

HDAC1 regulates pluripotency and lineage specific transcriptional networks in embryonic and trophoblast stem cells

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ABSTRACT

Epigenetic regulation of gene expression is important in maintaining self-renewal of embryonic stem (ES) and trophoblast stem (TS) cells. Histone deacetylases (HDACs) negatively control histone acetylation by removing covalent acetylation marks from histone tails. Because histone acetylation is a known mark for active transcription, HDACs presumably associate with inactive genes. Here, we used genome-wide chromatin immunoprecipitation to investigate targets of HDAC1 in ES and TS cells. Through evaluation of genes associated with acetylated histone H3 marks, and global expression analysis of Hdac1 knockout ES and trichostatin A-treated ES and TS cells, we found that HDAC1 occupies mainly active genes, including important regulators of ES and TS cells self-renewal. We also observed occupancy of methyl-CpG binding domain protein 3 (MBD3), a subunit of the nucleosome remodeling and histone deacetylation (NuRD) complex, at a subset of HDAC1-occupied sequences in ES cells, including the pluripotency regulators Oct4, Nanog and Klf4. By mapping HDAC1 targets on a global scale, our results describe further insight into epigenetic mechanisms of ES and TS cells self-renewal.

INTRODUCTION

Eukaryotic organisms solve the immense challenge of packaging 2 m of DNA into the nucleus of each cell in a conformation that is conducive for transcriptional regulation by compacting DNA into chromatin. Nucleosomes, which are the core unit of chromatin, consist of DNA wrapped around an octamer of histones. Post-translational modifications of histone tails, such as acetylation, play critical roles in regulating

transcription and chromatin structure. The expression state of a gene is largely correlated with its histone acetylation state. Histone deacetylases (HDACs), which negatively control histone acetylation by removing acetyl groups from histone tails, are generally thought to be transcriptional repressors. HDACs have been found to act as co-repressors in complexes such as the nucleosome remodeling and histone deacetylation (NuRD), SIN3 complexes and CoREST (1,2). HDAC1 is also required for embryo development (3), where HDAC1 null embryos die before E10.5, and HDACs have been shown to play an important role in embryonic stem (ES) cell differentiation (3). While HDAC1-deficient ES cells exhibit abnormal differentiation (4), a loss of either HDAC1 (4,5) or HDAC2 (5) does not seem to affect ES cell proliferation. In mouse genetic studies, deletion of both HDAC1 and HDAC2 is required to produce a phenotype in a tissue-specific manner, suggesting that the activities of HDAC1 and HDAC2 are largely redundant (6,7). HDAC inhibitors such as trichostatin A (TSA) have been used to address the overarching roles of Classes I and II HDACs in ES cell function, where treatment of ES cells with TSA inhibits differentiation (8). HDAC inhibitors also aid in the reprogramming process, where TSA treatment of cloned embryos improves heterochromatin remodeling (9), and TSA, valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) significantly improve the efficiency of generating induced pluripotent stem (iPS) cells (10). Recent evidence also demonstrates that HDACs are located at active and inactive genes (11). HDACs were found to bind more active than inactive genes, suggesting that HDACs may function in removing acetyl groups added by histone acetyl transferases during transcriptional initiation and elongation to assist in transcriptional elongation and prevent indiscriminate initiation (11).

To further understand epigenetic phenomena that contribute to chromatin structure and regulate gene expression, we evaluated promoter occupancy of HDAC1 in ES and trophoblast stem (TS) cells using genome-wide

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chromatin immunoprecipitation followed by microarray analysis (ChIP-chip). By evaluating genes marked with acetylated histone H3 (AcH3), and transcriptome analysis of Hdac1 knockout ES cells and ES and TS cells treated with TSA, we observed HDAC1 binding to active and inactive genes in ES and TS cells. Surprisingly, we observed HDAC1 binding to predominantly active genes in ES and TS cells, including genes whose expression is enriched in ES cells such as the core pluripotency-related factors Oct4, Sox2 and Nanog and genes whose expression is enriched in TS cells such as Cdx2, Elf5, Eomes and Sox2. We also identified a fewer number of inactive genes bound by HDAC1, without AcH3 marks, suggesting that HDAC1 binds a reduced number of inactive genes. In addition, we evaluated global promoter occupancy of MBD3, a component of the NuRD complex, in ES cells. Our results show that MBD3 co-binds a subset of HDAC1 targets in ES cells, including the pluripotency regulators Oct4, Nanog and Klf4. Overall, our results demonstrate that HDAC1 occupies critical genes involved in maintaining self-renewal of ES and TS cells.

MATERIALS AND METHODS

ES cell culture

R1 ES cells, obtained from ATCC (Manassas, VA, USA), were cultured as previously described (12). Briefly, R1 ES cells were cultured on gelatin-coated dishes without feeder cells in serum-free media containing LIF and BMP4 (ESGRO Complete Clonal Grade Medium, Chemicon) at 37°C with 5% CO₂. ES cells were passed by washing with PBS, and dissociating with Accutase (Millipore). For TSA treatment, ES cells were transitioned to feeder-free conditions and cultured for 24 h with 0, 10, 50 or 100 nM TSA (Sigma).

TS cell culture

TS cells were cultured as previously described (13). TS cells (TSC-BK12) were cultured in dishes containing γ -irradiated MEFs and RPMI 1640 medium supplemented with 20% FBS (Gibco), 1 mM sodium pyruvate (Gibco), 100 μ M β -mercaptoethanol (Chemicon), 2 mM L-glutamine (Gibco), 25 ng/ml recombinant human FGF4 (1 \times) (R&D Systems) and 1 μ g/ml heparin (1 \times) (Sigma). For ChIP-chip experiments, TS cells were passed twice to remove feeder cells in TS cell media containing 70% iMEF-conditioned medium, 1.5 X FGF4 and heparin at 37°C with 5% CO₂.

qRT-PCR expression analysis

RNA isolation and qRT-PCR were performed as previously described with minor modifications (12). ES cells were harvested using Accutase to dissociate cells. Total RNA was harvested from ES cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and DNase treated using Turbo DNA-free (Ambion) for 30 min at 37°C. Reverse transcription was performed using 1 μ g total RNA and a SuperScript III kit with random

hexamer primers (Invitrogen, Carlsbad, CA, USA). qPCR was performed using TaqMan probes and TaqMan Universal PCR Master Mix reagents (Applied Biosystems), or non-labeled primers and SYBR Green PCR Master Mix reagents (Applied Biosystems). Primers used for qPCR with SYBR green reagents were designed using Primer3 (<http://frodo.wi.mit.edu/>).

ChIP-chip analysis

ChIP-chip and ChIP-PCR experiments were performed as previously described with minor modifications (12,13). The polyclonal HDAC1 (ab7028) and MBD3 antibodies were obtained from Abcam. Briefly, 1 \times 10⁸ mouse ES (R1) and TS cells (TSC-BK12), all feeder-free were harvested and chemically cross-linked with 1% formaldehyde (Sigma) and subsequently sonicated. Sonicated cell extracts equivalent to 2 \times 10⁶ cells were used for immunoprecipitation assays. Five micrograms amplified ChIP-enriched DNA (ES cell HDAC1-ChIP, ES cell MBD3-ChIP, TS cell HDAC1-ChIP and corresponding Input whole-cell extracts for ES and TS cells) were DNase treated, labeled and hybridized to Affymetrix mouse promoter 1.0R tiling arrays. Mouse promoter 1.0R DNA tiling arrays contain 4.6 million 25-mer oligonucleotide probes spanning a distance of -6 to +2.5 kb relative to the transcriptional start site (TSS) for 28 000 mouse promoter regions, providing a resolution of 35 bp with 10-bp gaps between probes. At least three biological replicates were used for each ChIP-chip analysis. Quantile normalization, including probe intensity computation and log₂ adjustment, was applied to the tiling array data using CisGenome (14). Peak detection and gene annotation were performed as previously described (12,13,15). Briefly, peak detection was performed using the TileMap (16) application in CisGenome (14). MA statistics was applied to analyze the tiling array data. Post-filtering included discarding peaks if the total length was <100 bp, or if there were less than three continuous probes passing the cut-off, and merging two adjacent peaks if the gap between the two peaks was <300 bp and if there were less than five probes that did not pass the cut-off between the two peaks. Peaks with an FDR <0.10 were also discarded. Enrichment peaks were annotated with the closest gene using build 36 of the mouse genome.

Clustering analysis of promoter binding data

Promoter-binding patterns for HDAC1 were centered on enrichment peaks [-2 kb, +2kb] and visualized by hierarchical clustering analysis (HCA). Promoter regions where >25% of probes were absent were not included in this analysis. Hierarchical clustering was performed using Spotfire software.

Global expression analysis of TSA-treated ES and TS cells

Total RNA was isolated from the ES and TS cells cultured in the presence of 0, 10, 50 or 100 nM TSA for 24 h using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). For each duplicate biological replicate, 100 ng of RNA was amplified using a MessageAmp Premier RNA

Amplification kit (Ambion), and 13 μ g of fragmented aRNA was hybridized to Affymetrix mouse 430 v2.0 microarrays. Analysis was performed as previously described with minor modifications (13). Briefly, CEL files were loaded into ArrayAssist (Stratagene, La Jolla, CA, USA) and probes were normalized using the GC-RMA algorithm. Analysis of variance (ANOVA) was performed on all groups using a Benjamini and Hochberg FDR correction. Genes whose expression differed by at least 2-fold between any two groups with a P -value $< 5\%$ were considered as differentially expressed. Validation of microarrays was performed using qRT-PCR with Taqman probes as described above.

Global microarray expression analysis of public data

Analysis was performed as previously described with some modifications (13). Briefly, CEL files were loaded into ArrayAssist (Stratagene, La Jolla, CA, USA) and probes were normalized using the GC-RMA algorithm. ANOVA was performed on all groups using a Benjamini and Hochberg FDR correction. Genes whose expression differed by at least 2-fold between any two groups with a P -value $< 5\%$ were considered as differentially expressed. Validation of microarrays was performed using qRT-PCR with Taqman probes as described above.

RESULTS

Global mapping of HDAC1 binding in ES and TS cells

To interrogate genome-wide promoter binding of the HDAC1 in ES and TS cells, we used ChIP-chip analysis, using an antibody specific to HDAC1. Chromatin was prepared from ES and TS cells as previously described (12,13). HDAC1 ChIP-enriched DNA sequences were amplified and hybridized to high-density DNA promoter tiling arrays encompassing regions of 28 000 murine promoters ('Materials and Methods' section). Genomic binding peaks of HDAC1 in ES and TS cells were analyzed using Tilemap (16) and annotated to the nearest TSS using CisGenome (14) (Supplementary Tables S1 and S2). HDAC1-bound regions are enriched near the TSSs of target genes (Figure 1A). Only a small number of HDAC1-bound regions were found within proximal promoter regions [< 300 bp upstream or downstream of TSS; ES cells (51 genes), TS cells (103 genes)], while the majority of bound regions occupied distal regulatory regions (> 300 bp-upstream or downstream from TSS). To further evaluate binding patterns of genes bound by HDAC1, we clustered the binding profiles of HDAC1 using a 4-kb window around the enriched peak, and found that the intensity and length of HDAC1-binding profiles were similar between ES and TS cells (Figure 1B). We identified 2315 genes bound by HDAC1 in ES cells, 2538 genes bound by HDAC1 in TS cells, and 1032 genes co-bound by HDAC1 in ES and TS cells (Figure 1C).

Using Ingenuity Pathway Analysis (IPA) to functionally annotate HDAC1 target genes, we found that HDAC1 target genes are located at a similar frequency in subcellular locations including cytoplasm, extracellular

space, nucleus and plasma membrane, in ES and TS cells (Figure 1D). For example, 33% of HDAC1 targets in ES cells were located in the nucleus compared with 29% in TS cells (Figure 1D). Moreover, IPA identified several biological processes overrepresented in genes bound by HDAC1 in ES and TS cells including gene expression and cellular-, organismal-, embryonic- and tissue development (Figure 1E and F). To further compare Gene Ontology (GO) terms associated with genes bound by HDAC1 in ES and TS cells, we evaluated pair-wise correlations between GO terms associated with HDAC1 bound genes in ES and TS cells using AutoSOME (17), and clustered these results using Spotfire. Hierarchical clustering revealed the overrepresentation of related GO terms associated with HDAC1 targets in ES and TS cells including processes such as development and disease (Figure 1E and F), implicating a role for HDAC1 in regulating genes involved in common biological processes in ES and TS cells. To functionally categorize genes bound by HDAC1 in both ES and TS cells, we analyzed pair-wise correlations between GO terms associated with HDAC1 targets in both ES and TS cells using AutoSOME. Next, to identify clusters of GO terms represented in HDAC1-occupied genes, we generated a network map using Cytoscape (18) (Figure 1G). Using this approach, we identified clusters of GO terms belonging to multiple biological processes, which are overrepresented in genes bound by HDAC1 in ES and TS cells, including development (connective tissue, embryonic, nervous system, organismal and tissue) and disease (cancer, genetic disorder, reproductive system and respiratory), suggesting that many HDAC1 targets in ES and TS cells are functionally related to one another.

Next, we validated a subset of HDAC1 targets in ES cells by performing chromatin immunoprecipitation followed by qPCR analysis (ChIP-PCR). In this analysis, we confirmed that HDAC1 binding was enriched at active genes (Klf4, Oct4, Zfp42, etc.) and inactive genes (Notch1, Tead4, etc.) in ES cells (Figure 1H).

HDAC1 occupies core pluripotency-related genes in ES cells and trophoblast-specific genes in TS cells

Our genome-wide promoter analysis revealed that HDAC1 binds many genes in ES cells, which are important in maintaining pluripotency including the core transcription factors Oct4, Sox2 and Nanog and other genes whose expression is enriched in ES cells including Fgf4, Mbd3, Rest, Sox2, Tbx3 and Zfp42 (Figure 2A; Supplementary Table S1). Our results also show that HDAC1 binds many genes in TS cells, which are expressed in the TE and trophoblast lineage including Bmpr1a, Cdkn1c, Cdx2, Elf5, Eomes, Ets2, Gata2/3, Hand1, Msx2 and Tcfap2c (13) (Figure 2B and C; Supplementary Table S2). Additionally, HDAC1-occupied genes that are highly expressed in both ES and TS cells including the *de novo* DNA methyltransferase Dnmt3b, Eed, Jmjd3/4, Klf5, Lin28, Myc, Nr4a1/5a1, Sox2 and Tbx3, demonstrating that HDAC1 occupies epigenetic regulators and genes involved in maintaining

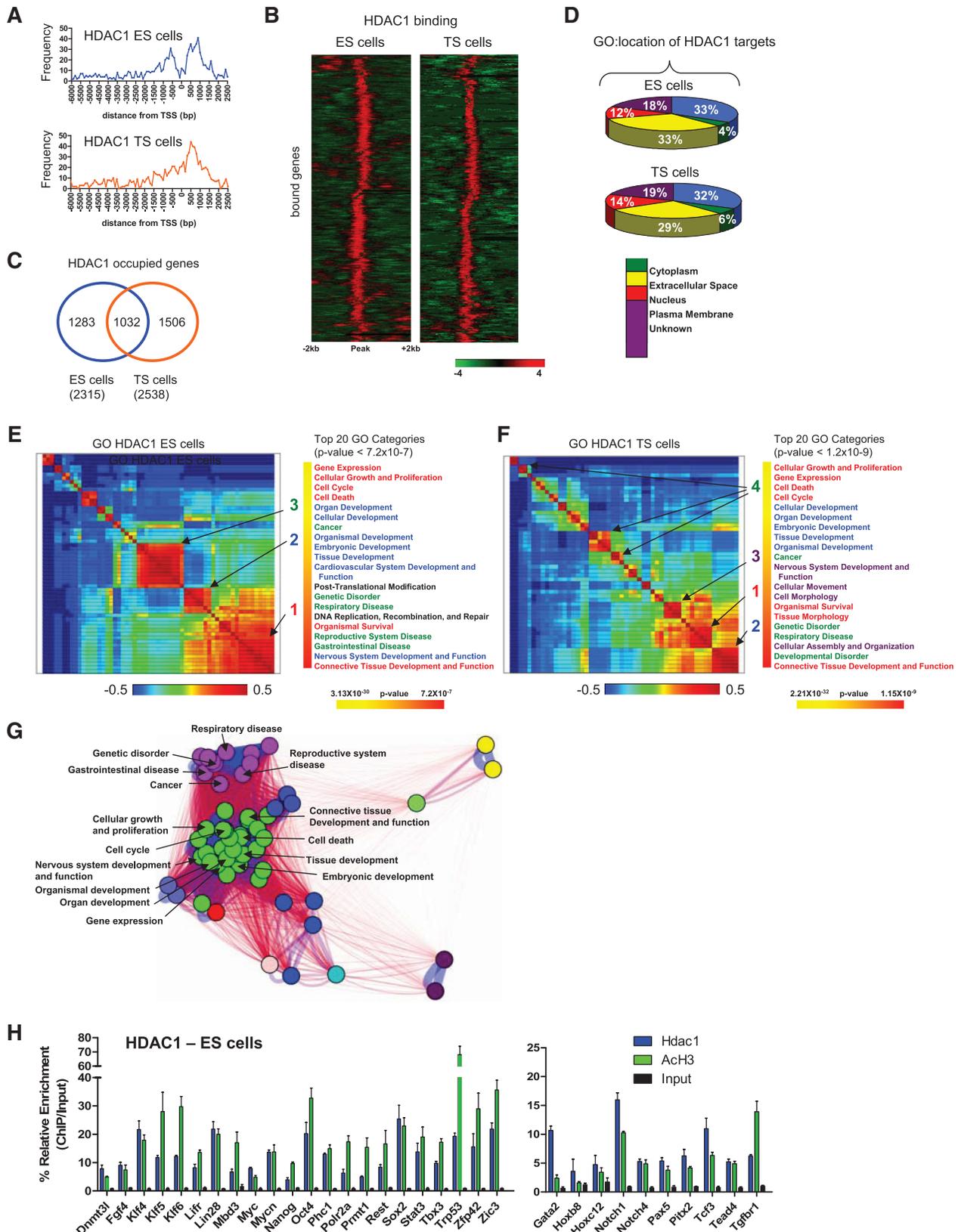


Figure 1. HDAC1 binding profiles and functional annotation of target gene in ES cells and TS Cells. (A) HDAC1 binding profiles near TSSs in ES and TS cells. (B) HDAC1 binding profiles were centered on enrichment peaks [−2, +2 kb] and clustered. (C) Venn diagram showing overlap of HDAC1-bound genes in ES and TS cells. (D) GO functional annotation of HDAC1-bound genes in ES and TS cells was performed using IPA. GO terms for cellular location were evaluated using IPA and represented as a percentage of total genes bound by HDAC1 in ES and TS cells. (E and F) Hierarchical clustering heat map of pair-wise affinities between any two GO terms associated with HDAC1-bound genes in (E) ES and (F) TS cells.

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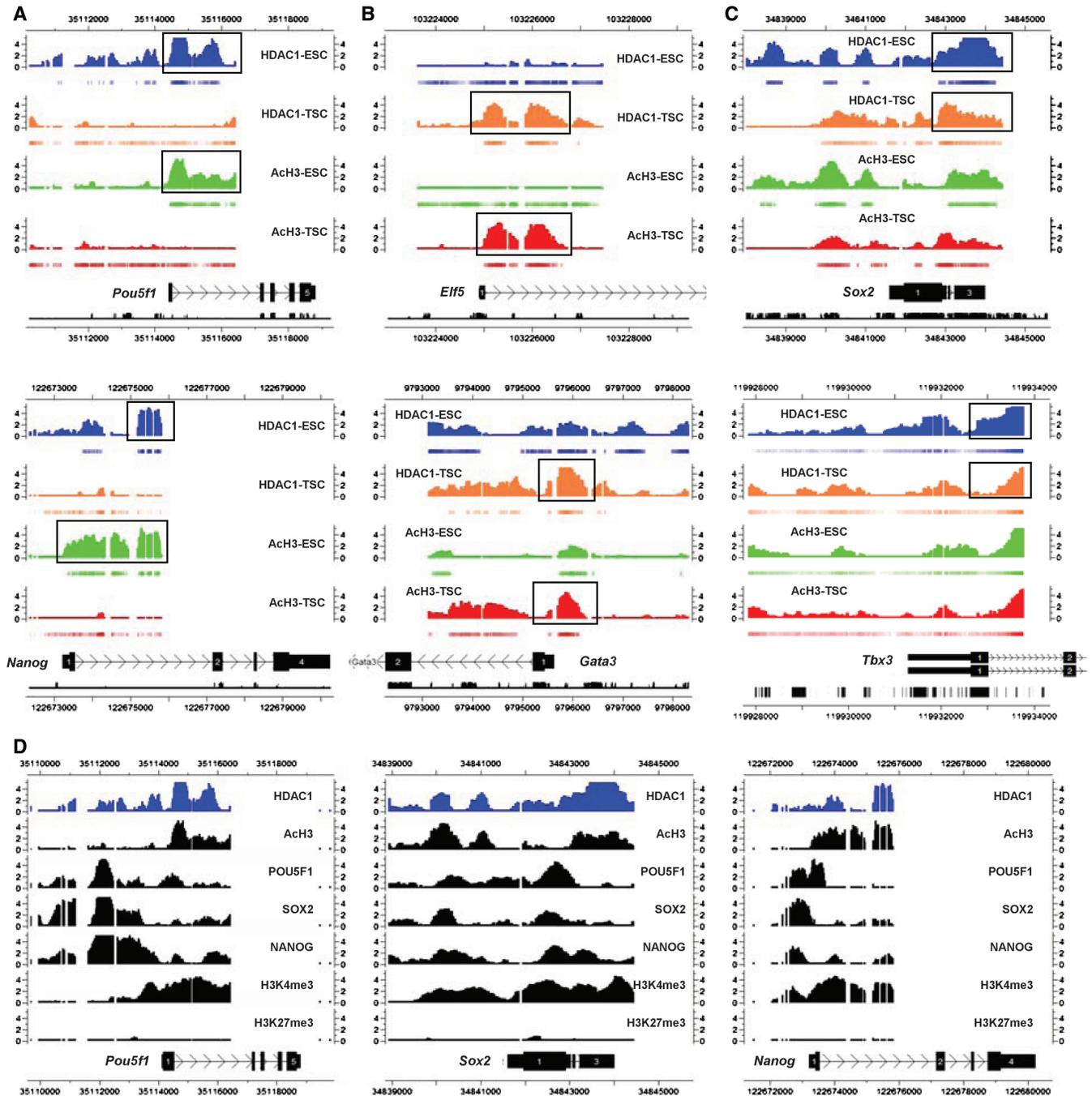


Figure 2. Genome-wide binding of HDAC1 and histone H3 acetylation (AcH3) demarcates ES and TS cells self-renewal. HDAC1 occupancy and AcH3 at (A) pluripotency-related genes in ES cells (*Pou5f1*/Oct4, *Nanog*), (B) genes whose expression is enriched in the trophoblast lineage (*Elf5*, *Gata3*) and (C) genes whose expression is enriched in ES and TS cells (*Sox2*, *Tbx3*). (D) HDAC1 occupancy and transcription factor (*Oct4*, *Sox2* and *Nanog*) binding, and histone marks (AcH3, H3K4me3 and H3K27me3) at core transcription factor loci in ES cells (*Pou5f1*, *Sox2* and *Nanog*). MA enrichment values (\log_2) are shown.

Figure 1. Continued

IPA was used to functionally annotate HDAC1 bound genes. AutoSOME was used to generate pair-wise association values. (G) Network map illustrating GO term analysis of HDAC1-occupied genes in both ES and TS cells. Nodes are colored by clusters of GO terms, where correlated terms are grouped by color. Edges represent pair-wise affinities between any two GO terms, as clustered by AutoSOME (0 = GO terms never co-cluster; 1 = GO terms always co-cluster). Cytoscape was used to generate the network using the edge-weighted spring embedded layout algorithm. (H) Confirmation of HDAC1 ChIP-chip enriched regions in ES cells using ChIP-PCR. Results are shown as percent enrichment relative to Input.

self-renewal in both ES and TS cells (Figure 2C; Supplementary Tables S1 and S2). These results are surprising because HDAC1 has been traditionally shown to localize with repressed or inactive genes.

Because HDAC1 was found to occupy promoters of many pluripotency-related genes in ES cells, we were interested in comparing the HDAC1-binding profiles to those of OCT4, SOX2 and NANOG and profiles of transcriptionally active and inactive histone modifications, in ES cells. Therefore, we compared HDAC1 binding profiles with OCT4, SOX2 and NANOG binding (19) and profiles of histone modifications including AcH3 (13), H3K4me3 (19) and H3K27me3 (19), at the core pluripotency-related genes Oct4, Sox2 and Nanog, in ES cells (Figure 2D). It is known that OCT4, SOX2 and NANOG co-bind their own promoters in ES cells (20,21), and the promoter regions of Oct4, Sox2 and Nanog are associated with the transcriptionally active histone modifications AcH3 and H3K4me3. While our results demonstrate that HDAC1 occupies Oct4, Sox2 and Nanog promoters in ES cells, HDAC1 binding patterns are aligned more with the profiles of active histone modifications (AcH3 and H3K4me3) compared to the binding profiles of OCT4, SOX2 and NANOG (19) (Figure 2D). These findings suggest that HDAC1 may regulate ES and TS cells self-renewal and chromatin structure by binding chromatin regions with specific histone modifications.

HDAC1 binding is associated with histone acetylation (AcH3) and gene activity in ES and TS cells

ES cells uniquely express Oct4 and Nanog, while TS cells uniquely express Cdx2 and Eomes, and ES and TS cells both express high levels of Hdac1, Sox2 and Tbx3 (Figure 3A) (13,22). Although expression of Oct4 and Cdx2 is uniquely expressed in ES and TS cells, respectively, HDAC1 binding to Oct4 in ES cells and Cdx2 in TS cells suggests a positive role for HDAC1 in supporting transcription of key target genes in both cell types. To further understand the expression state of HDAC1 targets in ES and TS cells, we used gene set enrichment analysis (GSEA) (23) to compare HDAC1 target genes with histone H3 acetylation (AcH3) marks in ES (15) and TS cells (13), and public microarray expression data from ES cells and differentiated embryoid bodies (EBs) (24), undifferentiated and retinoic acid (RA)-differentiated ES (22), ES and TS cells (25) and undifferentiated and differentiated TS cells (13). These GSEA results showed that the majority of HDAC1 target genes are active in ES and TS cells. Notably, expression of ~68% and ~58% of the HDAC1 targets was enriched more in ES cells compared with EBs (Figure 3B) and (RA)-differentiated ES cells, respectively (Figure 3C). Also, expression of ~61% of the HDAC1 targets in ES cells was elevated in ES cells compared with TS cells, and expression of ~74% of the HDAC1 targets in TS cells was enriched in TS cells compared with ES cells (Figure 3D). This analysis also revealed that HDAC1 profiles resemble AcH3 profiles, demonstrating that HDAC1 binding profiles are correlated with gene activity. Moreover,

expression of ~73% of the HDAC1 targets was enriched in undifferentiated TS cells compared with differentiated TS cells (Figure 3E). Altogether, these findings demonstrate that HDAC1 binds a higher proportion of active genes in ES and TS cells, and suggests that the frequency of binding is associated with retention of the pluripotent state.

Next, we evaluated the expression state of functional HDAC1 targets in ES and TS cells by ranking genes according to their expression change following ES and TS cells differentiation and evaluating the proportion of genes bound by HDAC1 using a sliding window of 500 genes (Figure 3F and G). We observed a greater proportion of genes bound by HDAC1, which were downregulated upon ES and TS cell differentiation compared with genes that were upregulated (Figure 3F and G), suggesting that HDAC1 binds mainly active genes in ES and TS cells. While this approach is useful for understanding the expression state of HDAC1 targets in ES and TS cells relative to their differentiated progeny, it does not directly address the absolute expression of HDAC1 targets. Therefore, we ranked genes according to their absolute expression in ES and TS cells, and then evaluated the proportion of genes occupied by HDAC1 (Figure 3H and I). Using this approach, we found that HDAC1 binds more genes that are highly expressed in ES and TS cells compared with genes that are expressed at a low level. Overall, these findings suggest that HDAC1 occupies genes that are active in ES and TS cells relative to differentiated cells, and genes that are highly expressed in ES and TS cells.

Previous studies demonstrated that Hdac1 knockout mice die during development before E10.5 (3), and Hdac1 null ES cells are only partially impaired in their ability to proliferate and maintain self-renewal. It is possible that the relatively normal phenotype of HDAC1 knockout ES cells may be due to elevated expression levels of other HDAC proteins such as HDAC2 and HDAC3 (3), which may have redundant functions in ES cells. To address this possibility, we evaluated the global expression of Hdac1-deficient ES cells and wild-type ES cells using public data (GEO accession number: GSE5583) (5). We identified 673 genes, including 18% (126 genes) of HDAC1 targets in ES cells identified in this study, which were differentially expressed between Hdac1-deficient ES cells and wild-type ES cells (>1.5-fold, <0.67-fold, FDR-corrected P -value < 0.05). When considering only genes with a P -value < 0.05, we identified 1270 differentially expressed genes, including 25% (322 genes) of HDAC1 targets in ES cells. Within this data set, a number of pluripotency-associated genes (e.g. Nanog, Sox2, Tbx3, Fgf4 and Zfp42) were upregulated in Hdac1-deficient ES cells (Figure 4A and B), suggesting that HDAC1 functional targets are active in ES cells. However, to further understand the expression state of HDAC1 targets in ES cells on a global scale, we used GSEA to compare HDAC1 target genes from this study with gene expression data from wild-type and Hdac1 knockout ES cells (5). The majority of genes bound by HDAC1 in ES cells and differentially expressed between wild-type and Hdac1 knockout ES cells ($P = 0.05$, FC

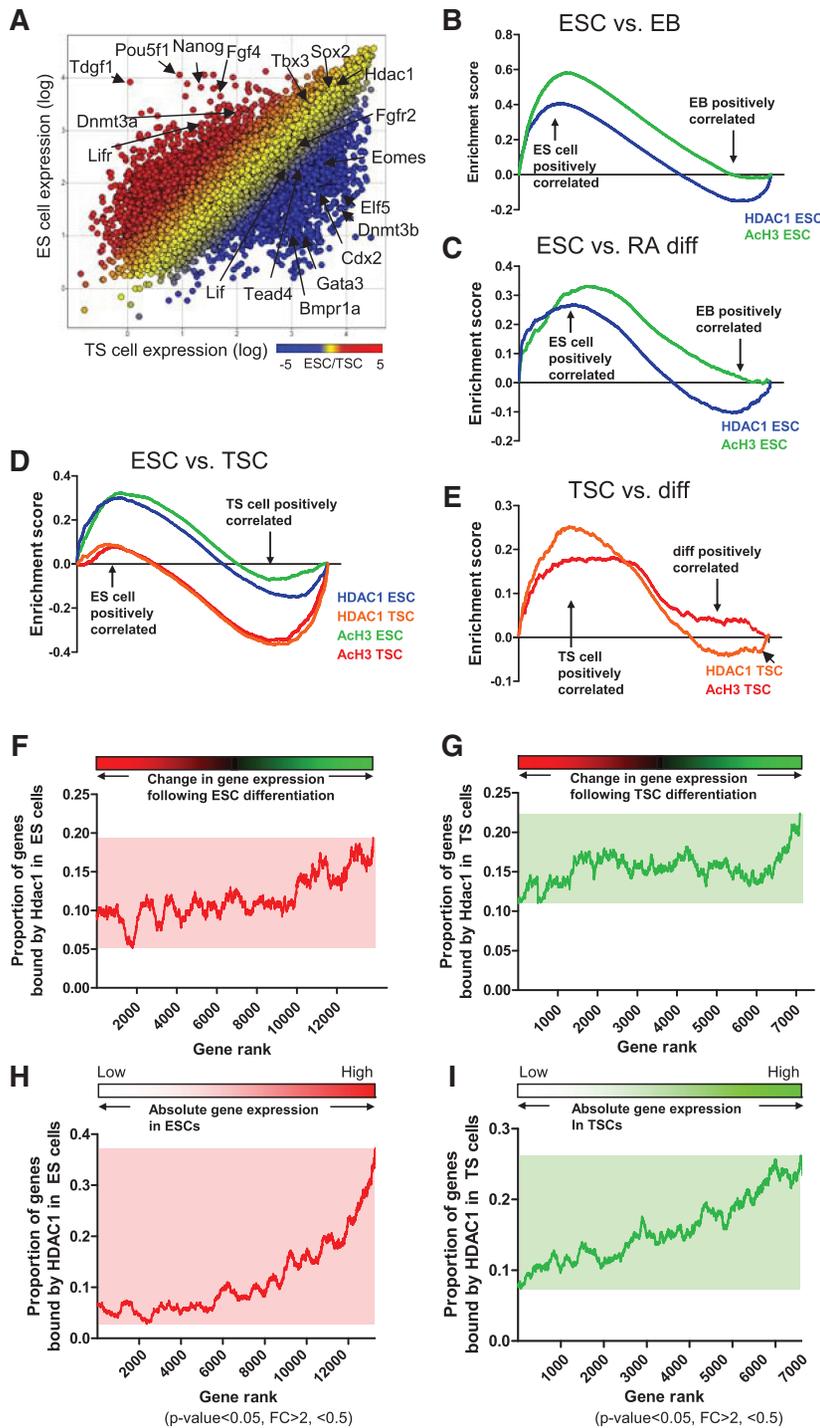


Figure 3. HDAC1 binding is positively correlated with histone acetylation (ACh3) and gene activity in ES and TS cells. (A) Comparison of global gene expression patterns in ES and TS cells. Genes are colored according to their expression ratio in ES/TS cells. (B–E) GSEA analysis of HDAC1 bound genes and histone acetylation (ACh3) in (B) ES cells and EBs, (C) ES and RA-differentiated ES cells, (D) ES and TS cells and (E) undifferentiated and differentiated TS cells. (F and G) Relationship between changes in gene expression following differentiation of (F) ES and (G) TS cells and the proportion of genes bound by HDAC1, using a sliding scale of 500 genes. HDAC1-bound genes are mainly active in ES and TS cells, as shown by the greater proportion of HDAC1-bound genes whose expression is downregulated following ES and TS cells differentiation compared with genes whose expression is upregulated following differentiation. (H and I) Relationship between the absolute expression of genes in (H) ES and (I) TS cells and the proportion of genes bound by HDAC1. Elevated gene expression in ES and TS cells is positively correlated with HDAC1 binding.

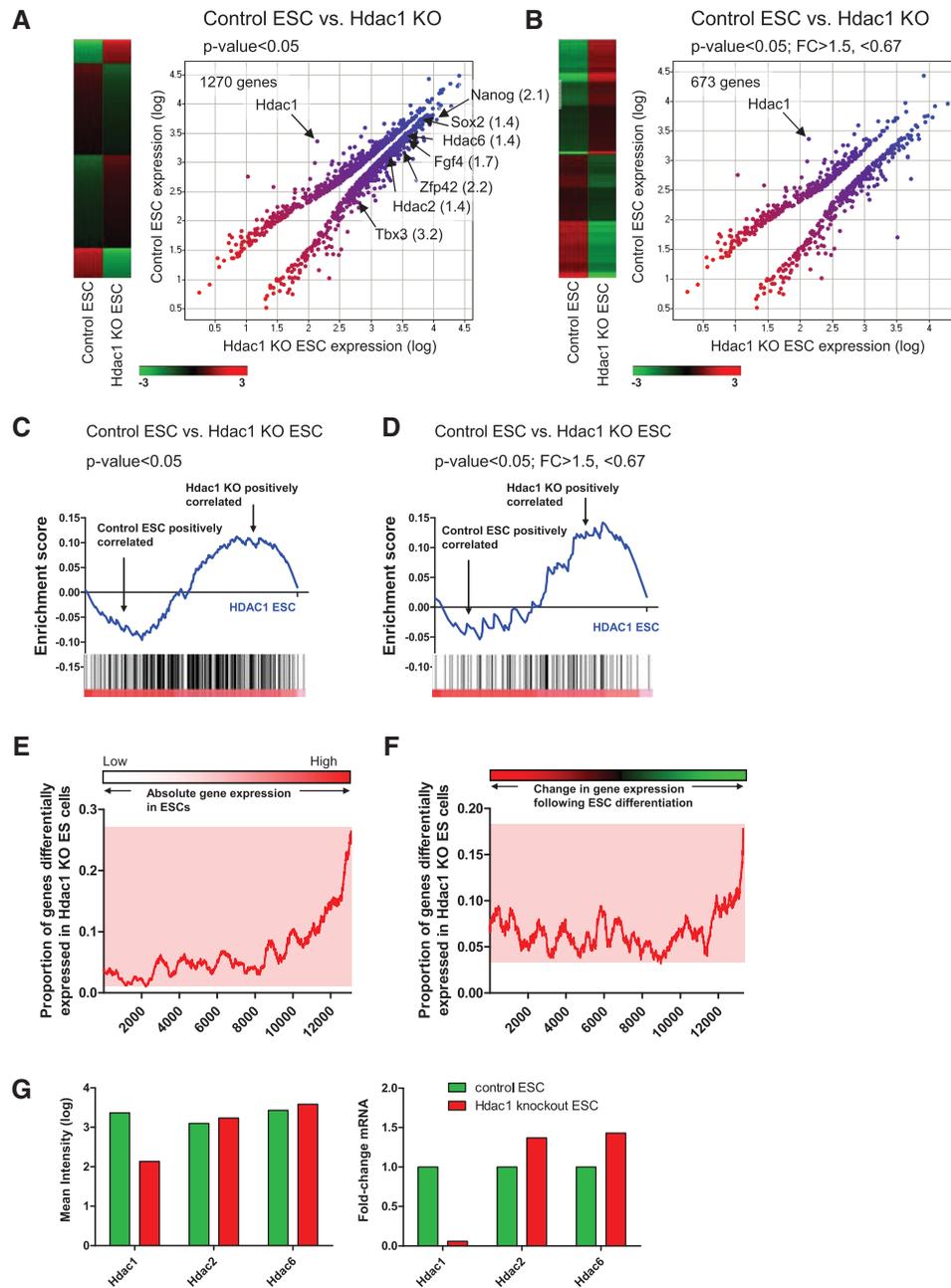


Figure 4. Hdac1 regulates expression of pluripotency genes in ES cells. (**A** and **B**) Global expression analysis of control and Hdac1 knockout (KO) ES cells. (**A**) Hierarchical clustering and scatter plot of genes with a (**A**) P -value < 0.05 (fold change in parentheses) and (**B**) a fold change > 1.5 or < 0.67. (**C** and **D**) GSEA analysis of HDAC1-bound genes in control and Hdac1 KO ES cells. (**E** and **F**) Relationship between (**E**) the absolute expression of genes in ES cells and (**F**) changes in gene expression following differentiation of ES cells and the proportion of genes differentially expressed in Hdac1 KO ES cells, using a sliding scale of 500 genes. (**G**) Log-adjusted mean intensity and fold-change expression levels of Hdac1, Hdac2 and Hdac6 in wild-type and Hdac1 KO ES cells.

>1.5, <0.67) were upregulated in Hdac1 knockout ES cells (Figure 4C and D), demonstrating that Hdac1 serves a predominantly repressive role in transcription. However, by ranking genes according to their absolute expression in ES cells, we observed a greater proportion of differentially expressed genes in Hdac1 knockout ES cells that were highly enriched in ES cells (Figure 4E), suggesting that HDAC1 functional targets are highly expressed in ES cells. In addition, by ranking genes

according to their expression change following ES cell differentiation, we observed a greater proportion of differentially expressed genes in Hdac1 knockout ES cells, which were downregulated upon ES cell differentiation (Figure 4F), further demonstrating that HDAC1 binds mainly active genes in ES cells.

Moreover, several HDACs, including those belonging to Classes I (Hdac2) and II (Hdac6), were upregulated in Hdac1-deficient ES cells (Figure 4G). Additional

HDACs represented in this data set (Hdac3, Hdac5 and Hdac7) were not expressed at significant levels. Interestingly, while the sum of the expression levels of Hdac2 and Hdac6 was similar to the expression level of Hdac1 in wild-type ES cells, the cumulative expression of Hdac2 and Hdac6 was upregulated 155% in HDAC1-deficient ES cells, suggesting that Hdac2 and Hdac6 may partially compensate for the loss of Hdac1 (Figure 4G). While extensive knockdown or knockout studies of multiple HDACs would address the potentially redundant roles of HDACs in ES cell self-renewal, an alternative to these technically challenging methods involves inhibiting Classes I and II HDACs using TSA.

Therefore, to better understand the positive or negative control of HDAC1 versus other HDAC target genes in ES and TS cells, we compared HDAC1 targets identified in our study with global mRNA expression data from ES and TS cells treated with the HDAC inhibitor, TSA. Total RNA was harvested from ES and TS cells treated with 10, 50 and 100 nM TSA for 24 h, and transcriptome analysis was performed using Affymetrix Mouse 430 v2.0 microarrays. Genes whose expression differed by at least 2-fold between any two groups with an FDR-adjusted P -value < 0.05 were considered differentially expressed. Treatment of ES cells with TSA for 24 h resulted in downregulation of pluripotency-related genes including Oct4, Esrrb, Nanog, Rest, Zfp42 and Utf1 and upregulation of lineage specific genes including Cdx2, Cebpa, Fgfr2, Gata3 and Mmp9. Moreover, we observed a loss of normal ES cell colony morphology upon treatment with TSA (Figure 5A). These results are in agreement with results from previous studies, which showed that HDAC inhibition leads to downregulation of pluripotency genes (5,26). Treatment of TS cells with TSA for 24 h resulted in downregulation of TS cell-specific genes including Bmpr1a, Elf5, Eomes, Gata3, Lin28 and Tead4 and upregulation of trophoblast-specific genes including Cdx1, Cebpa and Kitl. Also, we observed a loss of normal TS cell colony morphology, where TSA-treated TS cells lost their tight cell-cell contact, which is indicative of differentiation (Figure 5B).

A more detailed analysis of this whole-genome mRNA expression data using HCA, k -means clustering analysis and principal component analysis (PCA) indicated that pluripotency-related genes are downregulated following inhibition of HDACs in ES cells (Figure 5C), and trophoblast-specific genes are downregulated following inhibition of HDACs in TS cells (Figure 5D). Moreover, PCA analysis revealed that ES and TS cells treated with increasing concentrations of TSA (10, 50 and 100 nM) move through the first two components along the PC1 axis (Figure 5C and D). qRT-PCR was used to confirm our microarray results. Genes whose expression is highly enriched in ES cells including Dppa5, Esrrb, Pou5f1, Rest, Rex1/Zfp42 and Utf1 were downregulated, while lineage-specific genes including Cdx2, Cebpa, Csh2/Prl3b1, Fgfr2, Gata3, Hand1, Mmp9 and Tbx5 were upregulated following treatment with TSA (Figure 5E).

To strengthen the observed link between HDAC binding and gene expression of ES and TS cells in the presence or absence of TSA, we ranked genes according

to their expression change following TSA treatment, or absolute expression in ES or TS cells and then evaluated the proportion of genes bound by HDAC1 (Figure 5F and G). The number of genes downregulated following TSA treatment of ES and TS cells was greater than the number of genes upregulated in the population of genes bound by HDAC1. Moreover, HDAC1 binds more genes that are highly expressed in ES or TS cells compared with genes that are expressed at a low level (Figure 5F and G). These findings are unexpected because HDAC1 binding and subsequent gene expression occur more frequently at active versus inactive genes in ES cells.

The NuRD component MBD3 occupies a subset of HDAC1 targets in ES cells

The NuRD complex is one of the several complexes with which HDAC1 is associated. MBD3, along with HDAC1, is a core component of the NuRD complex, which is essential for embryogenesis and ES cell pluripotency (27). However, targets of MBD3 have not been investigated in ES cells. Therefore, to identify global promoter binding of MBD3 in ES cells, we used ChIP-chip analysis using an antibody specific to MBD3. Chromatin and MBD3 ChIP-enriched DNA was prepared as described above. MBD3 ChIP-chip data was analyzed using CisGenome (Supplementary Table S3). To compare the binding patterns of genes occupied by MBD3 and HDAC1, we clustered MBD3 binding profiles using a 4-kb window around the enriched peak, and found that HDAC1- and MBD3-binding profiles are similar in intensity and length (Figure 6A). We identified 1052 genes bound by MBD3 in ES cells, and 311 genes co-bound by MBD3 and HDAC1 (Figure 6B; Supplementary Table S3). Several genes that were co-bound by MBD3 and HDAC1 include pluripotency regulators such as Oct4, Nanog and Klf4 (Figure 6C).

Next, to understand the expression state of MBD3 targets in ES cells, we used GSEA to compare MBD3 targets with microarray expression data from ES cells and EBs (24). Our results show that MBD3 targets are expressed in both ES cells and EBs (Figure 6D). We further evaluated the relationship between MBD3 and HDAC1 binding and expression in ES cells and EBs by generating a terrain map of MBD3 and HDAC1 target gene expression values using gCLUTO (Figure 6E). This result shows that HDAC1 binds mainly active genes, while MBD3 binds both active and inactive genes. While these analyses are useful in understanding the expression state of MBD3 targets in ES cells, it does not address the absolute expression of MBD3 targets. Therefore, we ranked genes according to their absolute expression in ES cells, and then evaluated the proportion of genes occupied by MBD3 (Figure 6F). Using this method, we observed that MBD3 binds more genes that are highly expressed in ES cells compared with genes that are expressed at a low level. However, MBD3 does not bind as frequently to active genes compared to HDAC1. Altogether, these results suggest that MBD3 occupies active and inactive genes, including a subset of HDAC1-occupied genes and pluripotency regulators.

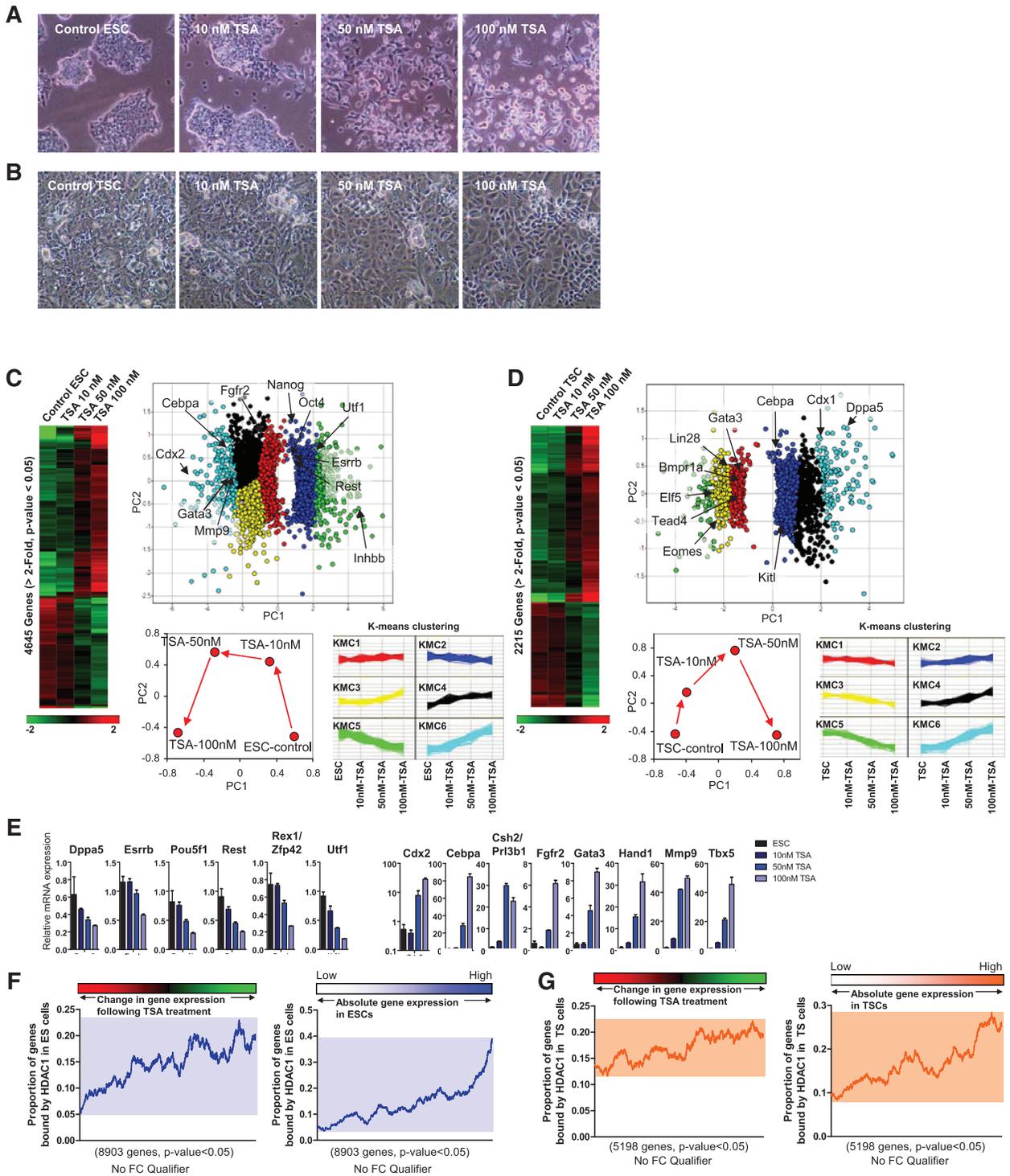


Figure 5. Global expression analysis of TSA treated ES and TS cells. (A) ES and (B) TS cells cultured in the presence of 10, 50 and 100 nM TSA for 24 h. Normal colony morphology was lost in ES and TS cells cultured with TSA. HCA of differentially expressed genes (>2 -fold; upregulated genes are red, downregulated genes are green) in (C) ES and (D) TS cells following treatment of TSA (10, 50 and 100 nM) for 24 h. PCA plots show differentially expressed genes clustered according to *k*-means. *K*-means clustered groups are shown in another plot (six groups of genes). The lower PCA plots of the first two components describe most of the data variability between the samples. (E) qRT-PCR expression analysis of ES cells treated with TSA for 24 h. (F and G) Relationship between changes in gene expression following TSA treatment, or absolute expression of these genes, and the proportion of genes bound by HDAC1 in ES and TS cells, using a sliding window of 500 genes. There is a greater proportion of HDAC1 bound genes whose expression is downregulated following TSA treatment compared with genes whose expression is upregulated upon TSA treatment, suggesting that HDAC1 binds to mainly active genes in ES and TS cells.

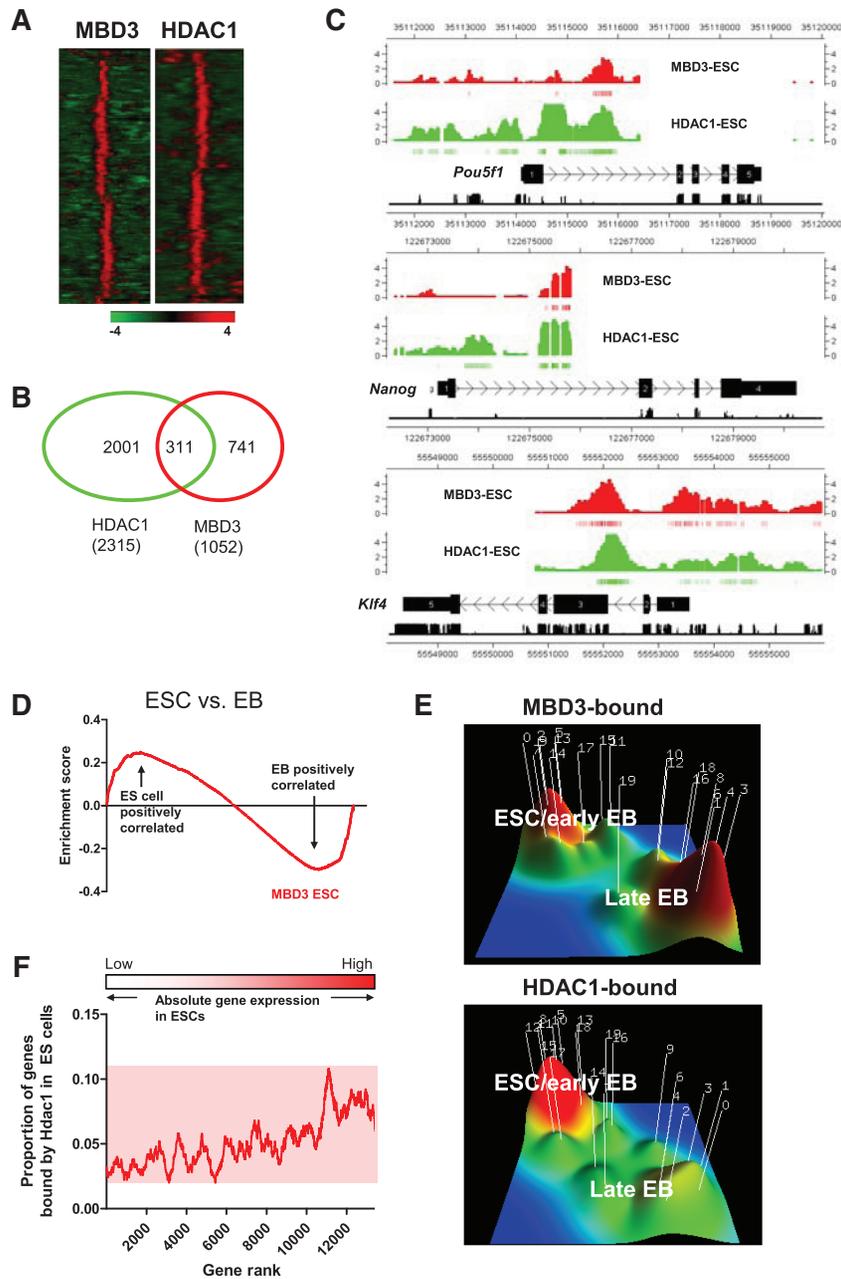


Figure 6. Global promoter occupancy of the NuRD component MBD3 in ES cells. (A) MBD3 binding profiles were centered on enrichment peaks [−2, +2 kb] and clustered. (B) Venn diagram showing overlap of MBD3 and HDAC1 bound genes in ES cells. (C) Co-occupancy of MBD3 and HDAC1 at pluripotency-related genes in ES cells (Pou5f1/Oct4, Nanog and Klf4). (D) GSEA analysis of MBD3-bound genes in ES cells and EBs. (E) 3D terrain map of MBD3 and HDAC1 target gene expression in ES cells and EBs. More HDAC1 targets are expressed in ES cells relative to EBs, while MBD3 targets are expressed in both ES cells and EBs. Peak height is proportional to the internal similarity of clusters and peak volume is proportional to the number of features within the cluster. (F) Relationship between the absolute expression of genes in ES cells and the proportion of genes bound by MBD3. Elevated gene expression is slightly correlated with MBD3 binding.

Functional analysis of HDAC1 targets

Recent genome-wide RNAi studies have provided significant insight into the genetic basis for ES cell pluripotency (28,29). Results from these studies describe genes that are positive regulators of Oct4 expression and ES cell pluripotency. To investigate the function of HDAC1 targets in ES cells, we evaluated the overlap between HDAC1 targets in ES cells identified in our study and

genes identified in two previous RNAi screens. Next, we ranked genes according to their expression change following differentiation and evaluated the proportion of genes bound by HDAC1 (Figure 7A and B). Genes whose expression was downregulated following differentiation, also bound by HDAC1, include positive regulators of Oct4 expression such as Oct4, Sox2, Dnmt3l, Nanog, Lin28, FoxD3, Klf4 and Zfp42 (Figure 7A and B), demonstrating that HDAC1 occupies pluripotency regulators that

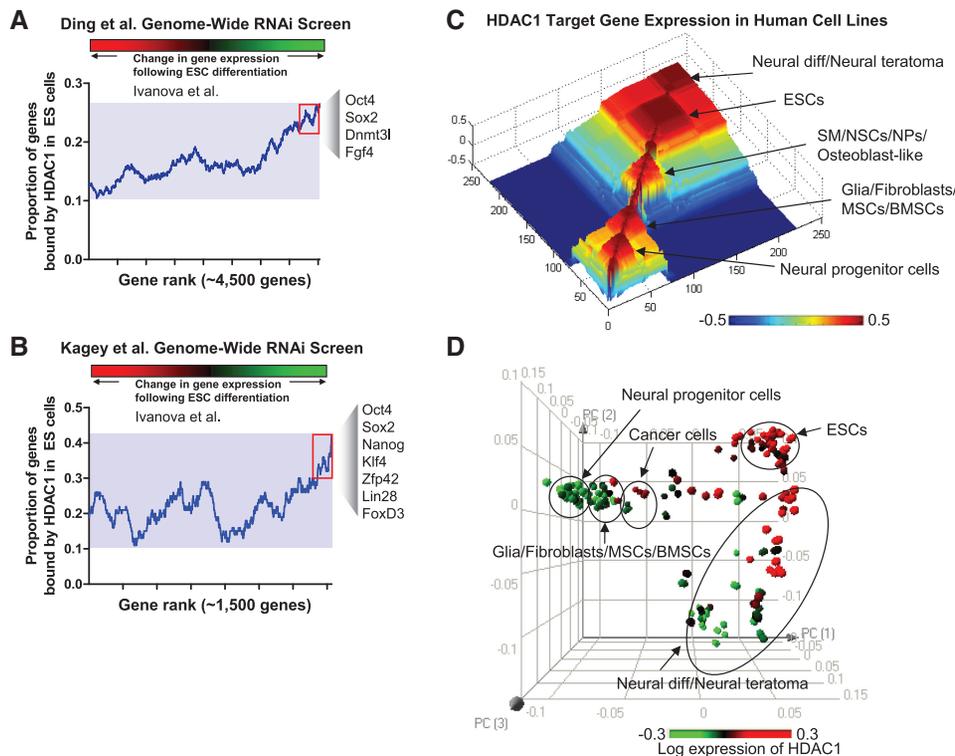


Figure 7. Functional analysis of HDAC1 targets. (A and B) Relationship between changes in gene expression during differentiation of ES cells and the proportion of genes bound by HDAC1, also identified as positive regulators of Oct4 expression in genome-wide RNAi screens, using a sliding scale of 500 genes. HDAC1 bound genes that are highly expressed in ES cells, and positive regulators of Oct4 expression, include pluripotency genes such as Oct4, Sox2, Nanog, Lin28, FoxD3, Klf4 and Dnmt3l. (C) Hierarchical clustering landscape plot of pair-wise affinities between expression values of any two human cell lines associated with HDAC1 bound genes in ES and TS cells. AutoSOME was used to generate pair-wise association values. Cell types are clustered according to their expression of HDAC1 target genes in ES and TS cells. (D) PCA analysis of HDAC1 target gene expression in a set of human cell lines.

support self-renewal of ES cells. These results demonstrate that a subset of HDAC1 target genes is important for ES cell pluripotency and Oct4 expression.

Genome-wide mRNA profiles have been recently compared between many human cell populations (30). These results describe regulatory networks that define distinct cell types, including ES cells and adult stem cells such as neural stem and progenitor cells. To understand the expression of HDAC1 target genes in an assortment of cell lines, we compared ES and TS cells HDAC1 targets with gene expression data from human cells (30). Then, we evaluated pair-wise associations between cell types using AutoSOME, and clustered these results in a landscape plot (Figure 7C). Our results show that cells are clustered according to their unique expression profile of HDAC1 target genes, demonstrating that the expression of HDAC1 targets demarcates human cell lines into functional groups of stem cell populations with varying potential. For example, expression of ES and TS cell HDAC1 targets in various human ES cell lines is more similar to one another compared to the expression of these genes in neural progenitors or smooth muscle cells (Figure 7C). These results were confirmed by PCA analysis of ES and TS cells HDAC1 target gene expression in human cell types. PCA showed that cell types with similar potential and level of HDAC1 expression are clustered together

(Figure 7D). In conclusion, by investigating the expression of ES and TS cells HDAC1 targets in many cell types, these results offer valuable functional insight into transcriptional networks of ES and TS cells.

DISCUSSION

Our genome-wide evaluation of HDAC1 occupancy in ES and TS cells lends new insight into epigenetic mechanisms that support self-renewal in pluripotent ES and multipotent TS cells. By mapping HDAC1 binding in ES and TS cells and the NuRD component MBD3 in ES cells on a global scale, and evaluating global mRNA expression of wild-type and *Hdac1* knockout ES and ES and TS cells cultured in the presence of the HDAC inhibitor, TSA, we found that HDAC1 distributions are present to a larger extent at active genes compared with inactive genes. We also observed upregulation of pluripotency-related genes in *Hdac1* knockout ES cells relative to wild-type ES cells, suggesting that *Hdac1* may limit the expression level of highly enriched genes in ES cells. Results presented here also show that in ES cells, HDAC1 binds key genes implicit in maintaining ES cell self-renewal such as Oct4, Sox2 and Nanog and in TS cells, HDAC1 binds important trophoblast-lineage genes such as *Cdx2*, *Elf5* and *Eomes*. Our results also show that

MBD3 and HDAC1 co-occupy several pluripotency regulators including Oct4, Nanog and Klf4. Moreover, a subset of HDAC1-occupied genes are overexpressed in both ES and TS cells such as Sox2 and Tbx3. Therefore, by identifying global HDAC1 binding sites in ES and TS cells, our work provides clues about epigenetic phenomena that may contribute to mechanisms of ES and TS cells self-renewal.

ES and TS cells, which are derived from preimplantation embryos, share the capacity to self-renew indefinitely *in vitro* in the presence of appropriate external signals including LIF and FGF4, respectively. During preimplantation blastocyst development, LIF produced by trophoblastic (TE) cells promotes ICM cell self-renewal and pluripotency, while FGF4 produced by ICM cells aids in TE cell proliferation and self-renewal. This paracrine signaling mechanism suggests a cooperative relationship between pluripotent ICM and multipotent cells of the TE, which persists upon formation of the blastocyst. Our results implicate a role for HDAC1 in propagating these signals, where HDAC1 was found to bind promoter regions of genes encoding components of the LIF and FGF4 signaling pathways in ES and TS cells. We observed HDAC1 binding to LIFR and FGF4 in ES cells, and HDAC1 binding to FGFR2 in TS cells (Supplementary Tables S1 and S2), demonstrating that HDAC1 occupies extracellular genes including growth factors and cytokines (LIF, FGF4), and receptors (FGFR2), which are critical for maintaining ES and TS cells self-renewal. These results suggest that HDAC1 occupancy of target genes in ES and TS cells may serve to reinforce signaling cascades that participate in the regulation of self-renewal.

The ability of ES cells to differentiate into cells of the three germ layers, and TS cells to differentiate into cells of the trophoblast lineage is determined in part by their distinctive epigenetic programs, where epigenetic modifiers and transcription factors participate in regulating chromatin structure by modifying histones or recruiting histone modifiers. For example, trithorax and Polycomb group proteins (PcGs) regulate regions of histone modifications H3K4me3 and H3K27me3, respectively, which are associated with developmentally repressed genes in ES cells that are primed for activation upon differentiation (31–33). Additional studies have demonstrated that chromatin remodeling proteins such as BRG1 are important in regulating ES (12,34) and TS cells self-renewal (13), and BRG1 was found to associate with genes involved in ES and TS cells self-renewal. We observed co-binding of HDAC1 and BRG1 at a number of target genes in ES and TS cells (data not shown), suggesting that multiple epigenetic regulators may co-regulate transcription of target genes. These results are in agreement with previous findings, which demonstrated that BRG1 cooperates with HDAC to regulate target gene expression (35). Moreover, transcription factors including OCT4 and NANOG have been shown to associate with epigenetic regulators including HDAC1 (36). In this study, we observed co-occupancy of HDAC1 and OCT4, SOX2 and NANOG (37) at 347 target genes in ES cells, and co-occupancy of HDAC1 and BRG1, EOMES and

TCFAP2C (13) at 295 genes in TS cells, suggesting that co-binding of transcription factors and epigenetic modifiers at target genes may be important in maintaining ES and TS cells self-renewal.

HDACs are generally thought to act as transcriptional repressors, by removing acetyl groups at inactive genes. HDAC1 has been shown to function as a co-repressor in multiprotein complexes, after being recruited to DNA by proteins in complexes such as the NuRD, the SIN3 corepressor, the CoREST, the Nanog- and Oct4-associated deacetylase (NODE) and the SHIP1 complexes (38). In addition, HDAC1 has been shown to be recruited by additional complexes such as the Polycomb repressive complex 2, which catalyze histone H3 K27 trimethylation at PcG target genes (39). Because several of these transcriptionally repressive HDAC1-containing complexes, such as NuRD, NODE and PcG, have important roles in normal ES cell function (27,31,36), it is plausible that HDAC1 associates with predominantly inactive genes in ES cells. An alternative interpretation has been suggested from results in T cells where HDACs have been recently shown to bind mainly to active genes but exert regulatory functions on active and inactive genes (11). These results are unexpected because HDACs are well known for their transcriptionally repressive functions. While these findings may seem to suggest a paradigm shift for understanding HDAC function in somatic cells, the authors conclude that HDACs may be more prevalent at active genes compared with inactive genes to reset the acetylation state, whereas inactive genes may not require continuous binding of HDACs to maintain their inactive state or condensed chromatin structure (11).

Because HDACs have a repressive role, it is reasonable to assume that inhibition of HDACs would positively influence the expression state of HDAC target genes. Indeed, we observed upregulation of pluripotency genes in Hdac1 knockout ES cells relative to control ES cells. However, we found that treatment of ES cells with the general HDAC inhibitor TSA resulted in downregulation of pluripotency-related genes and upregulation of lineage-specific genes, which is similar to previous results (26), demonstrating that HDACs as a whole positively and negatively regulate expression of target genes. It has been also demonstrated that treatment with TSA increases chromatin accessibility by increasing acetylation levels (40). HDAC inhibitors have been also used to influence chromatin accessibility during reprogramming. Recent work has demonstrated that four transcription factors including OCT4, SOX2, KLF4 and MYC are sufficient to induce reprogramming of somatic cells to a pluripotent state (41). While the reprogramming process is relatively inefficient, treatment with HDAC inhibitors such as TSA, VPA and SAHA significantly enhances the rate of generating iPS cells (9,10). Our results show that HDAC1 binds the four reprogramming factors Oct4, Sox2, Klf4 and c-Myc in ES cells (Supplementary Table S1). Therefore, treatment of HDAC inhibitors during the reprogramming process may allow Oct4, Sox2, Klf4 and c-Myc to more efficiently activate or repress target genes by regulating chromatin accessibility and relaxing repressive chromatin regions.

HDACs are also thought to regulate cell cycle progression, where HDAC1 null ES cells have reduced proliferation rates and increased expression of the cyclin-dependent kinase inhibitor, p21 (also known as Cdkn1a) (3). Our results support a role for HDAC1 in regulating proliferation and cell cycle progression, where HDAC1 was found to bind cell cycle regulators such as cyclin-dependent kinase inhibitors (Cdkn1a/b), cyclin dependent kinases (Cdk4, -6), cyclin genes (Ccnd1/2, Ccne1/2, etc.) and Myc (Supplementary Tables S1 and S2), in ES and TS cells. We also observed HDAC1 binding to p53 (also known as Trp53), which induces p21, and Mdm2, another member of the p53 tumor suppressor family. p53 also plays a role in repressing Nanog expression during ES cell differentiation (42), and p53 is negatively correlated with reprogramming efficiency (43,44). By associating with DNA regions encoding cell cycle regulators and tumor suppressors, HDAC1 may regulate the tumorigenic growth properties of ES and TS cells.

In conclusion, results presented here describe a role for HDAC1 in occupying active and inactive genes, including key transcription factors that regulate ES and TS cells self-renewal and differentiation. Consistent with our findings, a recent study also demonstrated that HDACs associated with active and inactive genes, albeit in a different cell context (11). Through genome-wide identification of HDAC1 binding sites in ES and TS cells, which comprise the first two stem cells populations to form during development, our results describe epigenetic mechanisms of self-renewal and increase our understanding of pluripotency and multipotency.

ACCESSION NUMBERS

The microarray data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo>) under accession no. GSE26087.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3.

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