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H4K16 acetylation marks active genes and enhancers of embryonic stem cells, but does not alter chromatin compaction

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Compared with histone H3, acetylation of H4 tails has not been well studied, especially in mammalian cells. Yet, H4K16 acetylation is of particular interest because of its ability to decompact nucleosomes in vitro and its involvement in dosage compensation in flies. Here we show that, surprisingly, loss of H4K16 acetylation does not alter higher-order chromatin compaction in vivo in mouse embryonic stem cells (ESCs). As well as peaks of acetylated H4K16 and KAT8 histone acetyltransferase at the transcription start sites of expressed genes, we report that acetylation of H4K16 is a new marker of active enhancers in ESCs and that some enhancers are marked by H3K4me1, KAT8, and H4K16ac, but not by acetylated H3K27 or EP300, suggesting that they are novel EP300 independent regulatory elements. Our data suggest a broad role for different histone acetylation marks and for different histone acetyltransferases in long-range gene regulation.

[Supplemental material is available for this article.]
H4K16ac changes during the differentiation of ES cells

In Drosophila, the MOF/NSL complex is thought to be associated with housekeeping genes; however, KAT8 is required for murine development (Gupta et al. 2008; Thomas et al. 2008; Li et al. 2012), suggesting that H4K16ac may be associated with developmentally regulated gene expression. In order to investigate this we differentiated 46c ESCs into neural progenitor cells (NPCs) and purified the Sox1-GFP-expressing NPCs by fluorescence-activated cell sorting (FACS). Differentiation was also confirmed by expression microarrays: GO term analysis of up-regulated genes showed an enrichment of terms for neuronal genes. We performed native ChIP sequencing in ESC and NPCs, and the levels of H4K16ac across the TSS of all genes was separated by expression into four quartiles. Surprisingly, this revealed that, unlike in ESCs, there is no significant enrichment of H4K16ac around TSS compared with input, and no relationship to gene expression levels in NPCs (Fig. 2A). Western blot showed that this does not simply reflect a global loss of H4K16ac upon NPC differentiation, which has been reported during later stages of embryoid body or retinoic acid (RA)-induced ESC differentiation (Fig. 2B; Li et al. 2012).

Genes that lose expression upon differentiation to NPCs (e.g., Tcl1) lose peaks of H4K16ac around their promoters (Fig. 2C). Some genes that retain similar levels of expression between ESCs and NPCs (e.g., Actb) still retain a small peak of H4K16ac over their promoter (Fig. 2D), though this is much reduced, and is not true of all genes in this class. This was also confirmed by ChIP-chip (Fig. 2D). However, genes that gain expression upon differentiation to NPCs (e.g., Foxb1 in Fig. 2E) do not generally gain a peak of H4K16ac in NPCs.

To determine how H4K16ac changes genome wide, we used the expression profiles from undifferentiated (UD) ESCs and differentiated NPC cells to determine genes that have higher expression in UD than in NPC, and conversely genes whose expression increases during the differentiation to NPCs. In UD cells, there is a clear difference between these two classes of genes; levels of H4K16ac are much greater on genes that are more highly expressed UD ESCs than in NPCs. However, in NPCs, there is little difference in average levels of H4K16ac between the two gene classes (Fig. 2F).
Figure 1. Profile of H4K16 acetylation in undifferentiated ESCs. (A) Input normalized (average reads per million/RPM) H4K16ac native ChIP-seq tag counts around (+/−5 kb) the transcription start site (TSS) and transcription end site (TES) of genes separated into quartiles according to expression in ESC from high to low (Q1–Q4). (B, top) H4K16ac/H3K27ac/H3K4me3 profiles (RPM/bp), in 200-bp sliding windows with a 20-bp step, across the Actb (active) and Ifng (silent) loci. Exons are shown as boxes below the graph, and the direction of transcription is indicated. (Bottom) Log2 H4K16ac/input at Actb and Ifng established by hybridization of H4K16ac ChIP DNA to a custom microarray. (C) Average RPM/bp tag counts across gene bodies for 500 active (left) and inactive (right) genes (in intervals of 10% of gene length) and extending 2 kb upstream of the TSS, and 2 kb downstream (in 200-bp intervals). Data are for H4K16ac (blue), H3K4me3 (pink), and H3K36me3 (green). (D) Genomic distribution of H4K16ac, H3K27ac, and H3K4me3 peaks in ESCs, and 0.5 × 10⁷ peaks randomly distributed throughout the genome. (Left) Percentage of histone modification peaks found across each category of genomic sequence, relative to mm9 RefSeq genes. (Right) Schematic detailing the categorization (peaks classified as distal intergenic do not fall into any of the previous categories).
**Figure 2.** Gain and loss of H4K16 acetylation during ESC differentiation. (A) Normalized (average reads per million/RPM) H4K16ac (solid lines) or input (dotted lines) ChIP-seq tag counts around (~5 kb) the transcription start site (TSS) and transcription end site (TES) of genes separated into quartiles according to expression in ESC (left) or NPC (right) (from high to low [Q1–Q4]). (B) Western blot of H4K16ac in undifferentiated OS25 ESC, OS25 cells after 3 d of differentiation using retinoic acid (lanes 1 and 2), undifferentiated 46c ESC, and NPCs (lanes 3 and 4). Levels of H3 are shown for comparison. (C) UD H4K16ac, UD input, NPC H4K16ac, and NPC input profiles (RPM/bp), in 200-bp sliding windows with a 20-bp step, across the Tcl1 locus, which is silenced upon NPC differentiation. Exons are shown as boxes below the graph and the direction of transcription is indicated. (D) As in C, across the Actb locus, this maintains similarly high levels of expression in UD ESC and NPCs. At the bottom, log2 H4K16ac/input is shown at Actb established by hybridization to a custom microarray of ChIP DNA from 46c ESC and 46c NPC. Chromosome position and RefSeq gene annotations are used from July 2007 (mm9) mouse genome build (UCSC). (E) As in C, across the Foxb1 locus, this is silent in ESC, and highly expressed in NPCs. (F) RPM H4K16ac (solid lines) or input (dotted lines) ChIP-seq tag counts around (~5 kb) the TSS of genes differentially up-regulated in either ESC (ESC up genes, green lines) or NPC (NPC up genes, red lines). Data are shown for ESC (left) and NPC (right).
Figure 3. Loss of H4K16 acetylation does not correlate to chromatin compaction in vivo. (A) H4K16ac (RPM/bp in a 200-bp sliding window with a 20-bp step) across the Nanog, Sox2, and control (Hbq-Il9r) loci in undifferentiated ESC (UD, top row) and in differentiated cells (D3, bottom row). The position of fosmid probes (green and red boxes) used in FISH is indicated. Genomic maps are from the mm9 assembly of the mouse genome. (B) Example FISH images of nuclei from undifferentiated (UD; left) and differentiated (D3; left) ESCs, hybridized with probe pairs cross the Nanog, Sox2, and Hbq-Il9r loci. Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm. (C) Boxplots indicating the distribution of squared interprobe distances (d²) normalized to nuclear radius² (r²) for UD and D3 cells. Boxes show the median and interquartile range of the data; circles indicate outliers. n = 50 nuclei. Statistical significance of differences were examined by a Mann-Whitney U-test in R version 2.14.0.
Figure 4. (Legend on next page)
We performed the same analysis on ESCs differentiated for 3 d (D3) with RA. This revealed a similar reduction in H4K16ac peak height over active genes during differentiation, though here, active genes in the differentiated cell population could still be distinguished from inactive genes by H4K16ac tag density (Supplemental Fig. S2A). Similarly, genes that gained in expression in D3 cells showed modestly higher H4K16ac than genes that were more highly expressed in UD cells (Supplemental Fig. S2B). Additionally, Hox genes that are activated during RA-induced differentiation show a clear increase of H4K16ac during differentiation (Supplemental Fig. S2C).

**H4K16 acetylation does not impact upon large-scale chromatin compaction**

In vitro, acetylation of H4K16 has been shown to result in a loss of nucleosome–nucleosome interactions and a disruption of chromatin compaction in vitro (Dorigo et al. 2004; Shogren-Knaak et al. 2006; Robinson et al. 2008; Liu et al. 2011). The gross chromatin compaction defects reported in Kat8-deficient murine ESCs (Li et al. 2012) would be consistent with H4K16ac leading to chromatin decompaction in vivo. However, a direct role of H4K16ac in chromatin decompaction at specific loci has not been tested in vivo in mammalian cells.

To determine directly whether large domains of H4K16ac, and their loss during differentiation, correlate with visible changes in chromatin compaction, we analyzed genomic regions at which H4K16ac is spread over large (>100 kb) genomic domains, rather than in discrete promoter-associated peaks, in undifferentiated ESCs, and where it is lost from these regions upon differentiation. The regions chosen for analysis were those encompassing Nanog and the adjacent pluripotency gene Dppa3, and Sox2 (Fig. 3A). To assay chromatin compaction, we used FISH with pairs of probes 90–150 kb apart from each other (Fig. 3B). The mean-squared interprobe distance (d²) has a linear relationship with genomic separation (kb) over this size range and can be used to both measure changes in chromatin compaction during development and ESC differentiation (Chambeyron and Bickmore 2004; Morey et al. 2007). Moreover, such analysis can determine the role of specific epigenetic pathways and histone modifications in chromatin compaction (Eskeland et al. 2010a). Indeed, in this way we have previously demonstrated a role for polycomb repressor complexes in compacting chromatin, and a general role for elevated histone acetylation, induced by inhibiting class I and II histone deacetylases, in decompacting higher-order chromatin structure at polycomb-repressed regions (Eskeland et al. 2010a,b). Normalization to nuclear size (nuclear radius squared: r²) takes into account any global changes in the nuclear area between cell types.

Surprisingly, we detected no significant increase in chromatin compaction around the Nanog (P = 0.91) and Sox2 (P = 0.35) loci, as H4K16ac domains are lost from these regions during ESC differentiation (Fig. 3C). We therefore conclude that H4K16ac is not simply linked to the levels of higher-order chromatin compaction that are assayable by FISH.

**H4K16 acetylation marks active enhancers in embryonic stem cells**

While profiling the genome-wide location of H4K16ac peaks, we noticed that a proportion fell into intergenic locations and that the overall genomic distribution of H4K16ac is rather similar to that of H3K27ac (Fig. 1D). Given that previous studies have established certain histone modifications, including H3K27ac, as predictive factors for regulatory regions such as enhancers (Roh et al. 2005; Heintzman et al. 2007; Creyghton et al. 2010; Rada-Iglesias et al. 2011), we reasoned that the intergenic peaks of H4K16ac may predict enhancers. Indeed, a peak of H4K16ac downstream from Klf4 in ES cells (Supplemental Fig. S1E) seems to locate at the cis-regulatory sequence that corresponds to a DNase I hypersensitive site (DHS) in the UCSC Genome Browser, which has been reported to be marked by H3K4me1 (a mark considered indicative of both active and inactive enhancers), H3K27ac, and EP300 in ES cells (Shen et al. 2012).

To investigate this more widely, we identified active and inactive enhancers in ESCs by previously established criteria, using publicly available genome-wide ChIP-seq data for H3K4me1 and H3K27ac (Creyghton et al. 2010; Rada-Iglesias et al. 2011). The H4K16ac profile over active enhancers is very similar to that of H3K27ac (Fig. 4A), and enhancers generally defined as active (H3K4me1+/H3K27ac+) have considerably more H4K16ac than inactive ones (H3K4me1+/H3K27ac−) (Fig. 4B). H3K4me3, which is known to mark promoters rather than enhancers (Rada-Iglesias et al. 2011), and MNase digested input DNA, were used as negative controls.

At the genetically defined ESC active enhancer for Igll1/lambdab5 (Liber et al. 2010), located between the Igll1 and Vpbe1 genes, there is no H3K4me3, but there is a peak of H4K16ac along with the previously defined enhancer marks, H3K4me1 and H3K27ac, in the Igll1–Vpbe1 intergenic region, coincident with a DHS (Fig. 4C). Conversely, at the Mnx1 locus, the inactive motor neuron-specific enhancer (Nakano et al. 2005) is marked by H3K4me1, but not H4K16ac or H3K27ac.

Globally, H4K16ac tag density and peak width are higher on promoters compared with enhancers, but at most pluripotency genes the H4K16ac level on enhancers is as high as it is on the promoters of these genes (Supplemental Fig. S3).

We noticed that a number of enhancers with lower levels of H3K27ac showed little to no H4K16ac in ESCs, while other enhancers defined as “inactive” due to their lack of H3K27ac showed H4K16ac. This suggests that some enhancers may be marked as...
active by one histone acetylation mark or the other. To quantify this, we defined distal H3K4me1+/H3K4me3– regions that overlapped either H3K27ac or H4K16ac, giving a total of 16,947 putatively active enhancers. A total of 37% of these regions overlap, but 2180 regions are unique to H4K16ac and 8420 are unique to H3K27ac (Fig. 4D). Of the 2180 H3K27ac independent regions, 74% are also acetylated at H4K16ac in the replicate data set from an independent ES cell line. The number of H4K16ac+/H3K27ac– regions was significantly enriched at enhancers compared with the genome-wide level (P < 0.01, Fisher’s exact test). An example of an H4K16ac+/H3K4me1+/H3K27ac– region at the Sgsm1 locus is shown in Figure 4E.

Finally, we wanted to examine whether the presence of H4K16ac on active enhancers was also a feature of differentiated cells. We therefore identified active and inactive enhancers using publicly available data for H3K4me1 and H3K27ac in NPCs (Meissner et al. 2008; Creyghton et al. 2010). We do not find that H4K16ac is present on active or inactive enhancers in this cell type (Fig. 4F). In addition, we looked at the NPC histone modification profile of a genetically defined active neural Sox10 enhancer (Antonellis et al. 2008). The region shows clear peaks of H3K27ac and H3K4me1, but has no enrichment of H4K16ac (Fig. 4G).

H4K16ac and KAT8 define a class of enhancers independent of H3K27ac and EP300

Although most H4K16 acetylation is thought to be mediated by KAT8 (Smith et al. 2005; Taipale et al. 2005; Li et al. 2012), it is possible that other enhancer-localized HATs, such as EP300, could catalyze H4K16ac at regulatory regions. Therefore, we used publicly available ChIP-seq data (Wang et al. 2008; Li et al. 2012) to compare KAT8 and EP300 binding profiles across the ES cell genome. As expected from the H4K16ac profile, and similar to EP300, KAT8 shows a much higher peak over the TSS of active genes than for inactive genes (Fig. 5A). Moreover, like EP300, KAT8 is also generally enriched around enhancers defined as active (H3K27ac+), but not inactive (H3K27ac–) (P < 2.2 × 10–16 for KAT8 and EP300) (Fig. 5B). Genome wide, peaks of H4K16ac have a greater overlap with KAT8 (71%) than with EP300 (58%), with 53% of H4K16ac peaks overlapping both HATs (Fig. 5C). A small number of KAT8 independent H4K16ac peaks are overlapped by EP300 (5%), compared with 18% of EP300 independent H4K16ac peaks that overlap KAT8. However, the lower sequencing depth of the EP300 data set may result in some false negatives.

Figure 5. (Legend on next page)
In some cases, for example, the intragenic enhancer in Kndc1 upstream of Utf1, KAT8 binding mirrors the profile of H4K16ac, H3K27ac, and H3K4me1 better than does EP300 (Fig. 5D).

There is also a small enrichment of KAT8 over enhancers that would have been defined as inactive based upon the absence of H3K27ac-defined peaks. Figure 5E shows an example of a KAT8 peak in an intron of Emi1, which overlaps peaks of H4K16ac and H3K4me1 and a DHS, whereas an EP300 peak was not detected, and the level of H3K27ac does not reach the threshold for peak calling.

To demonstrate the enhancer function of KAT8 bound/ H4K16ac acetylated regulatory regions, we cloned regions with peaks of H4K16ac and H3K4me1, but not H3K27ac, into a luciferase reporter vector with a minimal promoter. As a positive control, the genetically defined (Jiang et al. 2008) ESC enhancer of Nanog (gray-shaded box in Fig. 6A) was used. The H3K4me1+/H4K16ac+/H3K27ac– regulatory region downstream from Gsx2 (AE3) and the enhancer at the 3′ end of Otop3 (AE4) are shown as examples of other tested regions (Fig. 6B,C). Regions with H3K4me1, but not H3K27ac or H4K16ac, were used as negative controls. The Nanog enhancer and all four tested distal regulatory regions that are H4K16ac+/H3K27ac– had two- to 28-fold higher luciferase activity in ESCs compared with the plasmid control. Regions with H3K4me1 peaks but not H3K27ac or H4K16ac show no activity (Fig. 6D). Therefore, we conclude that KAT8-mediated H4K16 acetylation may mark a new set of long-range regulatory elements in mammalian ESCs.

Discussion

We find a high H4K16ac level around the transcription start sites (TSS) of expressed genes in mouse ESCs (Fig. 1). Upon differentiation, H4K16ac is reduced at TSS, although the global abundance of this histone modification seems unchanged (Fig. 2). Few specific genes gain H4K16ac during differentiation: Hox genes appearing to be an exception (Supplemental Fig. S2). The genomic sites that gain H4K16ac upon differentiation of ESCs remains unclear. There may be a general redistribution across the genome so that there are no longer many “peaks” of H4K16ac evident, or there may be a redistribution toward repetitive elements. Our preliminary analysis suggests that there may be some redistribution toward intronic sequences, but this will require further investigation (Supplemental Fig. S2D).

The enrichment of H4K16ac and KAT8 that we have found at the TSS of active genes in ES cells is similar to that reported for MOF in the context of the NSL complex in female flies (Gelbart et al. 2009; Lam et al. 2012). We find no evidence for MEN1-containing MSL complex (Larschan et al. 2011).

KAT8 has been biochemically purified with several complexes associated with H3K4 methyltransferase activity—either directly with MLL1 (Dou et al. 2005) or in the context of the NSL complex (Cai et al. 2010) together with WDR5—which is required for global H3K4me3 (Wysocka et al. 2005) and is also found in MLL/SET COMPASS complexes. Histone tail cross-talk between the KAT8–MLL complex and H3K4me3 has also been reported (Wu et al. 2011). However, we find that the distributions of H4K16ac and H3K4me3 around the TSS of active genes are distinct. Whereas H3K4me3 is much higher on the +1 nucleosome than the –1, H4K16ac is more symmetrically distributed on these two nucleosomes surrounding the nucleosome free region (Fig. 1C). H3K4me3 is also generally absent from regulatory domains, indicating that at these sites, KAT8 is unlikely to be in complexes associated with H3K4me3 activity, or that the catalytic activity of the H3K4 methyltransferase is altered in such contexts, or that an H3K4me3 demethylase activity is in operation.

Although there is substantial evidence for a direct role of H4K16ac in reducing levels of chromatin compaction mediated by nucleosome–nucleosome interactions in vitro (Dorigo et al. 2004; Shogren-Knaak et al. 2006; Robinson et al. 2008; Allahverdi et al. 2011; Liu et al. 2011), we did not detect any visible changes in higher-order chromatin compaction in vivo at loci (Nanog and Sox2), where large domains of H4K16ac are lost upon ESC differentiation (Fig. 3). However, we cannot rule out a role for H4K16ac in decompaction of chromatin at a smaller local nucleosomal level that is not amenable to analysis by FISH. Since H4K16ac has been shown to be important in mediating interactions between adjacent nucleosomes (Allahverdi et al. 2011; Liu et al. 2011), we suggest that the levels of chromatin compaction that can be assayed by FISH may instead be due to interactions between chromatin fibers. Our data suggest that the grossly aberrant chromatin compaction apparent in Kat8-deficient embryos and ESCs (Thomas et al. 2008; Li et al. 2012) may be an indirect effect of pancellular loss of this important factor, rather than a direct consequence of the loss of H4K16ac on chromatin compaction.

Surprisingly, like H3K27ac, we found that H4K16ac is enriched on active, but not inactive enhancers in ESCs (Figs. 4, 5). H3K27ac has generally been considered to be catalyzed by EP300 (Jin et al. 2011). However, we found enhancer elements where the KAT8 distribution more closely mirrors that of the H3K27ac than does EP300 (Fig. 5D,E). At these sites H3K27ac may therefore be catalyzed by another HAT (such as CREBBP/CBP), or by KAT8 in a context in which its substrate specificity has been relaxed (Cai et al. 2010).

Creighton et al. (2010) suggested the existence of H3K27ac marked enhancers devoid of H3K4me1 (18% of H3K27ac peaks >2 kb from a TSS). Similarly, we found that in ESCS 59% of similarly distal H4K16ac peaks are devoid of H3K4me1.

Importantly, we also found some co-occurrence of H3K4me1-modified regulatory elements with H4K16ac, but not H3K27ac, suggesting that these regions could be novel enhancers in ESCs marked.
by KAT8-mediated H4K16ac, but not EP300-mediated H3K27ac (Fig. 6B,C). We validated five of these newly identified sites and found that they are indeed functionally active regulatory elements in reporter assays in ESCs (Fig. 6D).

We suggest that H4K16ac represents a new marker of active enhancers in ESCs, which can be independent of H3K27ac. As H4K16ac is a very abundant histone modification in some cell types, it is unlikely to make an efficient/specific marker for enhancers in its own right, but only in combination with other histone modifications or the presence of specific DNA-binding proteins. We note that H3K9K14ac was reported at the Igll1/lambda5 enhancer (Szutorisz et al. 2005) in ESCs, and also that a recently studied histone mark—H3K122ac, catalyzed by EP300/CREBBP—is found at active enhancers in the MCF7 human breast cancer cell line (Tropperger et al. 2013). The KAT2A (GCN5)/KAT2B (PCAF) containing the ATAC complex has also been shown to be bound at enhancers—both those marked by EP300 and those that are EP300 independent (Krebs et al. 2011). Although in this later case a histone substrate for the HAT activity of ATAC was not defined at the enhancers, these studies, together with our data, all point to a much broader role for histone acetylation and for different HATs in long-range gene regulation.

Methods

Cell culture

46c (Sox1-GFP) ESCs (Ying et al. 2003) were cultivated without feeders in 10% FCS and differentiated into neural progenitor cells (NPCs), essentially as described previously (Pollard et al. 2006). On day 0 of differentiation, 46c cells were seeded onto gelatinized dishes in N2B27 medium (0.5×B27supplement, 0.5×N2supplement, 0.2 mM L-glutamine, 0.1 mM β-mercaptoethanol, Neurobasal medium:DMEM/F12 medium 1:1). Differentiating cells were trypsinized on day 5 and Sox1–GFP-positive+ cells were isolated using Fluorescence Activated Cell Sorting (FACS) on a BD FACSAriaII SORP (Becton Dickinson). BD FACSDiva software (Version 6.1.3) was used to control the instrument and analyze the data.

OS25 ESCs were cultured and differentiated by LIF withdrawal for 1 d and treatment with retinoic acid (RA) for 2 d (Morey et al. 2007).

Chromatin immunoprecipitation

The specificity of the anti-H4K16ac antibody (cat# 07-329 Millipore) was tested with a modified histone peptide array (Supplemental Fig. S1A) as described previously (Pradeepa et al. 2012). Native ChIP and assessment by hybridization to custom microarrays were as described previously (Eskeland et al. 2010a; Pradeepa et al. 2012). For the studies in OS25 cells, the following modifications were used to preserve histone acetylation levels. Cells were treated for 2 min with Sirtinol (5 μM) prior to trypsi-
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ChIP and sequencing analysis

ChIP-sequencing and image processing pipeline for OS25 H4K16ac was carried out at the Gene Pool sequencing facility (University of Edinburgh). For 46c H4K16ac, sequencing data was produced by The Danish National High-Throughput DNA-Sequencing Center. In addition, we obtained the following publicly available data sets in SRA lite format from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/): ESC H3K4me1, H3K27ac, H3K4me3, EP300, NPC H3K27ac (GSE24164) (Creighton et al. 2010), NPC H3K4me1 (GSE11172) (Meissner et al. 2008), NPC H3K4me3 (GSE12241) (Mikkelsen et al. 2007), and ESC KAT8 (GSE37268) (Lii et al. 2012). SRA-lite format files were converted to FASTQ using the SRA-toolkit fastq-dump tool, and all FASTQ files were aligned to the NCBI mm9 genome assembly using Bowtie (Langmead et al. 2009), allowing a maximum of two mismatches per read. Only sequences that mapped to one location were kept. Reads aligned for H4K16ac ChIP were, in millions; UD OS25 Input, 105; D3 Input 57; UD OS25 H4K16ac 79; D3 H4K16ac 36. UD 46c Input 142, UD 46c H4K16ac 124, NPC Input 140, NPC H4K16ac 111. Peaks were detected using SICER (Zang et al. 2009), utilizing M-Nase-digested ChIP input DNA as background control for H4K16ac, and input DNA for KAT8 (window size, 200 bp; fragment size, 150 bp; false discovery rate, 0.01, gap size H4K16ac, H3K4me1, 600 bp, H3K27ac, H3K4me3, 200 bp). Wiggle tracks were generated in a WCB Edinburgh Galaxy server (http://galaxy.psu.edu/) using a 200-bp window with a 20-bp step normalized to reads per million reads per base pair (RPB). DNase I hypersensitive sites were taken from the UCSC Genome Browser mm9 (July 2007) assembly from the UW ENCODE group (Sabo et al. 2004).

Tag density graphs over TSS and TES were generated using custom perl scripts, and the coverageBed utility of the BEDTools suite, and normalized to RPM/bp. H4K16ac peak localization throughout the genome was analyzed using the CEAS suite (Shin et al. 2009).

Expression analysis

RNA was extracted from UD and D3 differentiated ESs and analyzed by RT-PCR as described previously (Eskeland et al. 2010a). Microarray data sets for UD ESCs were generated using Illumina Mouse WG-6 v2.0 Expression BeadChip. RNA was labeled using the Total Prep RNA Amplification Kit (Ambion), and array hybridization and data extraction were carried out by the Edinburgh Welcome Trust Clinical Research Facility. Probe intensities were averaged across three biological replicates. The top 500 genes with a detection P-value of <0.001 were used as a sample of active genes, while the 500 genes with the lowest intensities and a detection P-value of >0.9 were defined as a sample of inactive genes.

Differential expression between UD and D3 OS25s was analyzed using Agilent whole mouse genome 4 × 44 k oligo microarrays. TRIzol-extracted RNA was Cy3 labeled using the Amino Allyl Message Amp II kit (Ambion), hybridized to arrays, and scanned using a NimbleGen MS200 scanner. Spot intensities were extracted using an Array-Prod Analyzer, quantile normalization, and calculation of log2 fold change, and adjusted P-values were performed in Limma (Smyth et al. 2005). Genes differentially expressed with an adjusted P-value of <0.005 were used for H4K16ac tag density analysis. For differential expression between 46c UD ESCs and day 5 NPCs, RNA was extracted using a High Pure RNA Isolation kit (Roche), Cy3 labeled as above, hybridized to an Agilent whole mouse genome 8 × 60k oligomicroarray, and scanned as above. Spot intensities were extracted using Agilent Feature Extraction software version 11.5.1.1 and the data were analyzed as above.

Western blotting

The 0.5 × 10⁶ ESC, NPC, and D3 cells were washed in ice-cold PBS, lysed in NP-40 lysis buffer at 4°C for 30 min, then boiled in SDS loading dye. Proteins were loaded onto a 4%–20% gradient polyacrylamide gel and transferred to a Hybond-P membrane (GE Healthcare). Membranes were probed with antibodies to detect α H4K16ac (1: 10000, Millipore) and α H3 (1:10000, Abcam). Blots were detected using the ChemiGlow West Chemiluminescence Substrate Kit (Protein Simple) on an Image Quant LAS4010 (Version 1, Build 1.0.0.52; GE Healthcare).

FISH and image analysis

FISH for the analysis of chromatin compaction, image analysis, and statistical
evaluation of mean-squared interpropeptide distances were as described previously (Eskeland et al. 2010a). Details of fosmid probes used are given in Supplemental Table S1.

Luciferase reporter assay

H4K16ac +ve putative enhancer regions were PCR amplified from E14 ESC genomic DNA (primer details in Supplemental Table S2) and cloned into the pGL4.26 luciferase reporter plasmid (Promega). Sequences of all clones were verified. The plasmids were then cotransfected with pRL-TK into E14 ESCs using lipofectamine 2000 transfection reagent. Forty-eight hours after transfection, a Luciferase assay was performed using the Dual-Luciferase Reporter Assay System protocol (Promega) as described by the manufacturer.

Data access

Microarray and ChIP-sequencing data for this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE43103.

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Author contributions: G.T. conducted and analyzed the ChIP-sequencing data and did the FISH. B.H.B. did the 46C ES cell differentiation. M.M.P. carried out enhancer reporter assays. R.E., M.M.P., and W.A.B. conceived and designed the experiments. G.T., M.M.P., and W.A.B. wrote the manuscript.

References