

Human factors and pathways essential for mediating epigenetic gene silencing

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Abbreviations: GFP, Green Fluorescent Protein; SUMO, small ubiquitin-like modifier; HDAC, histone deacetylase; DNMT, DNA methyltransferase; CMV, Cytomegalovirus; siRNA, small interfering RNA; GO, Gene Ontology; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; HBSS, Hank's Balanced Salt Solution; HAT, Histone acetyltransferase

Cellular identity in both normal and disease processes is determined by programmed epigenetic activation or silencing of specific gene subsets. Here, we have used human cells harboring epigenetically silent GFP-reporter genes to perform a genome-wide siRNA knockdown screen for the identification of cellular factors that are required to maintain epigenetic gene silencing. This unbiased screen interrogated 21,121 genes, and we identified and validated a set of 128 protein factors. This set showed enrichment for functional categories, and protein-protein interactions. Among this set were known epigenetic silencing factors, factors with no previously identified role in epigenetic gene silencing, as well as unstudied factors. The set included non-nuclear factors, for example, components of the integrin-adhesome. A key finding was that the E1 and E2 enzymes of the small ubiquitin-like modifier (SUMO) pathway (SAE1, SAE2/UBA2, UBC9/UBE2L) are essential for maintenance of epigenetic silencing. This work provides the first genome-wide functional view of human factors that mediate epigenetic gene silencing. The screen output identifies novel epigenetic factors, networks, and mechanisms, and provides a set of candidate targets for epigenetic therapy and cellular reprogramming.

Introduction

Epigenetic processes direct heritable changes in gene expression without changes in DNA sequence.¹ “Epigenetic gene silencing” describes the transcriptional shutoff of specific genes during development and cellular differentiation.^{2,3} Such epigenetic regulation can be viewed as a layered process, with external signals initiating the silencing of specific genes.⁴ At the chromatin level, epigenetic control is mediated by proteins that modify DNA and chromatin, and remodel nucleosomes. Specifically, the active or silent gene states are controlled by enzymatic placement and removal of a complex set of chemical modifications on DNA and histones.⁵⁻⁷ The modifications include DNA methylation, and a variety of post-translational histone modifications (acetylation, methylation, phosphorylation, and others), which are placed by members of large families of enzymes. These chromatin modifications are in turn recognized by protein complexes that ultimately control the active or silent transcriptional gene states.⁸ Epigenetic gene silencing can be divided into three processes: initiation of gene silencing, maintenance of the repressive chromatin state, and inheritance through S-phase and mitosis.^{4,9,10} In addition to these processes, recent studies have demonstrated the

functional importance of spatial chromatin organization in epigenetic control.^{11,12}

Although the general models for epigenetic control are well supported, numerous complexities in the placement and reading of epigenetic modifications on nucleosomal histones and DNA have been detected: context dependence, combinatorial effects, temporal patterns with respect to gene activity, rapid dynamics, and histone modification-DNA methylation crosstalk.¹³⁻¹⁶ Such complexities have made it difficult to identify key drivers of epigenetic control. Further, a consequence of these complexities is the potential for misregulation. The epigenetic gene-silent state is of particular interest as errors in placement of epigenetic silencing marks are associated with cancer and other epigenetic diseases.^{17,18} However, a prominent feature of epigenetic control is reversibility. Unlike fixed genetic errors, epigenetic errors such as occur in cancer may therefore be reversible at the chromatin level. This reversibility led to the concept, now in practice, of “epigenetic therapy,” whereby the reactivation of epigenetically silent tumor suppressor genes may restore growth control in cancer cells.¹⁷⁻²¹ Currently, the major focus of epigenetic therapy efforts is to develop and refine inhibitors of two families of epigenetic silencing factors, the histone deacetylases (HDACs) and

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DNA methyltransferases (DNMTs).¹⁷⁻²¹ There is intense interest in identifying additional therapeutic targets for reactivation of epigenetically silent genes. It is therefore critical to identify the entire complement of human factors and networks that mediate epigenetic gene silencing. While much is known about epigenetic marking systems, there have been no studies that provide an unbiased genome-wide functional view of the epigenetic factors and pathways in human cells. Understanding the entire spectrum of epigenetic mechanisms that regulate gene silencing is important with respect not only to cancer therapeutics, but to fundamental mechanisms of cellular differentiation and reprogramming.²²

We have developed and validated a robust high-throughput cell-based GFP reporter system to identify human factors and pathways that maintain epigenetic gene silencing. Here we describe the results of an unbiased genome-wide siRNA screen in which 21,121 human genes were interrogated to reveal roles in epigenetic gene silencing. The set of 128 factors identified in this screen, as described herein, was derived through a combination of biological readout, technical validation, and statistical analyses. The results of this screen demonstrate that siRNA knockdown of numerous individual factors, including known epigenetic repressors, is sufficient for release of epigenetic gene silencing. The factor set showed enrichment for functional categories, known protein-protein interactions, and also included 18 previously uncharacterized factors thereby identifying them as novel epigenetic silencing mediators.

Results

siRNA screen for epigenetic silencing factors and pathways

We previously described a cell-based reporter system that consists of a HeLa cell population harboring epigenetically silenced GFP-reporter genes under control of the CMV promoter.²³ This system is based on the principle that epigenetic gene silencing is reversible, and that a silent reporter gene can be reactivated by inhibition of key epigenetic silencing factors (Fig. 1A). The reporter cell population harbors chromosomally dispersed, epigenetically silent GFP genes, thus favoring detection of multiple classes of silencing mechanisms, rather than idiosyncratic, locus-specific mechanisms. This reporter cell system was used previously to carry out an siRNA knockdown screen with a biased epigenetic factor set (200 genes/factors).²³ The GFP signal after target gene knockdown was used as a readout, providing a quantitative measure of reporter gene reactivation (Fig. 1A). We showed that it is possible to reactivate silent reporter genes by siRNA-mediated knockdown of epigenetic factors involved directly in gene silencing (e.g., HDACs and DNMTs).²³ In the current study, we use this system to perform an unbiased genome-wide, gene-by-gene siRNA screen. The unbiased nature of the screen provided the potential to detect proximal chromatin silencing factors, upstream intracellular pathways, and novel, unstudied epigenetic factors.

The overall design of the genome-wide screen is outlined in Figure 1B. The Dharmacon siRNA SMARTpool genome-wide library was used, which targets 21,121 human genes. The

primary screen consisted of testing each gene/factor individually with siRNA SMARTpools containing a mix of four individual siRNAs. The details of the genome-wide siRNA screening procedure are outlined in Figure 1C, and are described in the Methods section. The assay was optimized for siRNA transfection in a 96-well plate format. SiRNAs targeting GAPDH and HDAC1 were used as negative and positive controls, respectively. The primary screen was performed using the complete genome-wide siRNA library. The reporter cells were transfected with siRNAs, and 96 h post-transfection, the level of GFP reporter gene reactivation was quantitated using a plate reader. Results of the primary genome-wide screen are shown as distribution of the ratio of GFP fluorescence relative to the negative control (Fig. 1C). A distinct set of 470 factors with high signal-to-noise ratio were selected for validation (see Methods section for details).

Validation testing of the 470 primary hits was performed using an siRNA library of deconvoluted pools of four single siRNAs per target. Reporter cells were transfected with individual siRNAs and reactivation of GFP reporter genes was measured by quantification of the percentage of GFP-positive cells using flow cytometry. Shown are flow cytometry profiles of GFP signal intensities, and gates, of cells transfected with siRNAs targeting the negative and positive controls, GAPDH and HDAC1, respectively (Fig. 1C). A gene/factor was considered validated if at least two individual siRNAs produced GFP reactivation according to the parameters outlined in the Methods section. A pie chart indicates that 128 genes from the 470 primary hit set were validated with two or more individual siRNAs (Fig. 1C). The complete distribution of the number of individual siRNAs from each pool of four that promoted reactivation is shown in Figure S1. The overall validation rate for each primary hit fits well with previous studies that used the same siRNA library to study other biological processes.²⁴ Thus, results of the validation step indicate that the overall screen output is of the expected quality for this siRNA library. Table S1 provides comprehensive data from both the primary and validation screens, and Table S2 provides a list of the 128 validated genes with descriptions.

Data analysis and factor hit representation

The set of 128 validated factors were organized according to the Gene Ontology (GO) database, for subcellular localization (Fig. 1D) and significantly enriched functional categories (Fig. 1E). Interestingly, factors in this set showed a broad spectrum of localization within the cell, indicating layers of epigenetic control.⁴ However, the validated targets were significantly enriched for factors localizing to the nucleus (2.2-fold more than expected by chance alone, $P = 0.0002$), and such factors are implicated as having a direct role in epigenetic gene silencing. Simple inspection of the gene list revealed that among these nuclear factors were those previously known to play a role in epigenetic gene silencing, including HDAC1, SETDB1/KMT1E, ATF7IP/MCAF1, and CHAF1A. Furthermore, several of these factors were detected in our previous biased screen²³ (see Discussion). Therefore, the screen output appeared to validate our unbiased approach, as both proximal and upstream factors were detected. We next set out to systematically analyze the screen output.

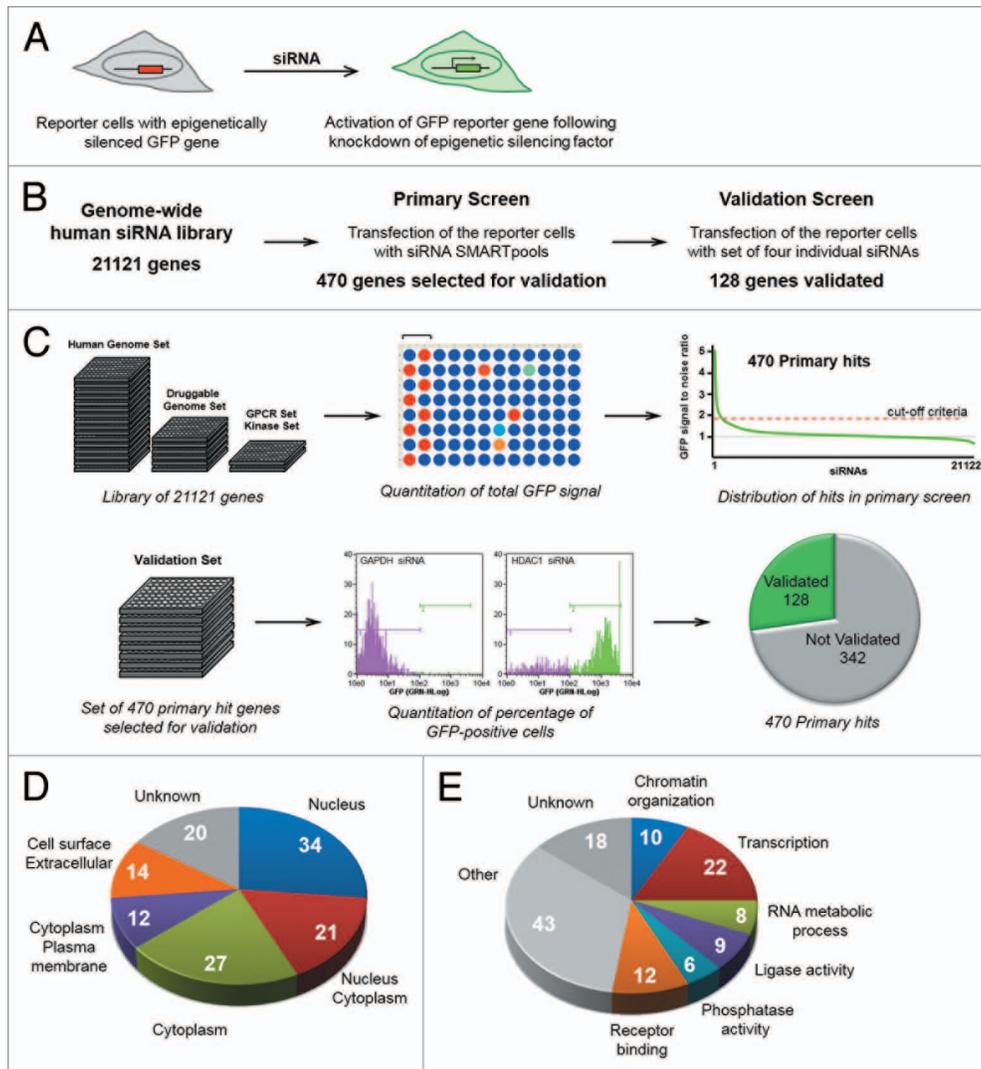


Figure 1. A Genome-wide siRNA screen identifies genes involved in epigenetic gene silencing. (A) A GFP reporter-based system for identification of cellular factors involved in epigenetic gene silencing. (B) Outline and summary of the two-step genome-wide siRNA screen. (C) A schematic view of the primary screen (top) and the validation screen (bottom). Bracket over wells indicates placement of alternating positive and negative controls. (D, E) Gene Ontology (GO) analysis of factors identified in the screen: subcellular localization (D) and pathway analysis (E). In Panel E, the colored slices indicate the 67 factors enriched in GO categories, as represented in Figure 2.

We tested the list of validated targets for statistical enrichment of GO categories and pathways, using the initial 21,121 screened genes/factors as a reference (Table S3). A total of 67 of the 128 factors were members of 6 enriched GO functional categories: “chromatin organization,” “transcription,” “RNA metabolic process,” “ligase activity,” “phosphatase activity,” and “receptor binding” (Fig. 1E). The detailed enrichment analysis for the 128 hits is summarized in Table S3. The 67 members of the enriched categories are illustrated in Figure 2, highlighting subcellular localization and refined annotated functions. In addition to organization based on enriched categories, we performed an unbiased analysis of the 128 factor set using EMBL String,²⁵ a database of known protein interactions. The set was found to be enriched for protein-protein interactions compared with a genome background ($P = 0.0001$). The network display map is provided in Figure S4, and will be referred to below to

highlight specific features of the 128 factor set. In general, the output shows a number of interactions, and features a “core” factor set that includes histone modifiers and factors involved in transcription.

Detection of the SUMO pathway as a mediator of epigenetic gene silencing

The screen revealed a significant role for the small ubiquitin-like modifier (SUMO) pathway in maintaining epigenetic silencing (Fig. 3; Fig. S4). The SUMO pathway includes an E1-E2-E3 enzymatic cascade similar to the ubiquitin pathway, as well as de-sumoylating enzymes.²⁶ Hundreds of cellular proteins are subject to SUMO modifications, which result in altered activities or localization.²⁷ The screen detected three key sumoylation pathway factors: two comprising the E1 enzyme complex (SAE1 and SAE2/UBA2) and the sole human E2 enzyme, UBC9/UBE2I (Fig. 3A). With respect to the representation of SUMO pathway

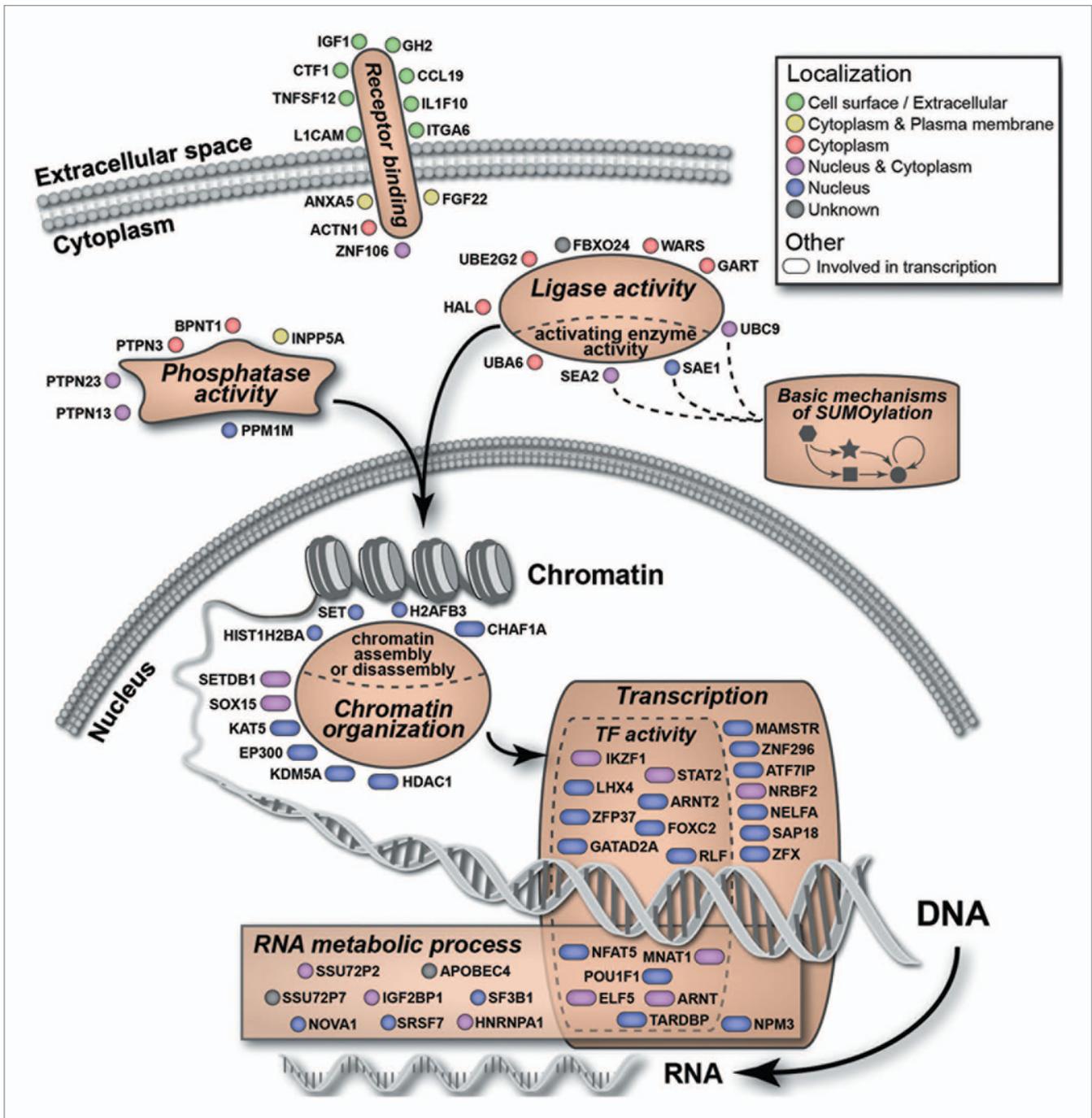


Figure 2. A summary of factors detected in the screen along with their membership in enriched GO categories. See Figure S2 for additional details.

factors within the entire library, the detection of SAE1, SAE2/UBA2, and UBC9/UBE2I represented a greater than 30-fold enrichment (Table S3). Overall, the siRNA pool deconvolution step performed during validation generally revealed a broad distribution in the number of positive siRNAs among the validated pools (i.e., from 2 to 4 siRNAs). Strikingly, all four siRNAs from each of the SAE1, SAE2/UBA2, and UBC9/UBE2I pools were positive (Fig. 3B), providing high confidence in the obtained results. As the E1 and E2 enzymes act in cascade, the identified

roles of both upstream (SAE1, SAE2/UBA2) and downstream (UBC9/UBE2I) factors provide compelling evidence for an essential role of this pathway in epigenetic gene silencing.

Temporal analysis of reactivation after factor knockdown

The roles of factors identified by siRNA knockdown may be either direct, i.e., on chromatin, or through indirect mechanisms, such as upstream triggers of nuclear events. A rapid release of GFP gene silencing after siRNA knockdown could indicate a direct role in the maintenance of epigenetic silencing for the

tested factor. Although the biological effect produced by siRNA knockdown is dependent largely on the half-life of the protein after the target mRNA is destroyed, a rapid effect is more readily interpretable. That is, a delay in reactivation could reflect either an indirect role for the factor, or simply the prolonged presence of the protein after mRNA knockdown. In contrast, very rapid reactivation would suggest both efficient depletion of the protein, as well as a more direct role for the factor, i.e., not involving a cascade of events.

To identify factors that might be acting directly to maintain epigenetic silencing, we performed analyses for a set of 20 factors to measure the rapidity of the response by monitoring GFP reactivation profiles (Fig. S3). Shown in Figure 3C are representative GFP reactivation profiles produced after knockdown of selected factors. The factor knockdown profiles extracted from Figure S3 (UBC9, HDAC1, FOXC2, CHAF1A, SETDB1) were chosen to represent members of different functional protein families (SUMO pathway, histone deacetylase, transcription factors, chromatin remodeling factors and histone methyltransferase, respectively). The knockdown of UBC9/UBE2I resulted in the most rapid reactivation of the GFP reporter gene among the 20 factors tested, with a maximum reached at 56 h post-siRNA addition (Fig. 3C; Fig. S3). In contrast, treatment with HDAC1 siRNA, targeting a known “direct” chromatin silencing factor, showed a GFP maximum at 72 h. Therefore, the very rapid reactivation of GFP genes after UBC9/UBE2I knockdown suggests a direct role for this protein in epigenetic gene silencing (see Discussion). Regardless of the difficulties in interpreting these results at the mechanistic level (as outlined above), these temporal analyses provide important relative measurements of the potency of factor knockdown.

Reactivation of epigenetically silent cellular genes

We next investigated whether factors identified in the GFP reactivation screen function in epigenetic silencing of cellular genes. Eight developmental and tumor suppressor genes that are epigenetically repressed in HeLa cells were tested for reactivation after knockdown of a functionally diverse group of 10 factors from the 128 factor set (Fig. 4). HeLa GFP reporter cells were transfected with the indicated siRNAs and, at 72 h posttransfection, mRNA levels were analyzed by qRT-PCR. Figure 4A shows a heat map of genes that displayed an increase in mRNA expression, with the reactivation criteria being 2-fold or greater (with a p-value < 0.05) compared with the siRNA negative control. GFP mRNA reactivation was monitored as a positive control. Knockdown of CHAF1A resulted in reactivation of all eight cellular genes tested (Fig. 4B). CHAF1A functions in the inheritance of repressive chromatin marks through S-phase,²⁸ and was detected in our earlier biased siRNA screen using the same reporter cells.²³ Of the ten factors tested, only SOX15 knockdown failed to reactivate any of the cellular genes. Knockdown of other factors resulted in reactivation of at least four genes, but in each case, a unique set was reactivated. These results suggest that the screen identified epigenetic silencing factors that act through distinct mechanisms. Notably, UBC9/UBE2I knockdown resulted in reactivation of only several genes, implying that the role of the SUMO pathway in epigenetic silencing is not universal (Fig. 4B).

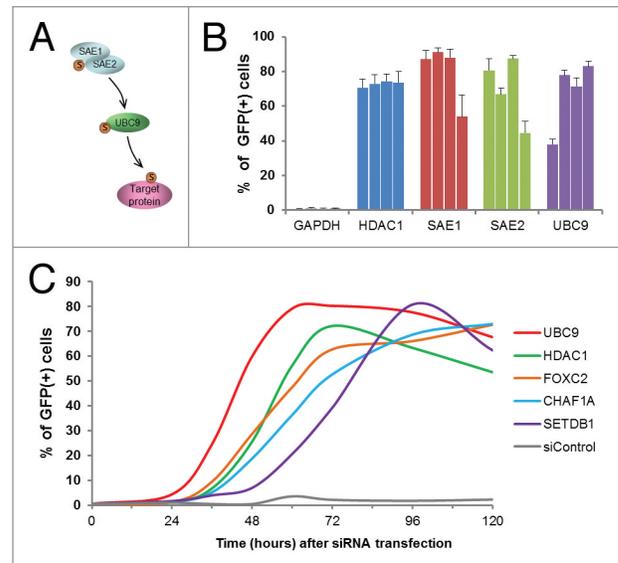


Figure 3. Detection of role for SUMO pathway components in epigenetic gene silencing. (A) A schematic representation of the SUMO modification pathway. (B) Reactivation of GFP reporter genes after knockdown of the indicated SUMO pathway components. The reporter cells were transfected with the indicated siRNAs targeting the SUMO pathway (four individual siRNAs per tested gene), and the percent of GFP-positive cells was measured 96 h post transfection. siRNA pools targeting GAPDH and HDAC1 were used as quadruplicate negative and positive controls, respectively. Error bars indicate standard deviation, n = 6. (C) A time course of GFP reporter gene reactivation after siRNA knockdown of indicated genes. siControl, negative control siRNA.

Discussion

Here we describe the results of a functional screen to identify human factors that maintain the epigenetically silent gene state. Our approach was built upon studies from our lab and others, which showed that epigenetically silent reporter genes could be reactivated by inhibitors of HDACs and DNMTs.^{23,29,30} These findings led to the development of a system in which silent GFP reporter genes could be reactivated in response to siRNA-based depletion of single factors. This system was then used to identify specific epigenetic silencing factors. Our initial biased siRNA screen targeting 200 candidates, identified a core set of 15 chromatin-based epigenetic silencing factors, each of which was critical to maintain the silent state.²³ Here, we describe the results of an unbiased, genome-wide siRNA screen, which revealed novel epigenetic silencing factors and pathways. Using the Dharmacon siGENOME library, we identified 128 factors (Fig. 2; Figs. S2 and S4) that included epigenetic silencing factors identified in our earlier biased 200-factor screen (CHAF1A, HDAC1, SETDB1/KMT1E, and others).

The essence of gene-by-gene siRNA screens is that knockdown of a single factor produces the tested phenotype. In the case of epigenetic silencing factors, this is of critical importance, as such single factors could thereby serve as potential targets for epigenetic therapy or cellular reprogramming. It is likely that our screen did not detect many factors that contribute to epigenetic silencing due to redundancies in their functions or the need for

UBC9/UBE2I.³⁹ Furthermore, sumoylation has been shown to increase HDAC1 activity.³⁹ Our previous work,²³ and results described here, demonstrate that HDAC1 is a key factor in epigenetic silencing in our system. Sumoylation of HDAC1 may therefore contribute to epigenetic silencing, and knockdown of factors participating in the sumoylation pathway (i.e., SAE1, SAE2/UBA2, UBC9/UBE2I) would result in loss of epigenetic silencing. To further test this idea, we used the LLO peptide of the bacterium *Listeria monocytogenes*, which has been reported to inhibit the sumoylation pathway by inducing degradation of UBC9/UBE2I.⁴⁰ Treatment of the GFP reporter cells with the LLO peptide resulted in robust reactivation of the GFP reporter (data not shown). Thus, interruption of the SUMO modification pathway by siRNA knockdown of enzymes, or by microbial protein-induced degradation, both lead to reactivation.

Methyl-binding domain protein 1 (MBD1) sumoylation has also been implicated in epigenetic silencing through promoting interactions with the SETDB1/KMT1E cofactor ATF7IP/MCAF1. Both MBD1 and ATF7IP/MCAF1 were identified as epigenetic repressors previously,^{41,42} as well as in the current study. Interruption of sumoylation has been shown to cause the dissociation of ATF7IP/MCAF1 and heterochromatin protein 1 (HP1) from MBD1-containing foci, along with the loss of the H3K9me3 heterochromatin mark.⁴³ Lastly, it has been reported that histone H4 is a direct target for sumoylation by UBC9/UBE2I, and this modification results in a repressive chromatin state.^{44,45} The rapid reactivation of the silent GFP gene after UBC9/UBE2I knockdown (Fig. 3C) implicates a relatively direct role of the SUMO pathway in silencing in this system.

Of the 128 factor set, eight (ARNT, C20orf112, EP300, IKZF1, LHX4, POU1F1, SEPT9, SET) are encoded by genes that have been associated with recurrent chromosomal aberrations in cancer according to the Mitelman Database (Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (2014), Mitelman F, Johansson B and Mertens F (eds.), <http://cgap.nci.nih.gov/Chromosomes/Mitelman>). The enrichment for this class of genes is 1.9-fold ($P = 0.08$) among the hits, and detailed descriptions of the eight genes are provided in Table S2. Of note, the *C20orf112* gene encodes a 436 amino acid protein with no known function. A fusion between the *C20orf112* and *RUNX1* genes has been identified in acute myeloid leukemia (AML), and a *PAX5-C20orf112* gene fusion was detected in acute lymphoblastic leukemia (ALL). IKZF1 is a widely expressed transcriptional regulator, interacts with HDAC1, and functions in lymphoid development.⁴⁶ The *IKZF1* gene promoter is translocated to the *BCL6* locus in B-cell non-Hodgkin lymphoma, and the *IKZF1* gene is deleted in other lymphoid diseases.⁴⁷ The *SEPT9* gene encodes a member of the septin gene family of filamentous structural proteins.⁴⁸ The *SEPT9* gene is involved in a translocation with the *MLL* gene in myelodysplastic syndrome (MDS).⁴⁹ Lastly, the screen identified SET, a known epigenetic silencing factor that appears to function by blocking acetylation of histones.⁵⁰ A SET-NUP214 translocation has been identified in both AML and ALL.⁵¹ Whether the putative epigenetic silencing roles for these eight factors are related to the described gene aberrations remains to be investigated.

The unbiased nature of the siRNA screen led to the detection of non-nuclear factors that are candidates for having indirect roles in epigenetic gene silencing. In particular, factors that participate in the integrin-adhesome complex were detected (Fig. 2; Fig. S4). Transmembrane integrin molecules on the cell surface attach to the extracellular matrix (ECM), and the integrin-adhesion complexes mediate associations with the actin cytoskeleton. One function of adhesions is to sense mechanical stimuli in the cellular environment and translate these stimuli into a broad range of responses, including changes in cell shape or cellular identity. These external stimuli may affect gene expression via physical connectivity between the ECM and chromatin, through the cytoskeleton and the nuclear lamina.⁵² Our screen detected five factors that are components of the integrin-adhesome complex (ACTN1, BCAR1, ITGA3, ITGA6, PPM1M),⁵³ representing a 1.93-fold enrichment ($P = 0.19$) (Fig. S4). More strikingly, 2 of the 18 human α integrins, $\alpha3$ and $\alpha6$ (encoded by the *ITGA3* and *ITGA6* genes, respectively) were detected in the screen. These results suggest that loss of integrin signaling can lead to reversal of epigenetic gene silencing in this system.

Implicit in the findings described here is that our reporter system is able to detect a wide range of cellular factors that impact epigenetic gene silencing. Clearly, the protein set includes general factors, rather than those unique for maintaining silencing of the CMV promoter. Indeed, several core cellular mechanisms have been shown previously to contribute to CMV promoter silencing, including histone modification and DNA methylation.⁵⁴ Our initial biased siRNA screen had detected roles for the histone modifying enzyme SETDB1/KMT1E and DNMT3A,²³ and these two factors were also detected in the current screen among the primary hits. As DNMT3A had been detected in the earlier screen,²³ it was not included for validation in the current study. Our experience with this reporter system is that detection of DNA methylation-based silencing is less robust, presumably due to the requirement for passive loss of DNA methylation after knockdown of DNMTs.⁵⁵ The focus of our current study was to use an unbiased approach to extend our understanding beyond the core silencing mechanisms. As such, we have not pursued technical improvements in the reporter system that might enhance the detection of components of the DNA methylation machinery in particular.

This screen also revealed potential roles in epigenetic silencing for previously uncharacterized or poorly characterized factors. We have investigated in detail the function of the factor, PRR14.⁵⁶ It was shown recently that the nuclear lamina plays an important role in maintaining an epigenetically silent heterochromatin compartment at the nuclear periphery.^{57,58} We demonstrated that PRR14 functions to tether heterochromatin to nuclear lamina, thus maintaining the peripheral nuclear heterochromatin compartment. Also, we showed that PRR14 may play a role in mitotic specification of H3K9me3-marked heterochromatin positioning at the nuclear lamina.^{56,59} Interestingly, PRR14 had not been identified as a component of the nuclear lamina or heterochromatin using other techniques, but was revealed in our functional screen for epigenetic silencing factors. To date, 18 factors detected in the screen remain uncharacterized (Table S4). Further study of these

factors could provide new insights into mechanisms of epigenetic gene silencing. For example, detection of the PRR14 nuclear lamina component along with integrin-adhesion factors suggests a functional linkage of the ECM to chromatin, via the nuclear lamina. This potential mechanism remains to be investigated in greater detail.

In summary, the findings described here have provided a broad functional view of human epigenetic silencing factors. The screen identified known, potent epigenetic repressors, as well as other factors not previously connected with epigenetic gene silencing. In addition, a set of uncharacterized factors was detected. We showed that one such factor, PRR14, functions in the maintenance of heterochromatin.⁵⁶ Future work may identify roles in biological processes, such as developmental gene regulation for novel factors discovered here, while other factors might be targetable for epigenetic therapy.

Methods

Reporter cells

The HeLa GFP-reporter cell line used for the screen was described previously.^{23,60} Cells were grown in 10% fetal bovine serum (FBS) Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics.

The high-throughput siRNA-based screen

The gene-by-gene high-throughput screening assay was designed using a robotic pipetting system for siRNA transfection, optimized in a 96-well format, with multiple readout options for measuring GFP expression. The transfection volumes and cell number were chosen according to the multi-well plate format using the manufacturer's recommendations. SiRNA and the DharmaFECT 1 (T-2001) transfection reagent were diluted in Hank's Balanced Salt Solution (HBSS), mixed, and then incubated for 20 min. Robotic pipetting was performed using the CyBi Well Vario (CyBio) liquid handler. A transfection mix containing siRNAs and transfection reagent was first dispensed into 96-well plates, followed by addition of the reporter cell suspension in complete medium without antibiotics (wet reverse transfection method). The final concentration of siRNA was 50 nM in 100 μ l of the transfection solution, with a cell number of 5,000 per well. After 48 h, the transfection medium was replaced with complete growth medium, and cells were incubated for an additional 48 h. After a total of 96 h post-transfection, cells were analyzed for reporter GFP gene reactivation as described below.

Data analysis

For the primary screen, data were analyzed as the ratio of sample signal to averaged negative and positive controls of the tested plate, signal-to-noise and signal-to-positive, respectively. Criterion for a positive candidate hit gene were signal to noise ratio greater than 1.8 ($S/N > 1.8$) and signal to positive ratio greater than 0.8 ($S/P > 0.8$). A total of 470 candidate genes were selected, excluding genes disconnected from the NCBI Reference Sequence (RefSeq) Database. The selected 470 candidate genes

were validated with an additional siRNA library containing four individual siRNAs per target gene (deconvoluted siRNA SMARTpools, Dharmacon). The validation screen data were analyzed as percentages of GFP-positive cells after transfection with single individual siRNAs. Raw values (percent GFP positive cells) for each single siRNA were averaged, and the standard deviation was calculated. A criterion for a positive single siRNA was an amount of GFP-positive cells greater than or equal to 20% after siRNA transfection with a coefficient of variation (%CV) less than 35%. This criterion was an equivalent of a fold-difference of at least 10 ($F.D. > 10$, $P < 0.001$) relative to the negative control. The background level of GFP-positive cells in negative control samples was 0.5–2.0% for the entire screen. A candidate gene was considered as validated if at least 2 out of 4 tested individual siRNAs were scored as positive. A total of 128 candidate genes were validated using these criteria. See **Table S1** for overall screening results.

Bioinformatics analysis of validated hit genes

The list of 128 genes was analyzed for enrichment of Gene Ontology (GO) terms and pathways (from KEGG and BIOCARTA databases) using DAVID software.⁶¹ Only results with a false discovery rate (FDR) $< 20\%$, and enriched at least 1.5-fold, were considered significant. All significant categories are shown in **Table S3** with hand-picked non-redundant categories demonstrated on the composite **Figures 2** and **S2**. Any additional gene annotations were added manually using literature searching and the Genecards (www.genecards.org) database. Annotation with subcellular localization was performed using the Genecards database and GO terms. The expression of the 128 identified hit genes in HeLa cells was checked using mRNA-seq data from the Morin et al. study⁶² and publicly available gene expression data from the study of Zhang et al.⁶³ obtained from GEO database (data set accession GDS3581, sample GSM410912). A gene was called as expressed in HeLa cells if it had coverage of more than three tags from mRNA-seq or more than two background levels from gene expression data. A total of 108 of 128 genes were detected by one of those two platforms (see **Table S2**).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:

www.landesbioscience.com/journals/epigenetics/article/32088

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