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A Synthetic DNA-Binding Domain Guides Distinct Chromatin-Modifying Small Molecules to Activate an Identical Gene Network**

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Abstract: *Synthetic dual-function ligands targeting specific DNA sequences and histone-modifying enzymes were applied to achieve regulatory control over multi-gene networks in living cells. Unlike the broad array of targeting small molecules for histone deacetylases (HDACs), few modulators are known for histone acetyltransferases (HATs), which play a central role in transcriptional control. As a novel chemical approach to induce selective HAT-regulated genes, we conjugated a DNA-binding domain (DBD) "I" to N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide (CTB), an artificial HAT activator. In vitro enzyme activity assays and microarray studies were used to demonstrate that distinct functional small molecules could be transformed to have identical bioactivity when conjugated with a targeting DBD. This proof-of-concept synthetic strategy validates the switchable functions of HDACs and HATs in gene regulation and provides a molecular basis for developing versatile bioactive ligands.*

Acetylation of histones is the critical event in transcriptional regulation and is maintained in an equilibrium state by histone acetyltransferases (HATs) and histone deacetylases (HDACs).[1] Small-molecule inhibitors for HDACs can induce global changes in the acetylation profile but in a sequence-independent manner.[2] We previously synthesized a class of sequence-specific small molecules called SAHA-PIPs, comprising the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and selective DNA-binding pyrrole–imidazole polyamides (PIPs). The biological evaluation showed that SAHA-PIPs can induce particular gene networks in mouse and human somatic cells.[3–6] We showed site-specific inhibition of HDACs and subsequent acetylation of histones as the mechanism behind the distinctive gene-activating ability of SAHA–PIPs.[7] Transcriptionally permissive marks are acquired more reliably by activating the epigenetic writers like HATs than by blocking the erasers (HDACs).[8] The CREB-binding protein (CBP)/p300 family are global transcriptional co-activating HATs that act as the master regulators of gene expression and play essential roles in cell-cycle control, differentiation, and apoptosis.[9,10] Several small molecules are known to be acetylation inhibitors.[11] By contrast, only a few HAT activators, such as pentadecylidenemalonate, *N*- (4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide (CTB), Nemorosone, and TTK21 are known.[12]

Considering the essential role of HATs in gene regulation, we chose to confer selectivity to the cell-permeable HAT activator CTB by conjugating it with a PIP. Recently, SAHA–PIP I or SAHA–I (Figure 1) was shown to target *OCT-3/4* and turn on the HDAC1-repressed core pluripotency genes.[7] The HATs p300/CBP-interacting protein are known to be the critical components of the pluripotency gene network.[13] As an innovative approach to induce HAT-regulated genes, we conjugated the predesigned DNA sequences in "I" to CTB to synthesize CTB–PIP I or CTB–I. NH_2-CTB and CTB were synthesized by following previously published procedures (Scheme S1 in the Supporting Information).[10] However, NH_2-CTB was not suitable for the solid-phase synthesis since the coupling monomer required carboxylic acid. For this reason, HO_2C-CTB, which can be synthesized by coupling CTB and adipic acid, was produced to optimize the synthesis of CTB-PIP conjugates (Scheme 1) and their purification (Figure S1 in the Supporting Information). Cytotoxicity studies showed that unlike SAHA, which kills 50% of cells at 1 μM concentration, CTB and CTB–I had no effect on HDF cells even at a concentration of 5 μM (Figure S2).

Biological evaluation of CTB–I was done by using a microarray with appropriate controls (CTB, SAHA, and SAHA–I) under optimized conditions[7] and according to preliminary studies. Remarkably, CTB–I activated a very similar cluster of genes to SAHA–I in HDF cells (Figure 1A; CTB–I and SAHA–I). CTB activated about 12 genes and SAHA activated a different set of 17 genes (Figure 1A and B;

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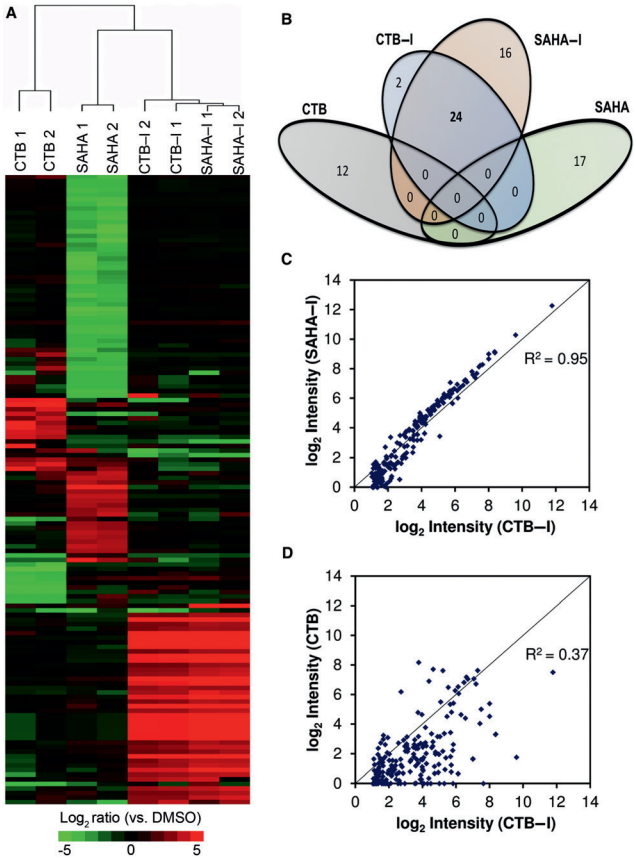
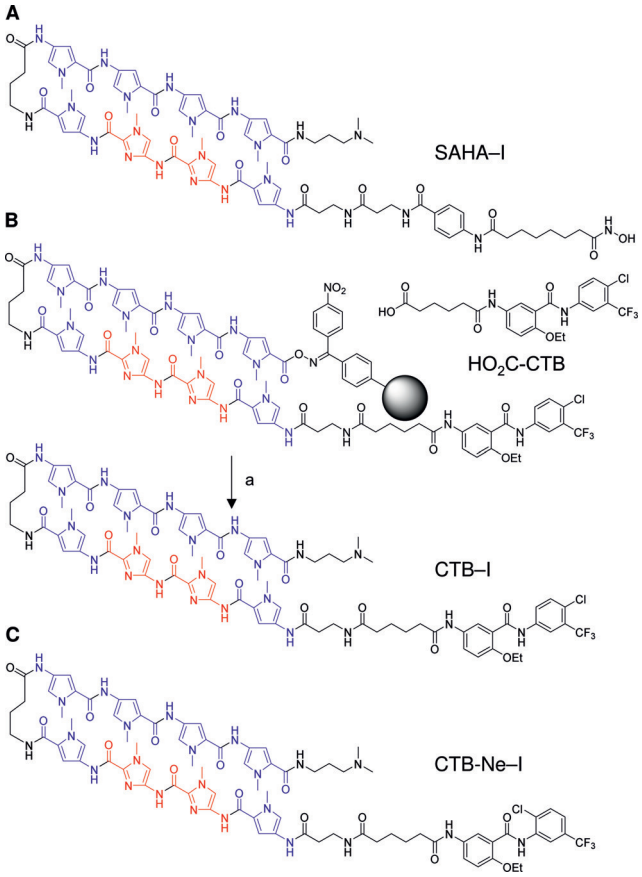


Figure 1. A) A heat map based on unsupervised hierarchical clustering analysis of the expression data derived from four wells for the top 138 genes in DMSO-, CTB-, SAHA–I-, and CTB–I-treated HDF cells (>10-fold change and $p<0.05$). B) A four-circle Venn diagram for the data (>5-fold increase and $p<0.05$) shows the number of genes upregulated in effector-treated HDF cells. C) A scatter plot of the data (>2-fold increase and $p<0.05$) shows a high correlation ($R^2=0.95$) between replicate samples of CTB–I-and SAHA–I-treated HDF cells, which suggests similar targets. D) Conversely, a plot for CTB–I-treated HDF cells vs. CTB-treated HDF cells showed a relatively weak correlation ($R^2=0.37$), which suggests distinct bioactivity.



Scheme 1. A) Structure of SAHA–I. B) Synthesis of CTB–I. General conditions (a): 3-dimethylaminopropylamine (Dp), 45 °C, 3 h. C) Structure of CTB analogue conjugate CTB-Ne–I.

CTB and SAHA). CTB–I and SAHA–I activated about 26 and 40 genes, respectively, including 24 common genes (Figure 1B; CTB–I, and SAHA–I). Interestingly, neither CTB nor SAHA shared any genes with each other or with CTB–I and SAHA–I. The scatter plot of the upregulated transcripts in CTB–I-treated HDF cells (>2-fold; $p<0.05$) produced a stronger linear correlation ($R^2=0.95$) with SAHA–I-treated HDF cells (Figure 1C) than with CTB-treated HDF cells ($R^2=0.37$; Figure 1D).

CTB–I favored transcriptional activation over repression because 48 genes were upregulated and 19 genes were downregulated (Table S1 in the Supporting Information). Conjugation to the PIP thus markedly altered and boosted the bioactivity of both CTB and SAHA by 2-fold and directed them to an identical gene network(s). Analysis of the upstream effectors showed that both SAHA–I and CTB–I activate *OCT-3/4*-regulated pluripotency genes (Figure 2A). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis validated the expression of the genes identified in Figure 2A, and the endogenous expression of *OCT-3/4* was significantly ($p<0.05$) induced by more than 50-fold in both SAHA–I- and CTB–I-treated HDF cells. CTB–I also significantly ($p<0.05$) induced *SOX2*, *NANOG*, *LIN28B*, *EPCAM*, and *SALL4* and markedly upregulated *ZIC3* and *DPPA4* (Figure 2B–I). This report is the first to show that directed HAT activity can be used to trigger the activation of key pluripotency genes and microRNAs like MIR302C[14] (Figure S3).

CTB–I and SAHA–I induced similar gene expression profiles, which suggests a common mechanism. To clarify the mode of action, we synthesized a CTB analogue conjugate termed CTB-Ne–I, where the relative positions of the $-CF_3$ and $-Cl$ in CTB are changed to abolish HAT activity according to Kundu and co-workers (Scheme S1c).[10] A HAT activity study in HDF cells showed that CTB–I retained the HAT-activating activity of CTB since HAT activity significantly increased in CTB- and CTB–I-treated HDF cells when compared to those treated with the inactive CTB-Ne–I (Figure S4). SAHA–I produced only a mild increase in HAT activity, which suggests an indirect effect caused by HDAC inhibition. This pattern was further substantiated through an enzymatic activity assay study carried out in nuclear extract from HeLa cells. Only CTB and CTB–I significantly increased HAT activity compared to SAHA–I and CTB-Ne–I (Figure S5). Unlike CTB–I and SAHA–I, the

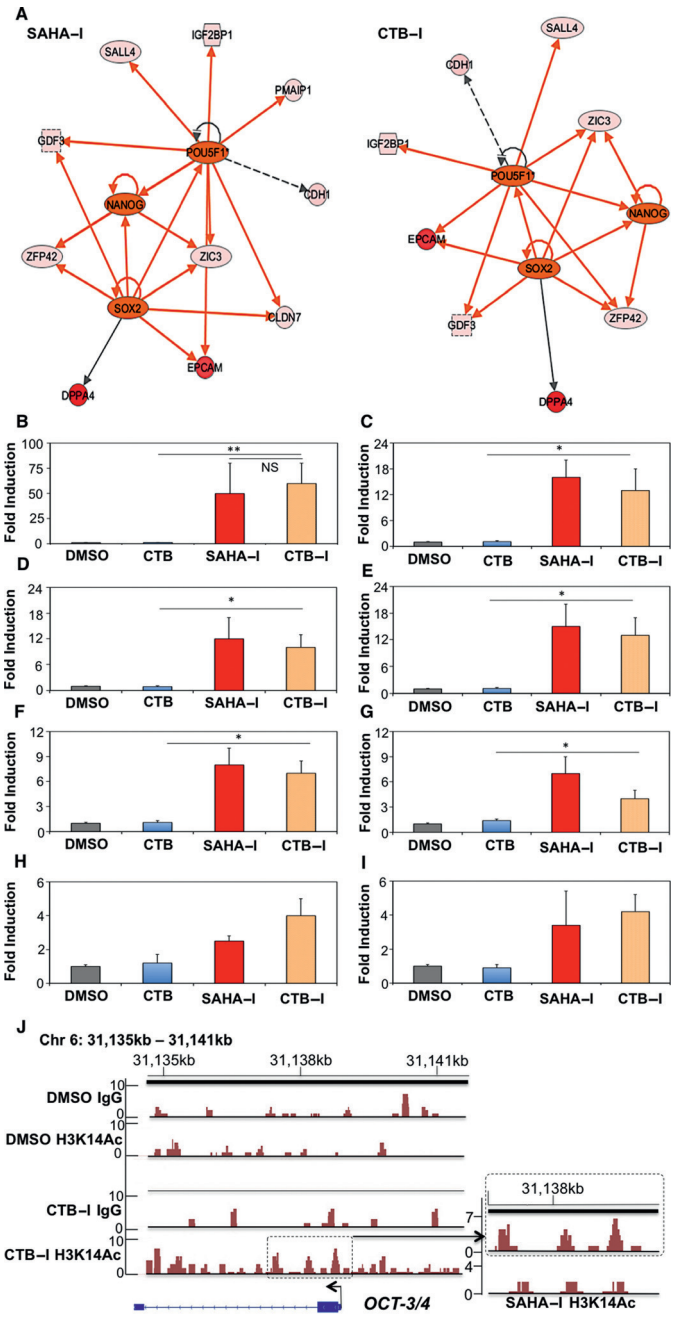


Figure 2. A) Analysis of the upstream regulators from the summary of data obtained from four wells shows that like SAHA–I[7], CTB–I activates the *OCT-3/4* pathway. Red shapes: substantially upregulated, pink shapes: somewhat upregulated, red arrows: leads to activation, black arrows: effect not predicted. qRT-PCR analysis of *OCT-3/4* (B), *SOX2* (C), *NANOG* (D), *LIN28B* (E), *SALL4* (F), *EPCAM* (G), *ZIC3* (H), and *DPPA4* (I) in the effector-treated (CTB, CTB–I, and SAHA–I) HDF cells. Mean ± SD from 18 wells, $p < 0.05$ (six biological replicates). J) ChIP-seq analysis after immunoprecipitation with H3K14ac antibody shows hyperacetylation in the promoter and transcribed regions (enlarged and indicated) of *OCT-3/4*, as with like SAHA–I[7].

inactive analogue CTB-Ne–I did not upregulate the pluripotency genes, which confirms the need for CTB-mediated HAT activity for gene induction (Figure S6). An HDAC activity assay in cells treated with individual effectors ruled out HDAC inhibitory activity of CTB as the possible gene induction mechanism since only SAHA and SAHA–I resulted in HDAC inhibition in cells (Figure S7). An in vitro assay carried out with nuclear extracts from HeLa and HDF cells further validated this pattern (Figure S8). Chromatin immunoprecipitation sequencing (ChIP-seq) analysis revealed hyperacetylation in the transcribed regions of *OCT-3/4* in CTB–I-treated HDF cells compared to DMSO-treated HDF cells (Figure 2J). Interestingly, like SAHA–I, CTB–I also resulted in enrichment in the acetylation level of H3K14 (Figure S9). Also, peaks around the putative promoter region with the "I" binding site were identical in CTB–I- and SAHA–I-treated HDFs (Figure 2J, dashed box).

Recently, high-throughput sequencing studies have substantiated the strong binding affinity of PIPs,[15] and in accordance with the gene expression pattern, SAHA–I showed superior binding specificity toward the promoter sequence of human *OCT-3/4*[7] than the germ-cell gene *PIWIL1*.[5] The in vitro binding association and ChIP–PCR studies verified that the pattern observed in the model oligonucleotide duplex can be extrapolated to the corresponding sequence within the chromatin.[7] CTB–I also triggered hyperacetylation marks in *NANOG*, *SOX2*, *DPPA4*, *EPCAM*, *LIN28*, and *ZIC3* (Figure S10), however, none had the notable acetylation profile observed in the promoter and transcribed regions of *OCT-3/4*. CTB–I did not produce a marked difference in the acetylation pattern observed in the germ-cell genes *PIWIL1* and *TDRD1* or the housekeeping gene *GAPDH* and *HMGB1* (Figure S11). The top five significantly enriched pathways verified the effect of CTB–I on the expression of the key pluripotency genes (Table S2, CTB-I-treated HDF cells). CTB induced an entirely different set of genes associated with liver X receptor (LXR; Table S2, CTB-treated HDF cells). HDAC1 and p300 bind directly to the overlapping regions of the histone H3 tail and compete with each other.[16] HDAC1 is known to regulate the pluripotency and lineage-specific transcriptional network.[17] A combination of CTB–I and SAHA–I resulted in the expression of *OCT-3/4*, with a similar expression profile to that obtained when the two constructs were used individuallly (Figure S12). Because p300 can acetylate HDAC1 upon activation and attenuate its deacetylase activity, these two synthetic histone modifiers could induce a similar pattern of gene expression. Although the actual mechanism underlying the identical bioactivity is not straightforward, site-specific acetylation triggered by SAHA-mediated HDAC inhibition or CTB-mediated HAT activation at a particular key sequence could cause the activation of *OCT-3/4* and its regulated genes.

As HAT activation is important in regulating the multigene networks associated cellular processes, our study could open new opportunities to modulate HAT-regulated genes. CTB–I produced similar HAT activity patterns in HeLa cells and HDF cells, thus indicating the potential of this strategy in p300 deficient cells. Since the achievement of chemically induced pluripotent stem cells,[18] bioactive ligands that aid cellular reprogramming are increasingly in demand. Our synthetic strategy could be harnessed to probe the functional interplay between histone-modifying enzymes in coordinating

the therapeutically important genes. Tuning of the functional module[19] and expansion of the recognition ability[20] could lead to the construction of robust epigenetic switches with versatile bioactivity.

Keywords: DNA recognition · epigenetics · gene expression · histone modification · synthetic biology

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