies the first repressive histone mark in Entamoeba and links it to RNAi.

**References:**

1. Bardees M. Foda and Upinder Singh.
2. The abbreviations used are: H3K27Me2, dimethylated histone H3 lysine 27; sRNA, small RNA; Eh, Entamoeba histolytica; RdRP, RNA-dependent RNA polymerase; Ago2-2, Argonaute 2-2; RPL21, ribosomal protein L21; Ap-A, amoebapore A; ROM, rhomboid protease; Pol II, RNA polymerase II; TGS, transcriptional gene silencing.

**Conclusion:** Transcriptional gene silencing and chromatin modification are coupled to maintain prolonged gene silencing.
silencing (P-TGS) by RNA degradation or translational repression (24, 25). RNAi-mediated TGS is facilitated by a complex of small RNAs bound to an RNA-induced transcriptional silencing complex. RNA-induced transcriptional silencing targets homologous DNA sequences where it induces recruitment of chromatin modifying factors and heterochromatin formation that result in silencing of target genes. The extensive interdependence between the RNAi machinery and heterochromatin assembly has been illustrated in Saccharomyces pombe, Tetrahymena thermophila, Drosophila melanogaster, Caenorhabditis elegans, and animals (26–31). In animals and fission yeast, fulfillment of both the siRNA pathway and repressive histone depositions are required for assembly of heterochromatin at appropriate genetic regions to achieve transcriptional repression (32).

Entamoeba histolytica is a protozoan parasite and an important human pathogen. Despite its position as a deep-branching eukaryote, E. histolytica has a distinct and complex endogenous RNAi pathway mediated by 27-nucleotide 5’-poly-P small RNAs that map antisense to genes and mediate TGS via a nucleolar-localized Argonaute protein (33, 34). We have recently developed a robust silencing tool in ameba by harnessing the endogenous RNAi pathway. With this approach, a gene with abundant antisense small RNAs can “trigger” silencing of a new gene fused to it through the generation of small RNAs to the fused gene (35). We were able to demonstrate stable gene repression, including ongoing silencing despite loss of the trigger construct hinting that the long term stable down-regulation may be mediated by epigenetic memory. Although many genes could be silenced using this approach, we also identified some genes that could not be silenced despite the generation of functional small RNAs (36). It has previously been demonstrated that RNAi-silenced loci have increased histone occupancy, however, given the sequence divergence of the amebic H3, commercially available reagents could not be applied to define the molecular modifications typically associated with heterochromatin (33, 37).

In the present study, we shed light on mechanisms of RNAi-regulated TGS in E. histolytica. We identified an amebic histone modification, dimethylated lysine 27 histone H3 (H3K27Me2), and generated and validated reagents for its characterization. Using custom antibodies to H3K27, H3K27Me2, and Argonaute 2-2 for chromatin immunoprecipitation (ChIP) characterization. Using custom antibodies to H3K27, H3K27Me2, and an irrelevant peptide (RNase III (Ac-RSSLEKYQT)) were blotted directly on a nitrocellulose membrane (Bio-Rad). The membrane was left to dry and then blocked with 5% milk in 1× Tris-buffered saline/Tween 20 (TBS-T) for 1 h. To block α-H3K27 or α-H3K27Me2 antibodies, increasing amounts of each peptide were used for blocking fixed amounts of the antibodies. We incubated 1 μg of H3K27 or H3K27Me2 antibody with increasing amounts of each peptide (10, 25, 50, or 100 μg). The mixtures were completed to 100 μl of 1× TBS-T and kept on a gentle rotator at room temperature for 2 h. To remove possible aggregates and avoid subsequent Western blot background, the peptide/antibody mixture was centrifuged for 15 min at 14,000 rpm at 4 °C. Next, we incubated each supernatant (i.e. blocked antibody), 1 μg of unblocked antibody, or preimmune serum with the peptides blotted on the membranes overnight then proceeded with the Western blot protocol. To test antibody specificity against E. histolytica histone H3, as in the peptide competition assays described above, we blocked the antibody with different peptide amounts (2, 4, 10, 20, 50, and 100 μg). Blocked or unblocked antibodies or preimmune serum were used to probe increasing amounts of whole cell lysates (10, 20, 40, and 80 μg).

**Experimental Procedures**

**Antibody Generation—**Rabbit polyclonal antibodies against two forms of E. histolytica histone H3 were custom generated by 21st Century Biochemicals, Marlboro, MA. Affinity purified antibodies were obtained by immunizing rabbits with H3K27 synthetic peptide (Ahx-VAFKAa(K)KMLSKD) for generating anti-H3K27 and H3K27Me2 peptide (Ahx-VAFKAa(KMe2)KMLSKD) for generating anti-H3K27Me2 (Fig. 1A). The percentage of dimethylated Lys-27 in H3K27Me2 peptide preparations was 99%. Anti-EhAgo2-2 and anti-EhRBP9 (or anti-Pol II) antibodies were generated in previous studies (33, 38). Commercial antibodies included a pan-specific mouse anti-actin antibody (MP Biomedicals, Burlingame, CA); secondary HRP-conjugated anti-mouse and anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) were also utilized.

**Peptide Competition Assays and Western Blot Analysis—**Increasing amounts (50, 100, 200, 500, and 1000 ng) of synthetic peptides H3K27, H3K27Me2, and an irrelevant peptide (RNase III (Ac-RSSLEKYQT)) were blotted directly on a nitrocellulose membrane (Bio-Rad). The membrane was left to dry and then blocked with 5% milk in 1× Tris-buffered saline/Tween 20 (TBS-T) for 1 h. To block α-H3K27 or α-H3K27Me2 antibodies, increasing amounts of each peptide were used for blocking fixed amounts of the antibodies. We incubated 1 μg of H3K27 or H3K27Me2 antibody with increasing amounts of each peptide (10, 25, 50, or 100 μg). The mixtures were completed to 100 μl of 1× TBS-T and kept on a gentle rotator at room temperature for 2 h. To remove possible aggregates and avoid subsequent Western blot background, the peptide/antibody mixture was centrifuged for 15 min at 14,000 rpm at 4 °C. Next, we incubated each supernatant (i.e. blocked antibody), 1 μg of unblocked antibody, or preimmune serum with the peptides blotted on the membranes overnight then proceeded with the Western blot protocol. To test antibody specificity against E. histolytica histone H3, as in the peptide competition assays described above, we blocked the antibody with different peptide amounts (2, 4, 10, 20, 50, and 100 μg). Blocked or unblocked antibodies or preimmune serum were used to probe increasing amounts of whole cell lysates (10, 20, 40, and 80 μg).

**Parasite Culture and Transgenic Strains—**E. histolytica trophozoites (HM-1:IMSS) were grown according to standard conditions (39, 40). E. histolytica strains containing trigger silencing or Myc overexpression constructs were previously generated (34, 35). Briefly, the 19-Trigger strains, 19T-ROM1, 19T-Ago2-2, and 19T-RdRP1, contain a silencing construct that has a short Trigger region fused to the full-length EhROM1, EhAgo2-2, or EhRdRP1, respectively. Similarly, overexpression parasite lines, Myc-ROM1, Myc-Ago2-2, and Myc-RdRP1, contain constructs with N-terminal 3× Myc tag preceding a full-length EhROM1, EhAgo2-2, or EhRdRP1 coding region, respectively. Both the silencing and overexpression constructs were built using the pKT plasmid as a backbone, which contains the 5’ and 3’ regulatory regions of the cysteine synthase gene; thus all generated constructs including the Trigger-silencing constructs were driven by the same cysteine synthase promoter (41). We maintained the cell lines at 12 μg/ml of
Dimethylated H3K27 Is a Repressive Histone Code in E. histolytica

G418 for EhAgo2-2 and EhRdRP and 6 μg/ml of G418 for EhROM1. A cell line, 19T-ROM1<sup>No plasmid</sup>, was generated by maintaining 19T-ROM1 trophozoites in TYI-S-33 medium without the G418 selection marker for greater than 2 years and loss of Trigger plasmid was confirmed by drug susceptibility and PCR (35).

**Immuno­fluorescence Microscopy**—Fluorescence microscopy was performed as described previously (33). Wild type *E. histolytica* trophozoites were grown for 48 h. Confluent cells were allowed to adhere onto a glass slide chamber (Thermo Fisher) for 3 h at 37 °C in TYI-S-33 medium. Cells were rinsed once with 1× PBS and fixed for 30 s with methanol/acetic acid (50:50) at room temperature. The cells were rinsed once with 1× PBS, permeabilized by 250 μl of PBS containing 0.1% Triton X-100 for 10 min at 37 °C, washed 3 times with 1× PBS, followed by blocking in 3% BSA-PBS for 1 h at room temperature. Blocked cells were incubated overnight at 4 °C with 1% BSA/PBS containing 1:100-diluted rabbit polyclonal H3K27Me2 antibody with gentle agitation. Following four washes with 1× PBS for 10 min each, the cells were incubated in the dark with 1% BSA/PBS containing a 1:200 dilution of fluorescent secondary antibody, anti-rabbit Alexa 488 (Cell Signaling) for 1 h at room temperature. Following antibody incubation, 80 μl of protein-A agarose/salmon sperm slurry beads (EMD Millipore) were added to each sample and incubated on ice for 10 min. Pelleted cells were suspended in 1 ml of ChIP cell lysis buffer (20 mM Tris-HCl, pH 8, 85 mM KCl, 0.5% Nonidet P-40, protease inhibitor mixtures) and incubated on ice for 10 min. The nuclei were isolated by spinning the cell lysate for 5 min at 1000 rpm. Isolated nuclei were incubated on ice for 10 min with nuclear lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 1.0% SDS, protease inhibitor mixtures). Chromatin was sonicated to an average length of 500 bp and centrifuged to remove residual insoluble materials. Supernatant was pre-cleared with 80 μl of protein-A agarose/salmon sperm slurry beads (EMD Millipore) for 30 min at 4 °C. Cleared lysate was diluted 5-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.1% EDTA, 20 mM Tris-HCl, pH 8.0, 167 mM NaCl). A supernatant volume representing 10% of total soluble chromatin was put aside at 20 °C as input for subsequent steps. Diluted lysate (equivalent to 2 × 10<sup>6</sup> cells) was incubated with each antibody overnight at 4 °C in a gentle rotator. Polyclonal antibodies used in ChIP experiments included 5 μg of each of *E. histolytica* anti-H3K27 and anti-H3K27Me2 antibodies (both customized by 21st Century Biochemicals), anti-EhAgo2-2 (customized by EZBiolab), anti-RBP9 (38), and IgG as a control (Cell Signaling). Following antibody incubation, 80 μl of protein A-agarose/salmon sperm slurry beads were added and incubated for 3 h at 4 °C. The beads were washed consecutively for 3 min on a rotator two times with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), twice with high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), one time with LiCl wash buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% Na-deoxycholate, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0) and two times with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Protein-DNA complex was eluted from protein-A or protein-G beads used with anti-RBP9 antibody by adding two times 250 μl of elution buffer (1% SDS, 2 mM EDTA, 50 mM Tris-HCl, pH 8.0) and incubating each for 15 min at 65 °C each time and vortexing every 5 min; 500 μl of eluate was subsequently collected. Protein-DNA cross-linking was reversed by adding NaCl to a final concentration of 200 mM and heating at 65 °C overnight. RNase treatment was performed using 20 μg of RNase A (Sigma) at 37 °C for 1 h followed by treatment with 128 μl of Proteinase K mixture (160 μg of Proteinase K, 625 mM Tris-HCl, pH 6.5, 156.25 mM EDTA) for 2 h at 45 °C. DNA was extracted using 500 μl of phenol/chloroform/isoamyl alcohol (Invitrogen), and enriched DNA was collected after 5 min of spinning at room temperature at 14,000 rpm. Collected DNA was cleaned by a PCR purification kit according to the manufacturer’s instructions (Qiagen) and eluted with 50 μl of DNsase-free water.
Dimethylated H3K27 Is a Repressive Histone Code in E. histolytica

ChIP-PCR Analysis—PCR (total volume 25 μl) were run using 4 μl of ChIP-eluted enriched DNA and 1 unit of platinum Taq DNA polymerase (Invitrogen) to amplify a DNA fragment of 100–200 bp size. To amplify only the episomal DNA fragment, forward primers were designed to specifically anneal to the episomal promoter (cytoeine synthase), Myc tag, or the 19-Trigger; the reverse primer was specific to the gene of interest. For identifying the chromosomal region of a target gene, the forward primers were designed to anneal specifically to the endogenous promoter of the target gene and the reverse primers were the same as used for amplifying the episomal fragments. The coding region primers anneal to the coding region of the target gene, in both the episomal and chromosomal copies, given that they have an identical sequence. The number of PCR cycles was set to 30 for amplifying the episomal or coding fragments and 35 for amplifying the chromosomal regions. PCR products were analyzed on a 1.5% agarose gel and results were analyzed using the Quantity One software (Bio-Rad) to measure the band intensity. The enrichment was calculated as the percentage of the input and compared with the steady state level of enrichment at the gene encoding ribosomal protein L21 (RPL21), a constitutively expressed gene. To calculate fold-enrichment, the data were normalized to that of RPL21 and subsequently the normalized average values were compared with a control cell line. The levels of enrichment are represented as the mean ± S.E. of a minimum of 3 independent ChIP experiments. ChIP-PCR primers designed as following: EHI_048600, 5’-ACACCCGGATGAAATTAGAATTAACC and 3’-ACA- CTAAGGCTGTGATCTGCAAATTIT; Ethap-A, 5’-AAAC- CAATCTAGAAAGCCATCG and 3’-GCTTTATTGCCAAC- GCTGTG; EhRPL21 (coding region), 5’-CTAACGGTGAAGA- TAGAGAACACG and 3’-TCCCAACACGTCCAGTCTTC- TT; EhRPL21 (promoter region), TAAATAACTAGAATTGT- GAACTACTGCCCTGCTTCTATTATTACCGTTAG; episomal promoter, 5’-CGTAAAAGATTATTGCTGACT- CATTT; EHI_197520: for 19-Trigger plasmids, 3’-CGTGG- TAGTGTGTTGGACTGAAACAT; Myc, for Myc-overexpression plasmids, 5’-GGGAGATGTTTCGGCTAC- GCCAT; EthRom1 (coding region), 5’-AGGCTCTACTC- CACCACTAAAATA and 3’-TAAATCTCATCACCATT- CTCTGTAAACA; EthAgo2-2 (coding region): 5’-GCTACCT- GCACAATCATTGAGATT and 3’-TTCCTTGGAAGAGGAC- GAGCAATATATAC; EthRdp1 (coding region), 5’-CCC- GGATGGATAATTTGATTATTGTC and 3’-GGTCAATC- TCCCAATATATACAGGCA; EthRom1 (chromosomal promoter region), 5’-GGTAAAGAGAGAACATGAAAT- AAAA and 3’-GGTCAATTACTCCAATCGAGATGTA; EthAgo2-2 (chromosomal promoter region), 5’-CCGATCTAA- TTAGCAATATTAT and 3’-GGTTTTATCTATTGATG- GTTATTAG; Episomal EthRdp1 (chromosomal promoter region), 5’-GGGACAACTATATATAACAAAAACAAAA and 3’-GGCCGGATATCCTCTAATATAATCAAGGCA.

**Results**

**Generation of E. histolytica Histone α-H3K27 and α-H3K27Me2 Antibodies—E. histolytica histone H3 has a highly divergent N terminus (Fig. A1) (42). However, conserved lysine residues, which are targets for post-translational modifications,

are maintained in the E. histolytica histone H3 tail. In higher eukaryotes, enrichment of methylated H3K27 is associated with gene repression (13, 14, 43, 44). Hence, we asked whether coupling of H3K27 methylation and transcriptional gene silencing is conserved in E. histolytica. We synthesized peptides to represent unmodified H3 (H3K27) and a dimethylated form of H3 (H3K27Me2) (H3K27Me2 peptide preparation had 99% dimethylated Lys-27), and generated rabbit polyclonal antibodies against each. Each antibody recognizes a single band at the expected molecular mass of 15 kDa using Western blot analysis (Fig. 1B). Reactivity of H3K27Me2 antibody strongly suggests the presence of a methylated form of lysine 27 in E. histolytica H3.

To determine the affinity of the α-H3K27 and α-H3K27Me2 antibodies, we immobilized increasing amounts of H3K27 and H3K27Me2 peptides on a nitrocellulose membrane and incubated it with anti-H3K27 or anti-H3K27Me2 antibody. Each antibody displayed a very strong affinity toward the peptide used for antibody generation (Fig. 1, C and D), whereas the preimmune serum did not (data not shown). Neither antibody reacted with the RNase III peptide, which served as an irrelevant peptide control (data not shown). To verify specificity of the antibodies, peptide competition assays were conducted in which we blocked each antibody with H3K27, H3K27Me2, or RNase III peptide (Fig. 1, C and D). We used increasing amounts of the peptides to block a fixed quantity of each antibody and then used the blocked antibody to probe different amounts of immobilized peptides. At antibody:peptide ratios of 1:10 and 1:25 μg, we noticed that the affinity of α-H3K27/H3K27- or α-H3K27Me2/H3K27Me2-blocked antibody decreased significantly. In contrast, both antibodies retained strong affinity when we used the same ratios to block the antibodies with nonspecific peptides (i.e. H3K27Me2 and RNase III peptides to block α-H3K27, or H3K27 and RNase III peptides for blocking α-H3K27Me2). Similar results were obtained when we increased the amount of blocking peptides to 50 or 100 μg (data not shown). Despite the specificity, each antibody showed some reactivity toward both peptide forms, H3K27 and H3K27Me2, which we attribute to the substantial similarity in peptide sequences. We extended the specificity test and probed increasing amounts of whole cell lysate with α-H3K27 or α-H3K27Me2 antibody each blocked with H3K27, H3K27Me2, and RNase III peptides (Fig. 1, E and F). Similar to results from the peptide competition assays, when we blocked either α-H3K27 or α-H3K27Me2 antibody with the cognate peptide, the Western blot signal was greatly reduced. In contrast, we observed strong immunodetection for α-H3K27 and α-H3K27Me2 antibodies when we blocked them with an irrelevant peptide. These results demonstrate that α-H3K27 and α-H3K27Me2 antibodies bind with greater specificity to E. histolytica H3K27 and H3K27Me2 histone forms, respectively.

**Dimethylation of Lysine 27 of Histone H3 Is a Repressive Epigenetic Mark in E. histolytica—Cross-talk between RNAi and H3K27 methylation leads to gene repression through heterochromatin condensation in multiple systems (14, 43). To determine whether H3K27Me2 associates with the condensed chromatin in E. histolytica, we performed immunofluorescence analysis. The tendency of heterochromatin to be visualized as
discrete foci is attributed to its condensed nature (45, 46). We stained wild type *E. histolytica* cells with H3K27Me2 antibody and identified discrete foci in the parasite nucleus, which co-localized with DAPI staining and that most likely represent the heterochromatin (Fig. 2A).

Previously, using a commercial α-H3 antibody we and others have demonstrated increased occupancy of H3 at loci affected by small RNA (sRNA)-mediated gene silencing in the *E. histolytica* G3 strain (33, 37). To more specifically investigate the mechanism of small RNA-induced transcriptional gene silencing, we asked if post-translationally modified H3 histone (H3K27Me2) contributes to small RNA gene silencing in *E. histolytica*. To answer this question, we performed ChIP assays to establish the correlation between small RNAs, gene silencing, and histone modifications in *E. histolytica*. To determine whether H3K27Me2 is enriched at silenced loci with abundant antisense small RNAs, we selected an amebic gene, *EHI_048600*, which has numerous antisense sRNAs and is

**FIGURE 1.** *E. histolytica* H3 may be methylated on lysine 27. 

**A.** Sequence analysis of N terminus of *E. histolytica* histone H3 aligned using ClustalW with homologous H3 sequence from different organisms. *Shaded bold lysine residue:* a highly conserved lysine, K27. *Underlined amino acids:* used for peptide synthesis. *:* identical amino acids; _/colon_ indicates conserved amino acids; _/period_ indicates semi-conserved amino acids. 

**B.** Western blot analysis for increasing amounts of whole cell lysate (WCL) prepared from wild type *E. histolytica* trophozoites. Total protein resolved by SDS-PAGE then transferred to PVDF membrane and analyzed for *E. histolytica* H3K27, dimethylated H3K27 (H3K27Me2), or actin. 

**C–F.** Both generated *E. histolytica* α-H3K27 and α-H3K27Me2 antibodies have high specificity. 

**C.** Peptide competition assays. Increasing amounts of H3K27 peptide were immobilized on dot-blot nitrocellulose membrane. The membranes were probed with α-H3K27 antibody alone or α-H3K27 antibody blocked as indicated with different amounts of H3K27, H3K27Me2, or irrelevant (RNase III) peptide. 

**D.** Same as C but the immobilized peptides are H3K27Me2 and membranes were probed with unblocked or blocked α-H3K27Me2 antibody in the same way as in C. 

**E.** Western blot analysis of increasing amounts of whole cell lysate from wild type trophozoites. Total protein was probed with unblocked α-H3K27 antibody or α-H3K27 antibodies blocked with 20 ng of each H3K27, H3K27Me2, or irrelevant (RNase III) peptide. Actin is used as a loading control. 

**F.** Same as E, however, the probing antibody is the unblocked or blocked α-H3K27Me2 antibody as in E.
silenced (35). Additionally, we included a highly expressed gene Amoebapore A (Ap-A) as a negative control. RT-PCR experiments highlight the different expression levels of the selected genes (very low expression of EHI_048600 and high levels of Ap-A) (Fig. 2B). To determine whether H3K27Me2 is enriched at silenced genes, we performed ChIP assays followed by PCR analysis to evaluate the abundance of H3K27Me2 at genes with distinct expression levels. Relative to the input DNA, we identified statistically significant enrichment of H3K27Me2 at the silenced gene (EHI_048600) compared with very low levels of H3K27Me2 at the highly expressed Ap-A gene (61.2% ± 5.1 compared with 15.7 ± 5.3%; p = 0.003) (Fig. 2C). To calculate the difference in enrichment between the two genes, we measured the levels of enrichment at a constitutively expressed gene, RPL21, and used it for normalization. We detected a 6-fold increase of EHI_048600 enriched with H3K27Me2 as compared with enriched DNA of Ap-A (Fig. 2D). The H3K27 antibody showed no significant enrichment at EHI_048600 (1.7-fold ± 2.7; p = 0.2), which agrees with the previous results of the commercial H3 antibody. Overall, these data imply that the majority of histone depositions at the silenced locus are H3K27Me2. These results indicate that H3K27Me2 is a repressive epigenetic mark in E. histolytica and may be responsible for maintaining transcriptional gene silencing.

RNAi-silenced ROM1 Gene Has Enriched Repressive Histone H3K27Me2 Deposition—Our laboratory has recently established a method for trigger-induced sRNA-mediated gene silencing in E. histolytica, which induces gene down-regulation by generating pools of antisense small RNAs to the target gene (35). In this manner, multiple genes including Rhomboid protease 1 (ROM1), Myb-transcription factor, and an H2O2-responsive transcription factor were down-regulated (47). These genes also displayed long term silencing despite removal of the trigger plasmid. However, other amebic genes such as Argonaute genes (EhAgo2-1, EhAgo2-2, and EhAgo2-3) and EhRNase III were resistant to gene silencing using the trigger approach, despite generation of abundant and functional small RNAs to these genes (36). The RNA-dependent RNA polymer-
Dimethylated H3K27 Is a Repressive Histone Code in *E. histolytica*

A schematic diagram for 19 trigger-Romer1 (19T-ROM1) silencing construct and Myc-ROM1 overexpression plasmid (Myc-ROM1). The two constructs have the same vector backbone and are driven by the same promoter and regulatory sequences. ROM1 antisense sRNAs are present in 19T-ROM1. Striped box, silent gene; PCR, amplified regions displayed in B. **, episomal specific region. B, RT-PCR analysis of RNA collected from 19T-ROM1 and Myc-ROM1 *E. histolytica* trophozoites. As diagramed in A, PCR primers were used to represent total levels of ROM1 transcribed from both ROM1 loci (episomal and chromosomal). Levels of episomal ROM1 transcript evaluated by primers designed specifically to amplify episomal transcript. Expression levels of the EHI _199600_ gene serve as a reference control. DNA template, genomic DNA or plasmid DNA (for episomal ROM1, only PCR product of 19T-ROM1 is shown).

To evaluate the level of H3K27, H3K27Me2, and Ago2-2 protein depositions at ROM1 loci in the two cell lines, we performed chromatin immunoprecipitation experiments followed by PCR analysis. First, we monitored the enrichment levels at the episomal ROM1 silencing construct. We found statistically significant enrichment of H3K27 (80.6% ± 5.2; p = 0.00001) and H3K27Me2 (54.9% ± 5.5; p = 0.0002) at the episomal locus containing the silenced episomal ROM1 in 19T-ROM1 cell relative to low abundance at the expressed RPL21 genomic locus (18.1 ± 4.1% and 9.8 ± 4.3%, respectively) (Fig. 4A). In contrast, the Myc-ROM1 cell line showed no significant histone enrichment at the episomal overexpression plasmid compared with RPL21 (p = 0.2 for H3K27; p = 0.1 for H3K27Me2) (Fig. 4B). H3K27 and H3K27Me2 histone enrichment at the silencing construct in 19T-ROM1 were 4-fold increased compared with the highly expressed plasmid in Myc-ROM1 cells (Fig. 4C). However, we could not detect significant enrichment of Ago2-2 at the episomal construct in 19T-ROM1 (p = 0.1). Deposition of H3K27Me3 on an episome has been reported recently in Kaposi sarcoma-associated Herpesvirus and linked to gene silencing (48).

Next we asked whether the profile of H3K27, H3K27Me2, and Ago2-2 deposition at the chromosomal ROM1 locus differs from that determined at the episomal plasmid. We amplified a chromosomal DNA fragment of ROM1 (including a promoter region and 5′ coding portion). We determined that in the 19T-ROM1 (ROM1 silenced), this chromosomal ROM1 region was significantly enriched for H3K27 (58.7 ± 3.9%; p = 0.0001), H3K27Me2 (28.5% ± 3.3%; p = 0.02), and Ago2-2 (47.5 ± 6.5%; p = 0.01) compared with the same locus in Myc-ROM1 cells, in which ROM1 is overexpressed (Fig. 4, D and E). Interestingly, the H3K27Me2 abundance was very high at this chromosomal ROM1 locus (10-fold enriched compared with the same locus in Myc-ROM1 cells) indicating that the silenced ROM1 locus was highly enriched with the repressive H3K27Me2 mark (Fig. 4F). Similar results were noted with the analyses of a 5′-coding portion of ROM1 (Fig. 4, G–I). The observed Ago2-2 enrichment at the chromosomal locus was in contrast to the results noted on the silenced episomal plasmid. Lack of substantial Ago2-2 enrichment at the coding region while detecting moderate enrichment at the chromosomal fragment, which includes a promoter region, may imply a transient role for Ago2-2 in an early stage of RNAi-mediated gene silencing. The results indicate that trigger-induced small RNA gene silencing in *E. histolytica* is associated with substantial deposition of repressive H3K27Me2 at silenced loci and a moderate amount of Ago2-2 at the chromosomally silenced locus when the genomic loci are transcribed from both the chromosomal gene and the episomal plasmid. We observed substantial reduction in ROM1 expression in 19T-ROM1 cells versus high levels in the Myc-ROM1 cell line (Fig. 3B). Next, we evaluated the level of ROM1 transcript generated only from the plasmid in each of the two lines (labeled episomal ROM1 to distinguish from ROM1 transcript expressed from genomic locus). We identified a very low expression level of episomal ROM1 in the 19T-ROM1 cell line as compared with the high expression level in the Myc-ROM1 cell line (Fig. 3B).
FIGURE 4. Both episomal and chromosomal silenced ROM1 genes are enriched in repressive marks. 19T-ROM1 and Myc-ROM1 cells were cross-linked with formaldehyde and the extracted nuclear lysate was immunoprecipitated with control IgG or antibodies specific for E. histolytica H3K27, H3K27Me2, or EhAgo2-2. Protein enrichment, with subtracted IgG background, is displayed as a percentage of the enriched region relative to input DNA. Likewise, enrichment levels measured at constitutively expressed RPL21. A, a significant increase of the 19T-ROM1 episomal region enriched with H3K27 and repressive H3K27Me2 as compared with RPL21. B, corresponding Myc-ROM1 episomal region with levels of protein deposits similar to RPL21. C, 4-fold increase of 19T-ROM1 episomal regions enriched with H3K27 and repressive H3K27Me2 as compared with Myc-ROM1. The chart displays the ratio of RPL21-normalized enrichment values at episomal region shown in A relative to normalized levels in B (i.e. normalized A/B). D, 19T-ROM1, significant increase in chromosomal ROM1 region enriched with H3K27, H3K27Me2, or Ago2-2. E, Myc-ROM1, chromosomal ROM1 enriched with similar protein levels as RPL21. F, significant increase in the amount of chromosomal ROM1 regions enriched with H3K27 (5-fold) and repressive H3K27Me2 (10-fold) as compared with the same region in Myc-ROM1 cells. The chart displays the ratio of the RPL21-normalized enrichment values at the chromosomal ROM1 region shown in D relative to normalized levels in E (i.e. normalized D/E). Data represent mean ± S.E. of at least three independent ChIP experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Dimethylated H3K27 Is a Repressive Histone Code in E. histolytica

under active process of trigger-silencing (i.e. silencing construct is under continued selection).

Long Term Gene Silencing of ROM1 Correlates with Elevated Levels of H3K27Me2 Deposition—It has been confirmed that distinct chromatin-remodeling complexes are responsible for maintaining long term gene repression at particular loci (49). E. histolytica trophozoites are able to maintain gene silencing for years after removal of the silencing construct as shown in both the G3 strain (37) and the trigger-ROM1 cell lines (35). We attribute this feature to possible inheritable epigenetics. To study the proposed inheritable epigenetics of prolonged gene silencing, we analyzed 19T-ROM1No plasmid compared with a control cell line where ROM1 is expressed at normal levels. RT-PCR analysis confirmed that the cells had stable down-regulation of ROM1 despite loss of the silencing construct, with ROM1 silencing at comparable levels to that when plasmid was present (Fig. 5, A and B). 19T-ROM1No plasmid cells maintained production of sRNA although at levels moderately lower than the cells with trigger plasmid (35). Using ChIP we determined protein depositions at the silenced ROM1 chromosomal gene locus by investigating a DNA fragment of ROM1 that included a promoter region and 5′ coding portion. ROM1 locus showed a significant enrichment of both H3K27 (61.6 ± 9%, p = 0.01) and H3K27Me2 (48.6 ± 11%; p = 0.02) relative to RPL21 (28.7 ± 7.6% and 16.4 ± 5%, respectively) (Fig. 5, C–E), the increased levels were 2-fold (for H3K27) and almost 3-fold (for H3K27Me2) compared with wild type cells. Interestingly, the same chromosomal region showed higher levels of histone enrichment in the presence of a silencing construct (Fig. 4F). Trigger-mediated silencing is transcription dependent with no silencing in a promoter-less Trigger-ROM1 construct (35). The chromosomal locus of ROM1 seems to be loaded with H3K27Me2 in a manner that permits continuous small RNA production. In contrast to the 19T-ROM1 cell line, we did not detect a significant Ago2-2 enrichment at the chromosomal ROM1 locus in 19T-ROM1No plasmid cells (57 ± 14.8%; p = 0.1). Furthermore, we looked at a coding region of ROM1 and found that the levels of H3K27Me2 were enriched to 20-fold relative to wild type cells and H3K27 deposition was 4-fold higher, whereas Ago2-2 did not show a significant increase (Fig. 5, F–H). The substantial higher enrichment of H3K27Me2 at ROM1 coding regions in the absence of a trigger-plasmid suggests that adaptation takes place after the silencing construct is removed. The data imply that Ago2-2 deposition may be needed for the initiation phase of silencing, but is not required for maintaining the silencing, whereas larger depositions of H3K27Me2 are needed in the absence of a trigger to maintain prolonged gene repression.

Trigger Silencing of Ago2-2 Is Incomplete: Silencing and H3K27Me2 Enrichment at the Episomal Plasmid but a Transcriptionally Active Chromosomal Locus with No H3K27Me2 Enrichment—The E. histolytica genome encodes several genes thought to be involved in the RNAi gene silencing pathway including three Argonaute proteins, Ago2-1, Ago2-2, and Ago2-3 as well as two RdRP genes and a single RNase III gene (50). All RNAi pathway genes we tested (Ago2-1, Ago2-2, Ago2-3, and RNase III) were resistant to trigger-mediated gene silencing despite generation of abundant and functional small RNAs (36). For our analysis, we focused on the Ago2-2 locus to determine whether there was differential silencing of the episomal and chromosomal copy of Ago2-2 and whether H3K27, H3K27Me2, or Ago2-2 deposits occurred at either the episome or chromosomal locations. To determine the levels of Ago2-2 transcript, we performed RT-PCR experiments in cells with the 19T-Ago2-2 silencing construct and compared the results to Ago2-2 expression levels in cells that contain Myc-Ago2-2 expressing plasmid (Fig. 6A). Interestingly, we found reduced levels of the episomal 19T-Ago2-2 transcript, which means the episomal Ago2-2 has lower expression compared with another episomal plasmid driven by the same promoter (Myc-Ago2-2) (Fig. 6B). When we tested the overall expression of Ago2-2 using primers that anneal to the coding region of Ago2-2 (thus amplifying signal from both episomal and genomic generated transcripts), we did not see a difference in gene expression in 19T-Ago2-2 cells compared with wild type cells. Our results indicate that in the 19T-Ago2-2 cells, whereas expression of the episomal copy is somewhat repressed, the genomic Ago2-2 locus is transcriptionally active. The epigenetic silencing of Ago2-2 and the abundant functional small RNAs to Ago2-2 raises the question about whether H3K27, H3K27Me2, and Ago2-2 proteins deposit on episomal Ago2-2 locus to induce epimorphic silencing. To answer this question, we performed ChIP experiments on 19T-Ago2-2 and Myc-Ago2-2 cell lines. ChIP-PCR analysis of the episomal 19T-Ago2-2 locus showed significant enrichment of H3K27 (77.3 ± 5.3%; p = 0.002) and H3K27Me2 (67.1 ± 6.5%; p = 0.007) as compared with RPL21 (24.6 ± 8.9% and 20.1 ± 9.9%, respectively), whereas Myc-Ago2-2 was not significantly enriched with histones (Fig. 7, A and B). Enrichment levels of H3K27Me2 at 19T-Ago2-2 were 6 times greater than that at Myc-Ago2-2 (Fig. 7C). Similar to the 19T-ROM1 cell line, we did not observe an epigenomic enrichment of Ago2-2. These results indicate that the 19-Trigger fusion to Ago2-2 was able to induce repressive H3K27Me2 deposits at the episomal locus of Ago2-2 to repress transcription. However, the chromosomal locus did not show any significant enrichment of H3K27, H3K27Me2, or Ago2-2, which matches the high transcriptional activity at that locus and is consistent with similar results of the Ago2-2 coding region (Fig. 7, D–I). Why the chromosomal copy of Ago2-2 does not silence despite generation of small RNAs to Ago2-2 and silencing of the episomal copy is not clear could be due to the essential nature of Ago2-2 or an inability of heterochromatin to assemble at the Ago2-2 locus. Taken together the trigger-generated Ago2-2 sRNA may induce recruitment of transcription repressive H3K27Me2 only at the episomal Ago2-2 locus but not at the chromosomal locus, which then retains its transcription activity. Collectively, we attribute the incomplete silencing of Ago2-2 to a small RNA-related factor or to the fact that the Ago2-2 genetic locus is protected against TGS.

The RdRP1 Locus Is Resistant to Small RNA Generation and Silencing and Lacks H3K27Me2 Enrichment—Applying the Trigger-mediated gene silencing method to down-regulate RdRP1 did not result in generation of small RNAs or gene silencing of RdRP1 (36). RdRP1 was the only gene tested to date in which the Trigger fusion did not result in small RNA generation. To further confirm the direct correlation between small
Dimethylated H3K27 Is a Repressive Histone Code in E. histolytica

FIGURE 5. Persistence of ROM1 silencing after removing the trigger-silencing construct coupled with enrichment of H3K27Me2. A, schematic diagram for transcriptionally inactive chromosomal ROM1 locus in 19 trigger-ROM1 cells that lost the silencing construct (19T-ROM1\(^{\text{No plasmid}}\)). Depicted is endogenously active ROM1 locus in wild type E. histolytica trophozoites. ROM1 antisense (AS) sRNAs are depicted in 19T-ROM1. Striped box: silent gene; arrows, transcription status; PCR: amplified regions in B. B, RT-PCR analysis of RNAs collected from 19T-ROM1\(^{\text{No plasmid}}\) and wild type E. histolytica cells grown for 48 h. The analysis shows prolonged down-regulation of ROM1 after removing the silencing construct. Transcript levels of the EHI_199600 gene serve as a reference control. DNA template, genomic DNA of wild type cells. C-H, ChIP-PCR analysis of 19T-ROM1\(^{\text{No plasmid}}\) and wild type E. histolytica cells shows levels of ROM1 regions enriched with E. histolytica H3K27, H3K27Me2, or EhAgo2-2. Enriched DNA with subtracted IgG background is displayed as a percentage of input and compared with enrichment at the constitutively expressed RPL21 gene. C, 19T-ROM1\(^{\text{No plasmid}}\) shows a significant enrichment of H3K27 and H3K27Me2 at ROM1-silenced locus. D, wild type cells display ROM1 expressed locus with enrichment levels similar to RPL21. E, increase in H3K27 (2-fold) or H3K27Me2 (3-fold) enrichment in cells with maintained ROM1 silencing as compared with wild type cells. The chart displays the ratio of RPL21-normalized enrichment values at the DNA region encoding ROM1 shown in C relative to normalized levels in D (i.e. normalized C/D). F, 19T-ROM1\(^{\text{No plasmid}}\) significant increase of ROM1 coding region enriched with repressive H3K27Me2 as compared with RPL21. G, wild type cells, levels of enriched ROM1 coding regions are similar to RPL21. H, silenced ROM1 coding regions enriched with repressive H3K27Me2 at 20-fold compared with wild type cells. The chart displays the ratio of the RPL21 normalized enrichment values at the ROM1 coding region shown in A relative to normalized levels in B (i.e. normalized F/H). Data represent mean ± S.E. of at least three independent ChIP experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
**Dimethylated H3K27 Is a Repressive Histone Code in *E. histolytica***

RNA and heterochromatin, we investigated the RdRPI locus in cells with an *RdRPI* silencing construct (19T-RdRPI) and cells containing a Myc-*RdRPI* expressing construct (Fig. 8A). RT-PCR analysis confirmed that the episomal *RdRPI* locus is highly transcribed, which fits the observation that no small RNAs are generated to the *RdRPI* locus (Fig. 8B). We conducted chromatin immunoprecipitation experiments followed by PCR analysis to investigate protein depositions at *RdRPI* containing plasmids and chromosomal *RdRPI* locus and did not observe any specific enrichment of repressive histone modifications (Fig. 8, C–E). The overall lack of histone enrichment confirms that histone repressive factors correlate with small RNA abundance and gene silencing.

**H3K27Me2 Deposits Interfere with RNA Polymerase II**—The differential pattern of H3K27Me2 depositions led us to analyze the sites that are enriched with the repressive mark H3K27Me2, for RNA Pol II. Active transcription takes place in two substantially different phases, the formation of an active initiation complex followed by an elongation process (51). Several studies reported the interaction between the yeast heterochromatinic gene silencing and transcription initiation factors as well as hindering RNA Pol II elongation by the repressive chromatin (52, 53). Here, we used *E. histolytica* strains with reduced expression of ROM1, whereas the silencing triggered plasmid is maintained (19T-ROM1) or removed (19T-ROM1*No plasmid*). We performed immunoprecipitation experiments using specific *E. histolytica* antibody raised specifically against EhPol II subunit RBP9, which is known to be involved in transcription elongation and initiation through the interaction with other Pol II subunits (38, 54). We targeted the ROM1 promoter and coding regions, which showed a distinguished pattern for H3K27Me2 enrichment (Figs. 4, F and I, and 5, E and H). We aimed to investigate the accessibility of those ROM1 regions to Pol II by ChiP experiments and compare the results to Pol II enrichment at the corresponding regions of the constitutively expressed *RPL21* gene. Additionally, we used wild type cells and a Myc-ROM1 overexpressing strain as control cells where Pol II enrichment at ROM1 and *RPL21* should be maintained at normal levels. Interestingly, we observed no significant difference of Pol II occupancy at the ROM1 promoter region in 19T-ROM1*No plasmid* cells as compared with wild type cells (1.7-fold; *p = 0.38*) (Fig. 9). In contrast, the coding region of ROM1 showed a significant reduction of Pol II (0.26-fold ± 0.1; *p = 0.0007*). These results are the opposite of what we noted for H3K27Me2 deposition where the promoter region showed 3-fold and the coding portion displayed 20-fold enrichment (Fig. 5, E and H). This inverse Pol II/H3K27Me2 abundance implies that transcription could be initiated for the purpose of producing sRNAs, but the elongation process is inhibited when Pol II collides with the H3K27Me2 deposits at the coding region of ROM1. The results of 19T-ROM1 cells with the silencing construct supported our conclusion because Pol II occupancy at the ROM1 promoter decreased significantly compared with Myc-ROM1 overexpressing strain (0.24-fold ± 0.07; *p = 0.03*). This low Pol II occupancy is consistent with the 10-fold increased deposits of H3K27Me2 at the ROM1 promoter region (Fig. 4F). As expected, the coding region of ROM1 showed a significant decrease in Pol II occupancy relative to Myc-ROM1 cells (0.32-fold ± 0.08; *p = 0.04*). The results suggest that during active trigger silencing the trigger plasmid is the primary source of transcription and sRNA production. However, in the absence of the trigger plasmid, stable silencing necessitates limited transcription of the chromosomal ROM1 locus to ensure sRNA production and continued silencing. Collectively, amebic cells appear to adapt sRNA production, transcription inhibition, and heterochromatin formation to maintain TGS.

**Discussion**

We describe the first molecular mechanism that couples transcriptional gene silencing and the RNAi pathway in the protozoan parasite *E. histolytica*. We identify H3K27Me2 as an amebic repressive epigenetic mark associated with silenced loci. We identify two distinct phases of RNAi-mediated transcriptional gene silencing (Fig. 10). In the initial phase, where the silencing Trigger is present, concurrent depositions of H3K27Me2 and Argonaute protein appear to repress gene transcription. In the second and prolonged gene silencing phase where the RNAi-Trigger is absent, efficient silencing is maintained apparently independent of Argonaute protein deposition, but by increased deposits of repressive H3K27Me2 on...
FIGURE 7. Repressive H3K27Me2 enriches only episomal Ago2-2. ChIP-PCR analysis of 19T-Ago2-2 and Myc-Ago2-2 cells demonstrate deposits of *E. histolytica* proteins H3K27, H3K27Me2, or EhAgo2-2. Enriched DNA is displayed as percentages of input DNA after subtracting IgG background and compared with enrichment at the constitutively expressed *RPL21* gene. A, significant enrichment of H3K27 and H3K27Me2 at the silenced episomal locus of Ago2-2. B, overexpressed episomal Ago2-2 gene is enriched with protein levels similar to *RPL21*. C, episomal silenced Ago2-2 enriched with repressive H3K27Me2 at 6-fold more than Myc-Ago2-2. The chart displays the ratio of the *RPL21*-normalized enrichment values at the DNA region encoding ROM1 shown in A relative to normalized levels in B (i.e. normalized A/B). D and E, results of ChIP-PCR analysis shows the absence of any significant protein deposits at the chromosomal Ago2-2 locus that is not silenced in 19T-Ago2-2 (D) or expressed in Myc-Ago2-2 cells (E). F, ratio of RPL21 normalized levels of D to E indicates similar protein enrichment at chromosomal Ago2-2 locus in both 19T-Ago2-2 and Myc-Ago2-2 cells. G, in 19T-Ago2-2 parasites, the coding region of Ago2-2 lacks repressive H3K27Me2 or Ago2-2 enrichment. H, in Myc-Ago2-2, the coding region of Ago2-2 was enriched with protein quantities similar to *RPL21*. I, the chart displays the ratio of the *RPL21* normalized enrichment at the Ago2-2 coding region shown in G, relative to normalized levels in H (i.e. normalized G/H). Data represent mean ± S.E. of three independent ChIP experiments. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.
coding regions. Thus, methylated histones appear to be the major mediator for prolonged transcriptional silencing. Moreover, we identified an inverse correlation between Pol II and H3K27Me2 enrichment at silenced loci indicating an associated impairment in transcription initiation and elongation. The Pol II enrichment at the promoter regions was determined only in the absence of the trigger-silencing plasmid indicating that low level transcription is maintained to ensure ongoing sRNA production. The repressive H3K27Me2 epigenetic mark characterizes the molecular interplay between the amebic RNAi pathway and heterochromatin-mediated transcriptional gene silencing in a deep-branching eukaryote.

In *E. histolytica* G3 strain it was previously noted that RNAi-silenced loci had increased histone H3 occupancy (33, 37). However, these studies could not address identification of specific repressive histone marks as the reagents for the study were not available. It has been reported in other biological systems that enrichment of methylated H3K27 associates with gene repression (14, 43). Using newly generated reagents, we have identified that *E. histolytica* also contains this repressive epigenetic mark, which deposits at RNAi-silenced loci. Identification of a repressive H3K27Me2 suggests the presence of a functional epigenetic machinery in *E. histolytica*, which reinforces the identification of potential histone methyltransferases, histone

**FIGURE 8.** Both the episomal and chromosomal RdRP1 loci are resistant to trigger silencing and are not enriched with repressive epigenetic marks. A, schematic diagram of constructs designed to silence RdRP1 (19T-RdRP1) or overexpress it (Myc-RdRP1). Both constructs are built from the same vector backbone and are under control of the same promoter and regulatory elements; **, episomal region; PCR, amplified regions of RdRP1 shown in B. 19T-RdRP1 cells do not have antisense sRNAs. B, RT-PCR analysis of RNAs collected from 19T-RdRP1 and Myc-RdRP1 *E. histolytica* trophozoites grown for 48 h. Up-regulation of the RdRP1 transcript shown with both primer sets designed specifically to anneal to cDNA synthesized from the construct-generated RdRP1 transcript (i.e. episomal RdRP1) or cDNA strands were made from episomal and chromosomal RdRP1 transcripts (total RdRP1). DNA template, genomic DNA or plasmid DNA (for episomal RdRP1; only PCR product of Myc-RdRP1 is shown). Expression levels of the EHI_199600 gene serve as a reference control. Data represent analysis of three independent biological replicates. C-E, ChIP-PCR analyses of 19T-RdRP1 and Myc-RdRP1 cells represent deposition levels of H3K27, H3K27Me2, or EhAgo2–2. Shown is the ratio of RPL21 normalized levels of the indicated enriched DNA of 19T-RdRP1 cells were calculated relative to that of Myc-RdRP1. Diagrams depict different amplified regions; 19T/Myc, plasmids of 19T-RdRP1 or Myc-RdRP1. Results of ChIP-PCR analysis showing the absence of any protein enrichment. Data represent three independent ChIP experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Dimethylated H3K27 Is a Repressive Histone Code in E. histolytica

modifying enzymes, DNA methylation components, and chromodomain containing proteins in the Entamoeba genome (55). Future efforts to characterize the RNA-induced transcriptional silencing complex will identify the molecular mediators of interactions between the Argonaute protein and histone modifying enzymes.

The ability of E. histolytica trophozoites to maintain silencing after trigger removal is a good opportunity to dissect the determinants for prolonged silencing. Our data indicate that the chromosomal ROM1 locus has different patterns of histone methylation in the presence or absence of the trigger-silencing construct. In 19T-ROM1 cells, where the silencing construct is present, we detected enrichment of 10-fold of H3K27Me2 at chromosomal regions containing a portion of the ROM1 endogenous promoter. However, after plasmid removal despite active silencing, the same chromosomal region had only 3-fold H3K27Me2 enrichment. Very interestingly, the opposite scenario was observed at ROM1 coding regions. The H3K27Me2 abundance at coding regions was increased 4-fold when the plasmid was present, compared with a 10-fold increase after plasmid removal. This observation indicates an inverse correlation between transcriptional activity and H3K27Me2 deposits, which is in agreement with the observation that trigger silencing is transcription dependent (35). In the absence of the silencing construct, there is a need for transcription of the gene locus as a source to produce small RNAs; thus, some accessibility of the promoter may be required to facilitate small RNA production. Our conclusion is supported by the results of Pol II ChIP experiments in which reduced levels of Pol II are in agreement with increased H3K27Me2 deposition. However, Pol II seems to access the promoter region easily in the absence of trigger plasmid, which may be due to a requirement for limited transcription to continuously generate sRNAs. In S. pombe, plants, and human, RNAi interplays with repressive H3K9Me-mediated heterochromatin assembly in a transcription-dependent manner (56–58). This differential pattern of H3K27Me2 enrichment demonstrates that amebic cells similarly modulate RNAi-mediated gene silencing. Heterochromatin spreading into neighboring regions has been described in several cases including yeast and plant (59–61, 72). Likely, the E. histolytica deposition of the H3K27Me2 mark in the promoter region and in the gene body might be an indication for heterochromatin spreading status.

The interplay between Argonaute proteins and heterochromatin-induced factors has been studied in multiple systems. In D. melanogaster, Argonaute proteins are implicated in heterochromatin-mediated silencing via a role in targeting and recruitment of histone modifying enzymes and chromodomain proteins (28, 62), and in S. pombe Ago1 deposits at genomic loci where other RNAi components enrich to contribute to heterochromatin silencing (63). Additionally, disruption of RNAi affects both establishment and maintenance of heterochromatin at centromeres (64). Human Argonaute proteins, AGO1 and AGO2, mediate TGS via interactions with lysine methyltransferases specific for methylation of H3K9 and H3K27 (65, 66). Our investigation of trigger-induced silenced genes demonstrated enrichment of Ago2-2 only at the silenced chromosomal ROM1 locus in the presence of the silencing construct. Despite silencing, the episomal plasmids lack Ago2-2 enrichment suggesting that targeting Ago2-2 is transient or highly specific.

Why the Ago2-2 silencing construct could not silence the chromosomal Ago2-2 locus despite generation of functional small RNAs is not known. One simple possibility is that Ago2-2 has an essential role and thus its genomic locus is protected from epigenetic modifications. Inhibition of euchromatin conversion to heterochromatin has been described in S. pombe and Arabidopsis thaliana, where it is mediated via Jumonji C domain-containing proteins (67, 68). Another possibility is that Ago2-2 small RNAs do not reach the Ago2-2 chromosomal locus due to a nonfunctional Ago2-2-sRNA complex. For example, the complex may fail to properly position thus failing to
facilitate recruitment of repressive chromatin factors. In cultured mammalian cells, a single-base difference in the positioning of a synthetic small RNA can activate or inhibit transcription (69). Alternatively, the Ago2-2 small RNAs may not load well into the silencing complex. Interestingly, Northern blot analysis in the 19T-Ago2-2 cells showed that the majority of Ago2-2 small RNAs appear to be of a larger size compared with small RNAs generated to ROM1 (36). The functional meaning of this small RNA size discrepancy needs further investigation. Given that each Argonaute protein has very specific requirements for small RNA association, it is possible that the larger small RNAs generated against Ago2-2 do not load productively into Ago2-2 and thus cannot mediate gene silencing (70). In Drosophila, it has been demonstrated that modifications of the 3′ terminus of siRNAs and miRNAs are coupled to efficient assembly into Ago2-RNA-induced silencing complex (71). Future studies to understand the role of Ago2-2 (and RdRP1) in the RNAi pathway as well the requirements for their activity may explain the higher degree of resistance of these genes to gene silencing.

Our efforts are the first to shed light on the molecular mechanism of RNAi-regulated TGS in E. histolytica. We introduce strong evidence for H3K27Me2 as a repressive histone mark in E. histolytica and point to a crucial role for heterochromatin assembly in RNAi-mediated TGS in ameba. Identification of factors that mediate the RNA-induced transcriptional silencing complex is of a high priority for future efforts.

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