Distinct DNA-based epigenetic switches trigger transcriptional activation of silent genes in human dermal fibroblasts

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The influential role of the epigenome in orchestrating genome-wide transcriptional activation instigates the demand for the artificial genetic switches with distinct DNA sequence recognition. Recently, we developed a novel class of epigenetically active small molecules called SAHA-PIPs by conjugating selective DNA binding pyrrole-imidazole polyamides (PIPs) with the histone deacetylase inhibitor SAHA. Screening studies revealed that certain SAHA-PIPs trigger targeted transcriptional activation of pluripotency and germ cell genes in mouse and human fibroblasts, respectively. Through microarray studies and functional analysis, here we demonstrate for the first time the remarkable ability of thirty-two different SAHA-PIPs to trigger the transcriptional activation of exclusive clusters of genes and noncoding RNAs. QRT-PCR validated the microarray data, and some SAHA-PIPs activated therapeutically significant genes like KSR2. Based on the aforementioned results, we propose the potential use of SAHA-PIPs as reagents capable of targeted transcriptional activation.
indicated that PIPs possess better selectivity than the natural DNA-binding proteins in mouse cells. Accordingly, distinct DNA binding PIPs could be directing SAHA to a set of silent genes and activate them.

To clarify this notion, we treated a library of thirty-two SAHA-PIPs to HDFs and evaluated their effect on the genome-wide gene expression. Here, we report the results of such extensive analyses to reveal the remarkable ability of unique SAHA-PIPs to impose unusual transcriptional activation of therapeutically important genes in a human somatic cell. Furthermore, we show that these targeted transcriptional activators could activate a different set of noncoding RNAs and suppress an identical set. RT-PCR studies validated the pattern observed with microarray analysis and some SAHA-PIPs activated the therapeutically important genes including the recently identified KSR2, the obesity gene and SEMA6A, the retinal 'ON' circuit factor. These DNA-based epigenetic switches could be developed to have the ability of modulating the transcription of therapeutically important genes and non-coding RNAs in a precise manner.

**Results**

**Effect of distinct SAHA-PIPs on genome-wide transcriptional activation in human dermal fibroblasts.** Firstly, distinct DNA sequence recognizing thirty-two different SAHA-PIPs (A to α19-21, termed here as 1 to 32) were synthesized and purified (Figure 1a) through Fmoc solid-phase synthesis using an oxime resin followed by conjugation with SAHA. Since SAHA-PIPs have the ability to permeate the nuclear envelope of the live cells without any transfection agents, they were simply treated with the HDFs seeded at 1.5 × 10^5 cells per dish. We chose 1 μM as the working concentration, and 48 h, as the time point to analyze gene expression based on the previous optimization studies. Global level changes in gene transcription were analyzed after the isolation of total RNAs from the effector (SAHA-PIPs 1 to 32, SAHA and DMSO) treated HDFs (Figure 1b). Screening of the number of genes up or down-regulated by more than ten-fold suggested that most of the PIPs dramatically increased the efficiency of SAHA to induce genome-wide transcriptional activation (Figure S1a and Table S1). In HDFs treated with SAHA-PIPs 1–11, 13–15, and 17–28, about 3 to 10 times more genes got up-regulated than that in SAHA treated HDFs (Figure S1a and Table S1). Interestingly, the analysis of the genes down-regulated by ten-fold indicated that the SAHA-PIP 1 to 32 down-regulate almost the same number (45-69) of genes as SAHA (Figure S1b and Table S1). In some SAHA-PIPs (12, 16, 29, 30, 31 and 32) treated HDFs, the number of up-regulated genes were lower than that in SAHA treated HDFs. Although, the reason behind this differential effect is unclear, it could be attributed to the imidazole content, a factor known to hamper the permeability and biological activity of some PIPs. Interestingly, analysis of the number of genes up-regulated by 2-fold suggested that almost the same number of genes got up- or down-regulated in both SAHA-PIP and SAHA treated HDFs (Table S1). Hence, it is reasonable to assume that most SAHA-PIPs trigger dynamic changes and induce transcriptional activation of developmental gene(s), which are usually conserved in HDFs. Microarray studies carried out with biological triplicate of a representative SAHA-PIP 9, DMSO and SAHA supported this notion and obviated the experimental differences. About twice the number of genes got induced in SAHA-PIP 9 treated HDFs than that in SAHA-treated HDFs (Figure 1c). It is important to note here that a similar pattern could also be observed in SAHA-PIP and SAHA treated MEFs.

SAHA-PIPs trigger differential transcriptional activation and undistinguishable transcriptional repression. A heat map of the top-100 up-regulated genes generated by normalizing the data from SAHA and individual SAHA-PIP (1 to 32) treated HDFs over the data obtained from DMSO treated HDFs revealed a remarkable pattern where each SAHA-PIP activated a unique cluster of genes (Figure 1d). The co-clustered genes observed in the data derived from biological triplicate of the representative SAHA-PIP 9 suggested the robustness of SAHA-PIP to activate unique set of genes (Figure 1d, a-c). Also, the clusters of SAHA activated genes were different from, not just one but also most of the thirty-two SAHA-PIPs, which suggested that the PIP could direct SAHA to different DNA sequences (Figure 1d, SAHA-a-c). To our knowledge, this is the first report to demonstrate the capability of a whole library of transcriptional activators to induce a unique set of genes. Among the SAHA-PIP activated genes, but for 5, 6, 7 and 9, only a minimal number of genes were common between each other (Table S2). On the other hand, in the case of the top-100 down-regulated genes, no such unique cluster of genes could be observed in individual SAHA-PIP treated HDFs (Figure S2). Also, the pattern of down regulation in the case of 1 to 32 was completely opposite to that of up regulation as among them, about 70–90% of down-regulated genes were the same (Figure S2 and Table S3). Few genes got down-regulated in 17 to 32 than that in 1 to 16 treated HDFs, and they were not common. This result could be due to the improved recognition of GC rich sequences by 17 to 32 owing to the presence of imidazole in their top arm. Nevertheless, the above-mentioned results clearly indicate that SAHA-PIPs only trigger differential transcriptional activation and not transcriptional repression in human fibroblasts. Although, there were commonly up-regulated genes in SAHA-PIPs 5, 6, 7 and 9 treated HDFs, most of them were developmental genes that co-activate each other. Analysis of the possible matching sites of these SAHA-PIPs may lead to the identification of key sequence(s), which are essential for the unusual unlocking of the usually conserved developmental genes.

**Remarkable ability of SAHA-PIPs and not SAHA to activate therapeutically important gene(s).** Functional analysis was performed using ingenuity pathway analysis (IPA) a web-based functional analysis tool with four-fold as the cut-off value to evaluate the comprehensive effect of SAHA-PIP. Consistent with our expectation, each SAHA-PIP displayed differential and significant (p < 0.005) functional annotations that were unique to themselves but those that are different from SAHA (Table 1). Although it is difficult to achieve targeted activation of singular transcription machinery with these 6 bp recognizing ligands, some SAHA-PIPs activated a distinctive set of genes. For example, SAHA-PIPs 1, 7 and 19 modulated a set of genes associated with glucose metabolism, heart, and ear development, respectively (Figure S3). Also, SAHA-PIPs 2, 13, 17, 18, 24 and 25 activated gene networks associated with hematological system, nervous system, hair and skin, respiratory, sensory system and digestive system, respectively (Figure S4). Since SAHA-PIPs distinctively activated some therapeutically important genes, we chose them as the candidate genes to validate the microarray data using qRT-PCR analysis. In accordance to the functional analysis of microarray data (Figure S3), SAHA-PIP 1 dramatically activated GRPR, a gene associated with insulin secretion and CD24, a surface marker for PDH1-positive pancreatic progenitors (Figure 2a and b). Likewise, SAHA-PIP 2 activated chronic lymphocytic leukaemia associated HLA-DOA and DPYSL5 (Figure 2c and d). Interestingly, SAHA-PIP 7 activated GPC3, a factor associated with cardiac and coronary vascular development and SEMA6A, which recently got identified as a critical gene for retinal development and motion sensing (Figure 2e and f). SAHA-PIP 10 activated PRSS8 and WNK2, a positive regulator of canonical Wnt/β-catenin signalling pathway (Figure 2g and h). SAHA-PIP 13 activated GPRCSB that got recently identified to contribute to neurogenesis (Figure 2i). In the case of second generation SAHA-PIPs, 17 activated PDLIM3, a gene belonging to the network shown in Figure S4c and 18 activated...
LEFTY1 and KSR2, the factors known to be associated with lung development32 (Figure 2j–l). Likewise, 19 activated TSTD1 and SMOC2, a factor known to be associated with hearing impairment33 (Figure 2m and n). Interestingly, ATCAY, a gene known to cause cerebellar ataxia got activated with 23 treatment34 (Figure 2o). Similarly, 24 activated the sensory system associated STT1 and 25 activated digestive system associated MYO7A35 and RBFOX3 (Figure 2p–r). Control studies carried out by treating HDFs with...
Table 1 | Top 6 Functions of SAHA-PIP modulated genes

| SAHA-PIP | Functions Annotation (p-Value) |
|----------|---------------------------------|
| 1        | Proliferation Of BMMC Cells (3.00E-05), Cough (4.94E-05), Proliferation Of Blood-Derived Mast Cells (6.79E-05), Whooping Chemotaxis Of Lymphatic Endothelial Cells (1.09E-04), Isomerization Of Lipid (1.09E-04), Regeneration Of Gastrocnemius (6.85E-06), Uterine Tumor (8.90E-06) |
| 2        | Degeneration Of Cholinergic Neurons (6.89E-06), Priming Of Cells (1.52E-05), Quantity Of Strialt Neurons (1.91E-05), Glucose Metabolism Disorder (3.77E-05), Growth Of Perikaryon (5.01E-05), Reduction Of Cholesterol (5.01E-05) |
| 3        | Uterine Cancer (3.56E-07), Uterine Serous Papillary Cancer (1.34E-06), Endometrial Cancer (2.33E-06), Abnormal Morphology Of Xiphoid Process (6.81E-06), Endometrial Carcinoma (6.85E-06), Uterine Tumor (8.90E-06) |
| 4        | Angina Pectoris (1.04E-04), Offactory Response Of Organism (1.28E-04), Lamellar Ichthyosis (1.62E-04), Bodily Balance (1.95E-04), Efflux Of L-Lactate (8.57E-04), Hyperplasia Of Stroma (8.57E-04) |
| 5        | Metabolism Of 9-Cis-Retinoic Acid (5.45E-06), Cytolsis Of Antigen Presenting Cells (8.56E-06), Cytolsis Of Phagocytes (2.12E-05), Expansion Of Tumor Cell Lines (2.50E-05), Metabolism Of Retinoid (4.40E-05), Cytolsis Of Fibroblast Cell Lines (7.28E-05) |
| 6        | Glucose Metabolism Disorder (9.72E-05), Quantity Of Bilirubin (1.22E-04), Diabetes Mellitus (1.55E-04), Familial Hyperaldosteronism (3.18E-04), Synthesis Of Triacylglycerol (4.26E-04), Lung Tumor (5.04E-04) |

SAHA-PIP Functions Annotation (p-Value)
SAHA-alone did not activate any of these therapeutically important genes (Figure 2 a–r, Bars SAHA). It is important to note here that the fold induction appeared very high with all SAHA-PIPs but not with SAHA treatment. This remarkable induction is attributed to the outstanding difference in the threshold cycle values of the analyzed SAHA treatment. This remarkable induction is attributed to the threshold cycle values of the analyzed SAHA-PIPs but not with SAHA-alone did not activate any of these therapeutically important genes. Nevertheless, it is reasonable to state that SAHA-PIPs trigger transcriptional activation of such key genes open up new vistas of opportunities in therapeutic gene modulation.

**Individual SAHA-PIPs trigger transcriptional activation of distinctive noncoding RNAs in HDFs.** Recent studies reveal that only one fifth of the transcription across the human genome gets associated with protein-coding genes, and a significant amount of the remaining fraction includes non-coding RNAs (ncRNAs), most of whose function remains unknown. The ncRNAs express in a development-specific manner, and they could also induce epigenetic regulation. Many functional revelations get attributed to the ever-increasing volume of newly characterized ncRNAs. For example, a long ncRNA termed 'Brave heart' was shown to activate the core cardiovascular gene network by functioning upstream of MesPI, a master regulator that establishes the cardiovascular lineage during mammalian development. Since transcriptional reorganization of ncRNAs could be linked to some common functional characteristics, we generated a heat map of the top 100 up-regulated ncRNAs. Consistent with the pattern observed with global changes in gene expression, unique clusters of ncRNAs were differentially up-regulated by individual SAHA-PIPs (Figure 3a). Again, a heat map of the top 100 ncRNAs down-regulated by individual SAHA-PIPs did not show such a unique cluster of ncRNAs (Figure S5). QRT-PCR studies again validated the microarray data and four of the uncharacterized noncoding RNAs got activated in HDFs after treatment with SAHA-PIP 9 and not SAHA (Figure 3b–e). SAHA-PIPs activating distinctive ncRNAs could be instrumental in assigning functional roles to uncharacterized segment of the human genome. Cytotoxicity did not influence the gene expression profile obtained with SAHA-PIP treatment as while SAHA alone killed about 50% of the cells, none of the SAHA-PIPs had cytotoxic effect on HDFs at 1 μM working concentration none of the SAHA-PIPs were cytotoxic, which suggests their potential use as therapeutic reagents (Figure S6b).

**Table 1 | Continued**

| SAHA-PIP | Functions Annotation (p-Value) |
|----------|-------------------------------|
| **31**   | Bleeding Of Kidney (5.10E-04), Chronic Large Plaque Psoriasis (1.27E-03), Chronic Small Plaque Psoriasis (1.27E-03), Induction Of Helper T Lymphocytes (1.47E-03), Swelling Of Ear (1.47E-03), Birthweight (1.64E-03) |
| **32**   | Diameter Of Blood Vessel (9.72E-04), Morphology Of Lung (1.16E-03), Alveogenesis Of Lung (2.19E-03), Abnormal Morphology Of Lung (2.26E-03), Antley-Bixler Syndrome Without Genital Anomalies Or Disordered Steroidogenesis (2.33E-03), Beare-Stevenson Cutis Gyral Syndrome (2.33E-03) |
| **SAHA** | Migration Of Tumor Cells (2.27E-08), Colorectal Cancer (5.80E-08), Gastrointestinal Tract Cancer (4.79E-07), Cell Movement Of Tumor Cells (7.70E-07), Cell Movement Of Cancer Cells (6.34E-06), Neoplasia Of Colon (1.75E-05) |

*Each result represents the sum of two individual culture plates. >4 fold up/down-regulated genes were analyzed using IPA as mentioned in methods. Top 6 functions are based on p-value derived from Fischer’s test. Data were analyzed through the use of IPA [Ingenuity® Systems, www.ingenuity.com].

**Methods**

Microarray studies and functional analysis with SAHA-PIPs. As mentioned before, HDFs were treated with 1 μM of SAHA, SAHA-PIPs 1–32 and 0.1% DMSO. After 48 h incubation, total RNA was isolated using RNAeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer’s instructions. The quality of the RNA samples was examined using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The mRNA from total RNA samples was amplified into dsDNA. T7 polymerase was used to generate Cy3 labeled cRNA. The labeled cRNA was purified using RNAeasy Mini kits and concentration was measured using Nanodrop ND1000 v3.5.2 (Thermo Scientific). The cRNA (825 ng) was fragmented and subsequently hybridized to SurePrint G3 Human GE v2 8 x 80K Microarray (Agilent Technologies, USA). The raw data and associated sample information were processed by GeneSpring GX v12.1.0 (Agilent Technologies, USA). For the biological replicate study using SAHA-PIP 9 and SAHA, Whole Human Genome Microarray 4 x 44 v2 (Agilent Technologies, USA) and Human Gene 2.1 ST Array Strip (Affymetrix, USA) were used. The microarray data and complete description of experimental procedure have been submitted to GEO for public access.
Figure 2 | Remarkable ability of SAHA-PIPs and not SAHA to trigger dynamic transcriptional activation of therapeutically important genes. Based on the microarray data, we chose therapeutically important genes distinctively activated by individual SAHA-PIPs. SAHA and DMSO were used as the control. The concentration of the effectors and incubation conditions were as mentioned in methods. QRT-PCR analysis of the expression level of (a) GRPR, (b) CD24, (c) HLA-DOA, (d) DPYSLS5, (e) GPC3, (f) SEMA6A, (g) PRSS8, (h) WNK2, (i) GPRC5B, (j) PDLIM3, (k) LEFTY1, (l) KSR2, (m) TSTD1, (n) SMOC2, (o) ATCAY, (p) SYTL1, (q) MYO7A and (r) RBFOX3. Fold changes relative to non-treated control (DMSO) are presented as induction values. Each bar represents the mean ± SD from 6 wells. Original Ct values are presented in Table S4.
been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE53319 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53319). The interpretation of the microarray data obtained from three individual plates is carried out by cluster 3.0 and Ingenuity pathway analysis (IPA®), which uses the dataset containing the gene identifiers and its respective fold change values. Fischer’s exact test is employed to measure the p-value that determines the association between the genes in the dataset and their functional annotation. The biological networks were generated based on these focus genes. QRT-PCR studies were done after cDNA synthesis using a ReverTra Ace qPCR RT Master Mix with gDNA Remover and amplifications with THUNDERBIRD SYBR qPCR Mix.

Figure 3 | Individual SAHA-PIPs trigger transcriptional activation of distinctive non-coding RNAs (ncRNAs). (a) An unsupervised hierarchical clustering analysis of top 100 ncRNAs in SAHA, SAHA-PIP 1–32 treated fibroblasts suggests that each SAHA-PIP also activate unique cluster of ncRNAs. Each result represents the sum of two individual culture plates. QRT-PCR analysis of the expression level of the uncharacterized gene probes (b) A_21_P0000813, (c) A_21_P0000821, (d) A_21_P0014207 and (e) A_19_P00319154 were carried out as mentioned in Figure 2 with SAHA and DMSO as control. Fold changes relative to non-treated control (DMSO) are presented as induction values. Each bar represents the mean ± SD from 6 wells. Original Ct values are presented in Table S4.
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Author contributions

Experiments were designed by H.S., J.T., T.B., H.N. and G.N.P.J.T., G.N.P., S.I. and S.S. performed research. G.N.P., S.I., H.L., C.A., T.V. and A.S. analysed the data. The manuscript was written by G.N.P., R.D.T. and J.T.

Additional information

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