Identification of a Functional Network of Human Epigenetic Silencing Factors*

Received for publication, September 9, 2009, and in revised form, October 29, 2009. Published, JBC Papers in Press, October 30, 2009, DOI 10.1074/jbc.M109.064667

Andrey Poleshko 1, Margret B. Einaron, Natalia Shalginskikh, Rugang Zhang, Peter D. Adams 2, Anna Marie Skalka, and Richard A. Katz 3

From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Epigenetic silencing is mediated by families of factors that place, remove, read, and transmit repressive histone and DNA methylation marks on chromatin. How the roles for these functionally diverse factors are specified and integrated is the subject of intense study. To address these questions, HeLa cells harboring epigenetically silent green fluorescent protein reporter genes were interrogated with a small interference RNA library targeting 200 predicted epigenetic regulators, including potential activators, silencers, chromatin remodelers, and ancillary factors. Using this approach, individual, or combinatorial requirements for specific epigenetic silencing factors could be detected by measuring green fluorescent protein reactivation after small interference RNA-based factor knockdown. In our analyses, we identified a specific subset of 15 epigenetic factors that are candidates for participation in a functional epigenetic silencing network in human cells. These factors include histone deacetylase 1, de novo DNA methyltransferase 3A, components of the polycomb PRC1 complex (RING1 and HPH2), and the histone lysine methyltransferases KMT1E and KMT5C. Roles were also detected for two TRIM protein family members, the cohesin component Rad21, and the histone chaperone CHAF1A (CAF-1 p150). Remarkably, combinatorial knockdown of factors was not required for reactivation, indicating little functional redundancy. Consistent with this interpretation, knockdown of either KMT1E or CHAF1A resulted in a loss of multiple histone-repressive marks and concomitant gain of activation marks on the promoter during reactivation. These results reveal how functionally diverse factors may cooperate to maintain gene silencing during normal development or in disease. Furthermore, the findings suggest an avenue for discovery of new targets for epigenetic therapies.

Epigenetic processes control the binary on-off states of specific gene sets, thereby creating heritable transcription patterns that drive development and maintain cellular identity. It is now well established that chromatin-based mechanisms underlie such epigenetic control, largely mediated by intricate chemical marks that are placed or removed by chromatin-modifying enzymes (1–8).

The prominent epigenetic regulatory marks on eukaryotic chromatin are histone modifications and DNA cytosine methylation (5meCpG), which are placed by enzyme complexes containing members of the histone modifying and DNA methyltransferase (DNMT) 4 families, respectively. The histone tails that protrude from nucleosomes are thus decorated by a variety of position-specific “histone code” marks, including acetyl, methyl, and ubiquitin lysine modifications. In contrast, DNA-based epigenetic regulation is limited to DNA methylation. Histone marks may be either activating or repressive, whereas the 5meCpG DNA mark is strictly repressive. The presence or absence of these chromatin marks provide cues for recruitment of downstream protein effectors that positively or negatively affect transcription or may also directly influence chromatin structure and function.

The heritable transcriotional off-state is denoted “epigenetic silencing.” The corresponding silent regions are generally characterized by hypoacetylated histones, histone H3 lysine 9 (H3K9) di- or trimethylation (H3K9me2/3), and DNA hypermethylation. These marks guide the formation of heterochromatic-like features over gene promoters or broader areas (2). Histone deacetylases (HDACs) are generally viewed as repressive epigenetic regulators that maintain the hypoacetylated histone state, thereby antagonizing the transcription-promoting activities of lysine acetyltransferases (KATs). The repressive H3K9 methyl histone mark is placed by lysine methyltransferases (KMTs) and is read by the effector heterochromatin protein 1 (2). Repressive methyl marks can potentially be antagonized by lysine demethylase (KDM) activities (8). The 5meCpG DNA marks, placed by DNMT enzymes, are read by methyl-CpG-binding domain proteins (MBDs) to promote silencing (3).

4 The abbreviations used are: DNMT, DNA methyltransferase; DNMTi, DNMT inhibitor; GFP, green fluorescent protein; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorting; LTR, long terminal repeat; CMV, cytomegalovirus; HDAC, histone deacetylase; HDACi, HDAC inhibitor; MBD, methyl-CpG-binding protein; KAT, lysine acetyltransferase; KMT, lysine methyltransferase; KDM, lysine demethylase; KMT, lysine methyltransferase; PcG, polycomb group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; TIF1, transcriptional intermediary factor 1; TSA, trichostatin A; 5-azaC, 5-azacytidine; TRIM, tripartite motif; CAF-1, chromatin assembly factor-1; PRC1, polycomb repressor complex; EF1, elongation factor 1α; H3K9, histone H3 lysine 9; pol, polymerase; TSS, transcriptional start site.
Both HDAC- and DNMT-based epigenetic silencing can sometimes be reversed by chemical inhibitors (9–15). HDAC inhibitors (HDACi) act by favoring KAT-mediated activating acetylation marks, whereas DNMT inhibitors (DNMTi) cause a passive loss of the repressive DNA methylation marks during cell division.

Deregulation of epigenetic silencing can lead to inappropriate shut-off of specific genes, a process that underlies a variety of human diseases, including cancer (10–13, 15, 16). In addition, silencing of viral genomes by epigenetic mechanisms can contribute to pathogenesis by promoting a latent viral state (17). In both cases, reversal of epigenetic silencing by inhibitors (e.g. HDACi) may provide therapeutic benefits (9–15). There is substantial interest in identifying functional networks of epigenetic silencing factors, because such knowledge may provide additional therapeutic targets. However, the processes surrounding enzymatic placement and removal, as well as decoding, of epigenetic marks are highly complex. For example, a variety of combinatorial, temporal, dynamic, and context-dependent histone modifications have been described (2–5, 7, 18–21). In view of these vast complexities, we have implemented a functional assay to identify silencing factor repertoires. This strategy has uncovered an epigenetic network in human cells and provides a general method for the identification of factors that may serve as targets for epigenetic therapies.

**EXPERIMENTAL PROCEDURES**

**Reporter Cells**—The primary GFP-silent reporter cells used in the screen comprises a HeLa cell population that harbors silent GFP-reporter genes under control of the CMV promoter (previously denoted TI-C) (22). Clones derived from this population were described previously (22, 23). In the secondary HDAC-silent reporter cell population, the GFP gene is under control of the weaker retroviral LTR promoter (previously denoted TI-L) (22). A third reporter population harbors the silent GFP under control of the EF1α cellular promoter.

Selection of 5-azaC-responsive reporter cells (denoted AI) was similar to that described previously for TSA-responsive GFP-silent reporter cells (denoted TI) (22). Briefly, HeLa cells were infected with an avian retroviral vector encoding the GFP reporter gene under control of the CMV promoter, and the population of GFP-negative cells was sorted at 7–10 days post-infection. These GFP-negative cells were treated with 5 μM 5-AzaC (Sigma-Aldrich) for 24 h to reveal HeLa cells harboring silent GFP genes, and the GFP-expressing cells were sorted after 48 h. After passage for 10 days in the absence of 5-azaC, there was a significant loss of GFP expression by re-silencing, and the resiling GFP-negative cells were purified by cell sorting. Treatment of these cells with 5-azaC resulted in reactivation of GFP, as expected (data not shown).

**siRNA Screening**—Screening was performed with an siRNA set encompassing the human epigenetics family (Qiagen) (Fig. 1B). This set comprises siRNAs that target 189 genes and was complemented with 11 additional siRNA targets (supplemental Table S1). Two independent siRNAs for each target were provided with the library. For follow-up validation analyses, a minimum of four, and up to seven independent siRNAs were analyzed (Fig. 2 and supplemental Figs. S1 and S2).

siRNA screening was performed using the “wet reverse transfection protocol” that was thoroughly optimized for use with HeLa cells in a 96-well plate format. Robotic pipetting was carried out using the CyBi Well Vario (CyBio). A solution containing siRNAs and DharmaFECT 1 (Dharmacon) transfection reagent diluted in Hanks’ balanced salt solution were first dispensed into 96-well plates, followed by addition of cells. The final concentration of siRNA was 50 nM, and the final transfection volume was 100 μl, with a cell number of 5000 per well. Plates were incubated at 37 °C in 5% CO₂ for 48 h, after which time the transfection medium was replaced by Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. After a total of 96 h post-transfection, cells were scored for GFP expression using a 96-well EasyCyte Plus FACS (Millipore-Guava Technologies). GFP(+) cells were measured using Guava EasyCyte Plus flow analyzer software (CytoSoft, Millipore-Guava). The data were scored as percent GFP-positive cells, and FACS profiles were analyzed for GFP mean fluorescence intensity. A mean fluorescence intensity cutoff was not used as a strict criterion, but most validated hits demonstrated intense GFP expression (supplemental Fig. S3). The positive and negative controls for the screen were an HDAC1 siRNA SMARTpool (Dharmacon) and a single siRNA targeting GAPDH, respectively, based on our previous study (23).

**Efficiency and Reproducibility of High Throughput Assay**—To evaluate the efficiency and reproducibility of the screening protocol, the Z’-factor was calculated (24). The Z’-factor was measured in three independent experiments using the primary reporter cell population. For this measurement, 96-well plates were used containing 48 positive control and 48 negative control siRNA-treated wells targeting HDAC1 and GAPDH, respectively. The Z’-factor was calculated as \((1 - (3 \times (s_p + s_s)/(\mu_p - \mu_s)))\), with the \(s_p\) and \(s_s\) being the standard deviation of positive and negative values and \(\mu_p\) and \(\mu_s\) the mean of positive and negative values. The average Z’-factor was 0.79, and the average \(r\) value for duplicate plates was 0.98.

**Data Analysis, Hit Validations with Secondary Assays, and Statistical Analysis**—We employed a low stringency approach for analysis of the initial screen results to overcome potentially false negative responses produced by any individual siRNA. Higher stringency was invoked during the validation steps. For the initial screen, a target was scored positive if two independent siRNAs caused an appearance of higher than 15% GFP-positive cells in the primary GFP-silent population, or 7.5% in the secondary reporter population. The different cutoff criteria reflect the generally weaker GFP response produced from the LTR promoter versus the CMV promoter. A target was also scored positive if only one independent siRNA fulfilled the criteria in both reporter cell populations.

Twenty-eight targets passed the initial criteria and were chosen for validation using a total of four independent siRNAs, which included a fresh set of the initial two siRNA replicates, and two additional independent siRNA replicates. The validation analyses included both the primary and secondary reporter cells. Three independent experiments were carried out, each in duplicate (supplemental Table S1). Raw values (percent GFP-positive cells) for each single siRNA were averaged, and the standard deviation was calculated. The -fold difference relative
Human Silencing Factor Network

to the negative control and the significance of change was analyzed with a two-tail t test. The Bonferroni correction was performed to adjust p values for multiple testing. An siRNA was scored as positive if, in the analysis of the primary reporter cell population, the -fold difference was higher than or equal to 10, and the p value was <0.0001. For the secondary reporter cell population with the weaker LTR promoter, an siRNA was scored positive if the -fold difference was higher than or equal to 3 and p was <0.01. Finally, the target was scored a hit if at least two of four siRNAs fulfilled these criteria (supplemental Fig. S1). We also employed a secondary assay to detect siRNAs that could potentially interfere with GFP expression and thereby produce false negative results (supplemental Fig. S4).

Overall, 15 out of 28 targets were validated with at least 2 independent siRNAs in one or both cell lines. A survey of published work indicated that proteins corresponding to all 15 validated hits are expressed in HeLa cells (supplemental Table S1).

Chromatin Immunoprecipitation—ChIP was performed using the EZ-Magna ChIP kit (Millipore Corp.) according to the manufacturer’s protocol. For immunoprecipitation procedures, antibodies against H3K9me3 (ab8898) and H4K20me3 (ab9053) from Abcam, and anti-acetyl histone H4 (06–866) from Upstate, were used. Quantification was performed using real-time PCR, with sets of primers for the GAPDH promoter region (sense, 5'-TACGGTGGGAGGTCTATATAAGCA; antisense, 5'-TCGAAACAGGAGGAGCAGAGAGCGA), the GFP CMV promoter (sense, 5'-TCGAACAGGAGGA-TACGGTGGGAGGTCTATATAAGCA; antisense, 5'-AAATCCCC-GTGAAGTCAAACC), and the GFP transcription start site (sense, 5'-TACGGTGGGAGGTCTATATAAGCA; antisense, 5'-CTGGCCCTTGCTACACCATT).

RESULTS

Rationale and Design of the siRNA Screen—Epigenetic drugs such as HDACi and DNMTi relieve epigenetic silencing by inhibiting or depleting enzymes that modify chromatin. siRNA-based knockdown of key silencing regulators would be expected to phenocopy such effects. We described previously a human reporter cell system that can detect functional roles for inhibiting or depleting enzymes that modify chromatin, and the activities of KDM4A and KDM2A may be expected to phenocopy such effects. We described previously a human reporter cell system that can detect functional roles for inhibiting or depleting enzymes that modify chromatin, and the activities of KDM4A and KDM2A may be expected to phenocopy such effects.

siRNA Screening Reveals a Diverse Factor Set—The initial analysis was performed using a multiwell format. Three different GFP-silent reporter cell populations that employ different promoters to drive the silent GFP gene were used (see “Experimental Procedures”). Two independent siRNAs for each epigenetic target were tested, and each plate included positive and negative control siRNAs, HDAC1 and GAPDH, respectively. Wells were treated with siRNAs targeting individual epigenetic regulators, and the percentage of resulting GFP-positive cells appearing after 96 h was measured by FACS. Transfection with only a small subset of siRNAs in the library produced robust GFP reactivation, i.e. in up to 80% of the cells in the well (Fig. 1C). The analysis detected roles for heterochromatin protein 1γ, and HDAC1 among the class I, II, and IV HDAC family members that are targeted by the siRNA set. As these two factors had been identified in our previous candidate study (23), they were excluded from further analysis. The remaining subset was further evaluated using multiple independent siRNAs, signal to noise criteria, statistical analyses, and testing in independent reporter cell populations (see “Experimental Procedures”). Using several detailed criteria, we validated 15 gene hits (“Experimental Procedures,” Fig. 2, Table 1, supplemental Fig. S1, and supplemental Table S1). Results from analyses of four independent siRNAs per target, with two different reporter cell populations, are shown in Fig. 2. As expected, all of the proteins encoded by the gene hits are present at detectable levels in HeLa cells (supplemental Table S1).

The gene hits include members of diverse functional classes of factors (Table 1). Four are members of the KMT and KDM families, which are predicted to mark chromatin for silencing by placement, or removal of methylation at specific lysines on histones H3 and H4: KMT1E (SETDB1), KMT5C (SUV420H2), KDM2A (FBXL11), and KDM4A (JMJD2A). The siRNA library coverage, and specific hits among the KMTs and KDMs that target the H3 N-terminal tail, are summarized in Fig. 3A. KMT1E (4, 8, 25, 26) is one of several KMTs that place the H3K9m2/3-repressive mark (Fig. 3A). Independent siRNAs targeting KMT1E produced robust GFP reactivation, whereas siRNAs targeting other known H3K9 KMT family members (4, 8, 25) did not score in this screen (Figs. 2, 3A, and 3B). These results suggest that KMT1E activity is required for maintenance of the repressive H3K9me2/3 mark, and that there are no functional redundancies among other H3K9 KMTs that are expressed in HeLa cells.

The reversal of silencing with siRNAs that target KDM2A, KDM4A, and KMT5C (Table 1 and Fig. 2) suggests that a specific constellation of methylation states of histones H3 and H4 is required for silencing maintenance in this system. The specificities and precise functions of these three enzymes remain to be fully elucidated, but our findings support their role in maintenance of a repressive chromatin state. The H3K36me2/3 modifications delineate active chromatin, and the activities of KDM4A and KDM2A may promote silencing by removal of these marks (25, 27). KMT5C places the repressive H4K20me2/3 methylation
mark, which is known to block acetylation of histone H4 (28). As indicated from the results shown in Fig. 2 and Table 1, the screen also identified roles for Polycomb-repressive complex 1 (PRC1) group proteins RING1 and HPH2. These proteins have been identified as components of the human silencing maintenance complex (hPRC1L) that places a repressive monoubiquitin mark on histone H2A at lysine 119 (29) (Table 1). Consistent with a role for these factors, we found that treatment with the proteasome inhibitor MG132 resulted in a depletion of monoubiquitinated histone H2A (30) and a concomitant reactivation of the GFP reporter gene (data not shown).

FIGURE 1. High throughput functional screen to detect epigenetic silencing factors. In A: Top, the siRNA library (Epigenetic family, Qiagen Corp.) was transferred to a sample plate along with sextuplicate or octuplicate control siRNAs targeting HDAC1 or GAPDH, as described previously (23). siRNA transfection of GFP-silent reporter cells is described under “Experimental Procedures.” Middle: stylized depiction of assay indicating typical responses measured with positive (green) and negative (black) controls, and test (yellow) siRNAs. Bottom: typical GFP FACS fluorescence profiles of GFP reporter cells after transfection with GAPDH and HDAC1 control siRNAs. Gating for GFP-negative cells (purple, gate 1) or GFP-positive cells (green, gate 2) is shown. B, pie chart describing the composition of the 200 gene siRNA library (Epigenetic family). Subfamilies are indicated: RITS, RNA-induced initiation of transcriptional silencing; HDACs, histone deacetylases; CHD, chromodomain/helicase/DNA-binding domain; HMG, high mobility group proteins; Swi/Snf2, switch/sucrose nonfermentable; ISWI, imitation SWI; Dnmt, DNA methyltransferase; Kmt, histone lysine methyltransferase; Kat, histone lysine acetyltransferase; PcG, polycomb group; Kdm, histone lysine demethylase; and Mbd, methyl-CpG-binding protein. A complete target list is provided in supplemental Table S1. C, representative results of initial screen. See “Experimental Procedures” for details of siRNA transfection and analysis. Results are scored as percentage of GFP-positive cells as measured by 96-well Millipore-Guava EasyCyte FACS analysis. Triplicate wells containing positive (HDAC1, black bars) and negative (GAPDH, white) controls are shown (at the far left). Results shown are duplicate samples, and error bars are indicated. Criteria for hit validation with independent siRNAs, and detailed statistical analyses, are presented in supplemental Fig. S1 and Table S1.
Evidence for functional cross-talk between the histone modification and DNA methylation machineries was also revealed. DNA methyltransferase 3A (DNMT3A) has been considered a de novo DNMT. However, this enzyme localizes to certain silent promoters (31), suggesting a role in the maintenance of silencing. As shown in Fig. 3C, siRNA knockdown of DNMT3A resulted in reactivation of GFP, whereas no significant activities were observed with siRNAs that target other DNMT family members. Treatment with DNMT inhibitors also caused GFP reactivation in these reporter cells (data not shown). Furthermore, the apparent requirements for methyl-binding domain proteins MBD1 and MBD3 to maintain silencing also support a role for the DNA methylation machinery (Fig. 2 and Table 1). Roles for non-enzymatic factors were also detected, including the histone chaperone CHAF1A (CAF-1 p150), two TRIM protein family members, and the cohesin component Rad21 (discussed below).

Analysis of Reporter Cell Clones Indicates That Silencing Factor Roles Are Largely Position-independent—The reporter cells used in this screen were derived as a population harboring chromosomally dispersed, silent GFP genes. siRNA-induced GFP reactivation in a large percentage of cells suggests that the potential roles for the identified factors are largely independent of the chromosomal location of the silent GFP genes. This interpretation predicts that the same set of factors (Table 1) would function in individual cell clones, in which the GFP-silent loci are at the same unique chromosomal position in all cells. Fig. 4 shows that four independent cell clones respond to siRNAs targeting six different factors. However, we note that differences in the degree of response indicate the potential for position-specific effects.

Isolation and siRNA Knockdown Analyses of Cells Harboring GFP Silent Genes Responsive to 5-azaC—The experiments described above utilized reporter cells in which the silent GFP gene was selected for HDACi responsiveness (denoted T). siRNA screening of this cell population also identified a role for DNMT3A, indicating a contribution of the DNA methylation machinery to silencing. To further address this finding, we devised a reciprocal selection strategy whereby we isolated reporter cells in which the silent GFP gene was responsive to the DNMTi, 5-azaC (denoted A) (see “Experimental Procedures”). This DNMTi-responsive GFP-silent cell population
was then challenged with a subset of siRNAs corresponding to the targets identified in the screen of HDACi-responsive reporter cells. Remarkably, knockdown of each siRNA target tested, including HDAC1, resulted in reactivation of the silent GFP gene (Fig. 5 and supplemental Fig. S5). These findings indicate that the identified factor set does not reflect the reporter cell selection procedure. Furthermore, the results support a role for functional cross-talk between the histone modifying and DNA methylation machineries.

**Analysis of Histone Modifications at the Silent Promoter**

The siRNA-based knockdown approach identified a candidate factor network, based on their apparent essential roles in silencing maintenance. To investigate the mechanisms of factor cooperativity, and the apparent lack of functional redundancy, we initiated studies to detect alterations in the chromatin status of the reporter gene after knockdown of factors. We began by determining if repressive histone modification signatures corresponding to the activities of the identified histone-modifying enzymes, KMT1E and KMT5C (as well as HDAC1), were associated with chromatin at the silent GFP reporter gene promoter. Using ChIP analysis, we characterized both the histone modification status and engagement of the transcriptional apparatus at the GFP gene promoter in GFP-silent HeLa reporter cell populations (Fig. 6A). As a control, we analyzed the resident GADPH gene promoter, which is transcriptionally active in HeLa cells. The results showed very low levels of RNA pol II occupancy at the silent GFP promoter and transcriptional start site (TSS), as compared with the active GADPH promoter.

Histone H3 and H4 N-terminal tail acetylation (H3ac/H4ac) was also reduced at the GFP promoter/TSS as compared with the GADPH promoter, a pattern characteristic of epigenetic silencing. The hypoacetylation pattern at the GFP promoter/TSS, as compared with the GADPH promoter. These marks correspond with the predicted enzymatic activities of KMT1E and KMT5C, respectively. The ChIP results, therefore, strongly support the functional roles detected for these enzymes in the siRNA screen.

**Knockdown of Functionally Diverse Factors Results in Coordinated Loss of Repressive Histone Marks and Appearance of Activation Marks**

To determine if the factors that we identified in the screen act through the maintenance of repressive marks, we performed ChIP analysis after siRNA knockdown of candidate factors. We carried out detailed analyses on two factors with diverse predicted functions, KMT1E and CHAF1A (CAF-1 p150) (Fig. 6B). As described, KMT1E is a histone-modifying enzyme, while CHAF1A is a histone chaperone. As in Fig. 6A, we measured the levels of two major repressive histone marks, H3K9me3/H4K20me3, two histone activation mark sets, H3ac/H4ac, as well as RNA pol II occupancy. After KMT1E or CHAF1A siRNA knockdown, RNA pol II occupancy increased dramatically at the GFP promoter and TSS regions. These results established that siRNA knockdown of either KMT1E or CHAF1A resulted in the recruitment of the transcriptional apparatus to the silent reporter locus, concomitant with GFP reporter protein accumulation. Treatment with KMT1E siRNA resulted in a decrease in the repressive H3K9me3 mark at the promoter/TSS, and a reciprocal increase in the H3ac and H4ac activation marks. These results confirm the predicted role for KMT1E in maintaining the H3K9me3-repressive mark in this system. Surprisingly, KMT1E knockdown also resulted in a reduction in the level of the H4K20me3 mark. Furthermore, CHAF1A knockdown caused a reduction in the levels of both the H3K9me3 and H4K20me3 marks, as well as an increase in the H3ac/H4ac marks. As knockdown of either KMT1E or CHAF1A resulted in a similar resetting of histone mark profiles, our overall results demonstrate interdependent, but non-redundant roles for these factors in maintaining both epigenetic silencing and the repressive marks.
In parallel with the siRNA knockdown experiments, we also measured changes in the histone modification profile after HDACi treatment. TSA caused the expected increases in levels of the H3ac/H4ac activating modifications and occupancy by RNA pol II. However, TSA treatment also caused the reciprocal loss of the H3K9me- and H4K20me-repressive marks. Thus, either knockdown of individual, functionally diverse factors, or HDAC inhibition appear to be sufficient to provoke broad changes in the epigenetic marks, including appearance of activation marks, loss of repressive marks and engagement of the transcriptional apparatus. Overall, these results indicate that, in this system, silencing maintenance requires a high level of factor cooperation, but there is little or no detectable functional redundancy.

Evidence for an S-phase-specific Role of CHAF1A (CAF-1 p150) in Epigenetic Silencing—The chromatin assembly factor 1 (CAF-1) is a three subunit complex (p150, p60, and p48) that mediates nucleosome assembly during DNA replication or DNA repair.
The CHAF1A gene encodes the p150 subunit, which binds H3.1ac/H4ac for assembly during DNA replication. The results described above show that depletion of CHAF1A results in the loss of repressive histone marks and reactivation of the silent reporter. Cell cycle analyses indicated that knockdown of CHAF1A did not cause S-phase arrest, although there was a small increase in the fraction of cells in S-phase (data not shown). Because CAF-1 has several functions, we asked if the identified role for CHAF1A in silencing required passage through S-phase. Cycling and S-phase-arrested reporter cells were treated with CHAF1A siRNA for 24 h, and GFP reactivation was measured after another 48 h. For comparison, we also treated cycling and arrested cells with several other siRNAs that promote GFP reactivation (HDAC1, KMT1E, MBD3, and TRIM33) (Fig. 6C). As expected from previous experiments (22), S-phase arrest did not suppress reactivation by HDCA1 siRNA. In fact, reactivation was enhanced, as was also seen after treatment with KMT1E, MBD3, and TRIM33 siRNAs. In contrast,
S-phase arrest significantly suppressed the ability of CHAF1A siRNA to reactivate the GFP reporter gene. This effect is consistent with a specific role for CHAF1A in maintaining silencing during S-phase.

**Promoter-specific Roles for Silencing Factors**—Epigenetic silencing can be mediated by promoter-specific corepressor complexes. In our analyses, we utilized GFP-silent reporter cells in which the GFP gene is under control of several different promoters (Table 1) (23). Factor roles appeared to be largely promoter-independent; indeed, some apparent differences may be due to reduced sensitivity when a weaker promoter is used (Table 1). To further compare the findings obtained with the viral CMV and LTR promoter-driven reporter genes, we also interrogated cells harboring the silent GFP gene under control of the cellular EF1α promoter, using the 15-member siRNA hit set (Table 1). As shown in Fig. 7, the pattern of reactivation in the EF1α reporter cells was indicative of a factor network similar to that identified with the other reporter cells. However, transfection with siRNAs targeting four of the factors failed to cause GFP reactivation when we employed statistical criteria similar to that used with the LTR-based reporter cells. Notably, KMT1E siRNA failed to reactivate GFP efficiently, suggesting that a different H3K9 KMT may play a role.

**DISCUSSION**

The mechanisms that underlie epigenetic control include DNA methylation and histone modification. Recent work has revealed that the placement and decoding of these modifications is a more intricate process than once believed, characterized by intra- and inter-histone modification cross-talk (32, 33), histone-DNA methylation cross-talk (32, 33), and dynamic histone modifications during transcription (18, 21). A detailed understanding of these processes is important, because deregulation of epigenetic modifications can cause disease. In particular, inappropriate epigenetic silencing may lead to various disease states, and the reversal of silencing appears to be an avenue for therapeutic intervention (9–15). Epigenetic drugs, such as HDACi and DNMTi, relieve epigenetic silencing by inhibiting (or depleting) enzymes that modify chromatin; however, current epigenetic therapy targets are limited to these two enzyme families. Our previous study provided a proof-of-concept for knockdown of essential silencing factors would result in reactivation of reporter genes. In this system, the GFP-silent reporter serves as a beacon to monitor the effects of global depletion of epigenetic regulators and enables a one-step, quantifiable assay. The results were validated at several levels, including analyses of up to four to seven independent siRNAs per target gene (supplemental Figs. S1 and S2). The readout was not binary, because the GFP signal produced during reactivation showed characteristic target-specific trends. For example, individual KMT1E and CHAF1A siRNAs consistently produced more robust GFP signals, as compared with DNMT3A siRNAs. These target-specific differences may reflect each factor’s particular function, abundance, or half-life after mRNA knockdown.

We considered whether experimental parameters might contribute to the factor set that we have identified. First, the silent GFP reporter genes in the cell population used in our study are widely dispersed (32, 33), presumably neutralizing position-specific factor roles. Indeed, analyses of cell populations harboring dispersed reporter genes, or individual cell clones harboring reporter genes at a limited number of sites (Fig. 4), indicate that the factor set is highly independent of the chromosomal position of the reporter gene. One interpretation of these results is that the set represents a functional repertoire of factors that predominates in HeLa cells. Our analyses included reporter cells that utilize different promoters to drive the silent GFP gene, and the factor hits were largely promoter-independent (Table 1); as such, the factor set does not appear to represent a promoter-specific transcriptional corepressor complex. However, some differences were noted, the most significant of which appears to be a reduced role for KMT1E for silencing of the EF1α promoter-driven GFP gene. Another feature of the reporter system that might contribute to factor roles was the selection of GFP-silent cells by transient treatment with an HDACi (TSA) or a DNMTi (5-azaC). These selection strategies provided a strong technical advantage, as a means to enrich for a cell population in which all cells harbored silent reporter genes. However, selection for either HDAC- or DNMT-mediated silencing revealed a similar factor set by siRNA screening. We conclude, overall, that the factor set that we have identified is promoter and position independent, and is not biased by the reporter cell selection proce-
dure. In fact, the similar factor sets identified using DNMTi and HDACi selection supports the idea of significant cross-talk between the DNA methylation and histone modification machineries (32, 33).

Our experimental strategy proved to be robust, because siRNA analyses of the 200 candidates yielded a validated set of 15 hits (Table 1 and Fig. 8). The hits included members of diverse protein families. To obtain an overview of the known relationships among the hits, we used STRING (available online), a data base of protein interactions. This analysis detected a core network of factor interactions that include CHAF1A, MBD1, MBD3, KMT1E, HDAC1, and DNMT3A, as well as others. In supplemental Fig. S6 we provide a depiction of these interactions. A general model for roles for these and other factors is shown in Fig. 8. The potential functional roles of factors, and their interactions, are discussed below.

Two factors identified in the screen, KMT1E (SETDB1) and KMT5C (SUV420H2), are enzymes that are predicted to place repressive histone methyl modifications, H3K9me3 and H4K20me3, respectively. H3K9 methylation recruits heterochromatin protein 1 to mediate silencing, while the H4K20 methylation may block an activating modification, acetylation of histone H4 (28). These marks were detected on the GFP-silent promoter by ChIP analysis (Fig. 6, A and B). We interpret these results to indicate that the activities of these enzymes are required to maintain these repressive marks on the GFP-silent promoter and, accordingly, the silent state of the GFP gene.

We also detected requirements for PRC1 proteins, RING1 and a partner HPH2, consistent with their predicted function in placement of the repressive monoubiquitin mark on histone H2A (H2Aub, Fig. 8) (29). Taken together with the findings described above, a constellation of repressive marks on histones H3, H4, and H2A appears to be required to maintain silencing (Fig. 8). Roles were also detected for two histone demethylase enzymes KDM2A and KDM4A that may promote silencing by removal of H3K36me2/3 modifications that are associated with the bodies of transcriptionally active genes (25, 27).

Numerous examples of bidirectional cross-talk between the DNA methylation and histone modification machineries have been described (32, 33), and such interactions are believed to reflect a self-reinforcing silencing mechanism (11). Here, we derived GFP-silent reporter cells that were selected by response to an HDACi. However, siRNA analyses revealed roles for KMT1E, DNMT3A, and repressive MBD proteins (MBD1 and MBD3) among other factors (Fig. 8). siRNA analyses of GFP-silent reporter cells that were derived by reciprocal selection with the DNMT inhibitor 5-azaC also revealed roles for DNMT3A and KMT1E, as well as several other factors common to the HDACi selection strategy (Fig. 5). These results provide strong support for cross-talk between the DNA methylation and histone modification machineries in maintaining silencing in this system.

The siRNA library targeted nine histone chaperones and chromatin assembly factors, including CHAF1A. Knockdown of CHAF1A, but not the two other CAF-1 subunits (p60 and p48), nor other chaperones, resulted in reporter gene reactivation. Knockdown of CHAF1A also provoked (i) a coordinated loss of the repressive H3K9me3 and H4K20me3 histone marks, (ii) an increase in histone acetyl activating marks, and (iii) an increase RNA pol II occupancy (Fig. 6B). Furthermore, reactivation by CHAF1A siRNA was inhibited in S-phase-arrested cells (Fig. 6C). In contrast, such cell cycle arrest slightly
enhanced GFP reactivation by siRNAs targeting several other factors. Taken together, our results favor a model whereby CHAF1A plays a role in maintenance of epigenetic silencing through S-phase. This role is seemingly not limited to the maintenance of the H3K9me3 mark, as we also observed loss of the H4K20me3 mark. Interestingly, mouse embryonic cells that are deficient or null for CHAF1A show a reduction of both H3K9me3 and H4K20me3 marks in pericentric heterochromatin (34). Here we found that CHAF1A plays a major role in maintenance of these marks in non-embryonic human cells. Importantly, our results demonstrate a correlation between CHAF1A function, histone marks, and maintenance of gene silencing through S-phase. The discrimination of such a role illustrates the ability of this system to detect highly diverse, but coordinated, functions of silencing factors. Indeed, three factors that we identified, MBD1, CHAF1A, and KMT1E, have been implicated in collaborating during S-phase to transmit the H3K9me mark to replicated chromatin, as guided by DNA methylation (Fig. 8) (35).

Based on our current findings, as well as previous studies (23, 36), we believe that the silent GFP transgenes described here may provide a nucleation site for heterochromatin formation, which in turn leads to reporter gene silencing. In particular, the identification of coordinated H3K9 and H4K20 methylation marks, and their apparent maintenance by CHAF1A, supports this notion. We have also detected widespread DNA methylation within and around the GFP transgene (data not shown), consistent with heterochromatin formation. The cues that initiate these processes are currently unknown. However, our experimental system has proven to be powerful for identification of factors that are required to sustain the silent state.

Of the remaining factors, TRIM24 (TIF1α) and TRIM33 (TIF1γ) are interacting members of the TIF1 family of transcriptional regulators that can function as repressors (37), and RAD21 is a member of the cohesin family that has been implicated in transcriptional control (38). Two factors, ZMYND8 (RACK7), a putative transcriptional regulator, and PBRM1 (BAF180), a component of the human SWI/SNF chromatin-remodeling complex PBAF, have not yet been directly implicated in epigenetic silencing. Our findings have revealed that each identified factor can be singularly targeted for depletion to promote reactivation of a silent gene. These results indicate a surprising lack of redundancy within, or between factor families. The apparent high level of interdependence among the functioning set may be explained, in part, by the fact that the presence of each factor is required to maintain one or more physical complexes. In this regard, it has been demonstrated that chromatin-modifying enzymes, in addition to their catalytic activities, can play a scaffolding role. For example, it has been shown that the HMT G9a (KMT1C) can contribute to DNA methylation in an activity-independent manner (39).

In summary, we have identified a set, or network, of specific factors that maintains epigenetic silencing in human cells. The composition of the set may reflect the particular functional repertoire of epigenetic silencing factors that are available in these cells. The experimental approach described herein may be extended to other human cell types, offering the possibility of identifying additional cell or disease type-specific factors. Because knockdown of any member of the set relieves silencing, such factors may serve as targets for the development of novel epigenetic therapies.

Acknowledgments—We benefited from the use of the following Fox Chase Cancer Center (FCCC) Facilities: Flow Cytometry and Cell Sorting, Translational Research, and Biostatistics and Bioinformatics. We thank Dr. Yan Zhou from the FCCC Biostatistics and Bioinformatics Facility for assistance. We are also extremely grateful to Dr. Andrew Kossenkov and Olga Tchuvatkina for providing critical advice related to statistical analyses. We also thank Drs. Tim Yen, Alfonso Bellacosa, Jeff Peterson, and Severin Gudima for comments on the manuscript. Lastly, we are grateful to Marie Estes for assistance in preparing the manuscript and Karen Trush for help with artwork.

REFERENCES
1. Bernstein, B. E., Meissner, A., and Lander, E. S. (2007) Cell 128, 669–681
2. Grewal, S. I., and Jia, S. (2007) Nat. Rev. Genet. 8, 35–46
3. Klose, R. J., and Bird, A. P. (2006) Trends Biochem. Sci. 31, 89–97
4. Kouzarides, T. (2007) Cell 128, 693–705
5. Martin, C., and Zhang, Y. (2007) Curr. Opin. Cell Biol. 19, 266–272
6. Strahl, B. D., and Allis, C. D. (2000) Nature 403, 41–45
7. Taverna, S. D., Li, H., Ruthenburg, A. J., Allis, C. D., and Patel, D. J. (2007) Nat. Struct. Mol. Biol. 14, 1025–1040
8. Shi, Y., and Whetstine, J. R. (2007) Mol. Cell 25, 1–14
9. Bolden, J. E., Peart, M. J., and Johnstone, R. W. (2006) Nat. Rev. Drug Discov. 5, 769–784
10. Esteller, M. (2006) Br. J. Cancer 94, 179–183
11. Feinberg, A. P., and Tycko, B. (2004) Nat. Rev. Cancer 4, 143–153
12. Hake, S. B., Xiao, A., and Allis, C. D. (2007) Br. J. Cancer 96, (suppl. R31)–R39
13. Jones, P. A., and Baylin, S. B. (2007) Cell 128, 683–692
14. Marks, P. A., and Breslow, R. (2007) Nat. Biotechnol. 25, 84–90
15. Yoo, C. B., and Jones, P. A. (2006) Nat. Rev. Drug Discov. 5, 37–50
16. Feinberg, A. P. (2007) Nature 447, 433–440
17. Lieberman, P. M. (2006) Trends Microbiol. 14, 132–140
18. Berger, S. L. (2007) Nature 447, 407–412
19. Ruthenburg, A. J., Allis, C. D., and Wysocka, J. (2007) Mol. Cell 25, 15–30
20. Ruthenburg, A. J., Li, H., Patel, D. J., and Allis, C. D. (2007) Nat. Rev. Mol. Cell Biol. 8, 983–994
21. Li, B., Carey, M., and Workman, J. L. (2007) Cell 128, 708–719
22. Katz, R. A., Jack-Scott, E., Narezkina, A., Palagin, I., Boimel, P., Kulkosky, J., Nicolas, E., Greger, J. G., and Skalka, A. M. (2007) J. Virol. 81, 2592–2604
23. Poleshko, A., Palagin, I., Zhang, R., Boimel, P., Castagna, C., Adams, P. D., Skalka, A. M., and Katz, R. A. (2008) J. Virol. 82, 2313–2323
24. Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999) J. Biomol. Screen 4, 67–73
25. Allis, C. D., Berger, S. L., Cote, J., Dent, S., Jenuwien, T., Kouzarides, T., Pillus, L., Reinberg, D., Shi, Y., Shiekhattar, R., Shilatifard, A., Workman, J., and Zhang, Y. (2007) Cell 131, 633–636
26. Schultz, D. C., Ayyanathan, K., Negorev, D., Maul, G. G., and Rauscher, F., J. (2002) Genes Dev. 16, 919–932
27. Frescas, D., Guardavaccaro, D., Kuchay, S. M., Kato, H., Poleshko, A., Basur, V., Elenitoba-Johnson, K. S., Katz, R. A., and Papano, M. (2008) Cell Cycle 7, 3539–3547
28. Latham, J. A., and Dent, S. Y. (2007) Nat. Struct. Mol. Biol. 14, 1017–1024
29. Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R. S., and Zhang, Y. (2004) Nature 431, 873–878
30. Groothuis, T. A., Dantuma, N. P., Neeffs, J., and Salomons, F. A. (2006) Cell Div. 1, 21
31. Li, H., Rauch, T., Chen, Z. X., Szabo, P. E., Riggins, A. D., and Pfeifer, G. P. (2006) J. Biol. Chem. 281, 19489–19500
32. Cedar, H., and Bergman, Y. (2009) Nat. Rev. Genet. 10. 295–304
33. Fischle, W. (2008) Genes Dev. 22, 3375–3382
34. Quivy, J. P., Gérard, A., Cook, A. J., Roche, D., and Almouzni, G. (2008) *Nat. Struct. Mol. Biol.* 15, 972–979
35. Sarraf, S. A., and Stancheva, I. (2004) *Mol. Cell* 15, 595–605
36. Narezkina, A., Taganov, K. D., Litwin, S., Stoyanova, R., Hayashi, J., Seeger, C., Skalka, A. M., and Katz, R. A. (2004) *J. Virol.* 78, 11656–11663
37. Peng, H., Feldman, I., and Rauscher, F. J., 3rd (2002) *J. Mol. Biol.* 320, 629–644
38. Peters, J. M., Tedeschi, A., and Schmitz, J. (2008) *Genes Dev.* 22, 3089–4114
39. Dong, K. B., Maksakova, I. A., Mohn, F., Leung, D., Appanah, R., Lee, S., Yang, H. W., Lam, L. L., Mager, D. L., Schübeler, D., Tachibana, M., Shin-kai, Y., and Lorincz, M. C. (2008) *EMBO J.* 27, 2691–2701