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Balancing of Histone H3K4 Methylation States by the Kdm5c/SMCX Histone Demethylase Modulates Promoter and Enhancer Function

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SUMMARY

The functional organization of eukaryotic genomes correlates with specific patterns of histone methylations. Regulatory regions in genomes such as enhancers and promoters differ in their extent of methylation of histone H3 at lysine-4 (H3K4), but it is largely unknown how the different methylation states are specified and controlled. Here, we show that the Kdm5c/Jarid1c/SMCX member of the Kdm5 family of H3K4 demethylases can be recruited to both enhancer and promoter elements in mouse embryonic stem cells and in neuronal progenitor cells. Knockdown of Kdm5c deregulates transcription via local increases in H3K4me3. Our data indicate that by restricting H3K4me3 modification at core promoters, Kdm5c dampens transcription, but at enhancers Kdm5c stimulates their activity. Remarkably, an impaired enhancer function activates the intrinsic promoter activity of Kdm5c-bound distal elements. Our results demonstrate that the Kdm5c demethylase plays a crucial and dynamic role in the functional discrimination between enhancers and core promoters.

INTRODUCTION

The methylation state of lysines in histones has been linked to the establishment and maintenance of epigenetic and transcriptional states of genes (Bonasio et al., 2010; Greer and Shi, 2012). Transcription start sites (TSSs) are marked by trimethylation of lysine 4 of histone H3 (H3K4me3), which facilitates access and assembly of RNA polymerase II (pol II) transcription complexes (Vermeulen et al., 2007; Wysocka et al., 2006). In contrast, H3K4me1 modification combined with acetylated H3K27 (H3K27ac) is predictive of active enhancers (Bonn et al., 2012; Buecker and Wysocka, 2012; Creyghton et al., 2010; De Santa et al., 2010; Ernst et al., 2011; Heintzman et al., 2007; Zentner et al., 2011), which can regulate the transcriptional activity of genes over larger genomic distances (Banerji et al., 1981; Bulger and Groudine, 2011; Ong and Corces, 2011). A variety of protein complexes capable of binding H3K4me3 have been identified, but specific H3K4me1 binders seem sparse (Jeong et al., 2011; Ruthenburg et al., 2007). Several SET-domain-containing enzymes, including the SET1/MLL proteins (Ruthenburg et al., 2007; Shilatifard, 2008), can achieve methylation of H3K4 to all three states. It was reported very recently that the Trithorax-related (Trr) protein from fruit flies, and by analogy the mammalian MLL3/4 proteins, are particularly important for maintaining global levels of H3K4me1 (Herz et al., 2012). H3K4 methylations can be reversed by members of the Kdm5/Jarid1 family (for K4me3/2 to K4me1) or Lsd/Kdm1 (for K4me2/1 to K4me0) family of histone demethylases (Cloos et al., 2008; Secombe et al., 2007). Genome-wide studies revealed that Kdm5a (also known as [a.k.a.] Jarid1a/Rbp2) and Kdm5b (a.k.a. Jarid1b/Plu-1) localize to promoter regions (Lopez-Bigas et al., 2008; Peng et al., 2009; Schmitz et al., 2011). In contrast, Lsd1/Kdm1a binds to both promoter and distal elements (Whyte et al., 2012), and Lsd2/Kdm1b colocalizes with H3K36 methylation in the gene bodies of actively transcribed genes (Fang et al., 2010). Recruitment of H3K4 demethylases to specific genomic loci could be mediated via intrinsic "reader" domains for modified chromatin (Greer and Shi, 2012), but gene-specific transcription factors also seem to be involved (Blair et al., 2011; Secombe et al., 2007; Tahiliani et al., 2007).

Members of the Kdm5 family have distinct biological functions (Blair et al., 2011; Varier and Timmers, 2011). Several studies have shown a direct involvement of Kdm5a and Kdm5b in cancer. Loss of Kdm5a inhibited tumorigenesis in a mouse model (Lin et al., 2011). Also, Kdm5a is overexpressed in gastric cancer and high levels of Kdm5b have been observed in breast and prostate cancer (Blair et al., 2011; Varier and Timmers, 2011). Kdm5a and Kdm5b are involved in maintenance of a slow-growing, drug-resistant population of cancer cells (Roesch

et al., 2010; Sharma et al., 2010). Other reports, however, showed that Kdm5a is required for induction of cellular senescence in several cell types (Chicas et al., 2012; Nijwening et al., 2011). Knockout of *Kdm5b*, but not of *Kdm5a*, results in early embryonic lethality in mice (Catchpole et al., 2011). The role of Kdm5b in embryonic stem cells (ESCs) and their differentiation is controversial. One study showed that Kdm5b knockdown triggers differentiation and loss of self-renewal of ESCs (Xie et al., 2011), whereas another study showed that although knockout of the *Kdm5b* gene does not affect ESC growth or morphology, Kdm5b is important for differentiation along the neuronal lineage (Schmitz et al., 2011). The *KDM5C* gene (a.k.a. *SMCX* or *JARID1C*) has been implicated in different human diseases, such as renal cancer and X-linked mental retardation (Blair et al., 2011; Dalgliesh et al., 2010). Based on their biochemical activity (me3/2 to me1), all of the Kdm5 members are expected to act as transcriptional repressors, and studies on Kdm5a and Kdm5b support this model (Blair et al., 2011; Dey et al., 2008; Iwase et al., 2007). In light of these studies, the genetic classification of the Kdm5 homolog from *Drosophila melanogaster*, *Lid*, as a Trithorax-group type of gene activator (Gildea et al., 2000) could be explained by a global increase of H3K4me3 and redistribution of H3K3me3 binding proteins such as CHD1 (Eissenberg et al., 2007).

To gain insights into the function of Kdm5c, we determined its genomic distribution in both mouse ESCs and neuronal progenitor cells (NPCs). In addition, we profiled messenger RNA (mRNA) expression and H3K4me1 and H3K4me3 chromatin marks before and after Kdm5c knockdown in ESCs. Our results indicate that transcriptional regulation by Kdm5c is dependent on the genomic element bound. At promoters, Kdm5c restricts transcriptional output, whereas Kdm5c binding to enhancers stimulates their activity. In addition, our results stress the importance of H3K4 methylation states in the control of gene expression. We propose that the functional identity of transcription regulatory regions in mammalian genomes is achieved through balancing enzymes that deposit and remove histone H3K4 methylations.

RESULTS

Genome Localization of Kdm5c in Mouse ESCs and NPCs Reveals Both Promoter-Proximal and Promoter-Distal Binding

The Kdm5c demethylase protein is expressed in mouse ESCs and NPCs. To determine the genomic localization of Kdm5c, we expressed a biotinylated version of this protein via BAC recombineering (Poser et al., 2008) in BirA-ESCs (Figures S1A–S1E). This IB10-derived ESC line coexpresses the bacterial BirA biotin ligase (Driegen et al., 2005), allowing highly selective chromatin immunoprecipitation (ChIP; van Werven and Timmers, 2006). NPCs were derived from the tagged ESC line and immunoblot analysis showed that the tagged Kdm5c protein is expressed to similar levels in NPCs compared with ESCs (Figures S1E and S2A). Streptavidin purification of Kdm5c crosslinked chromatin combined with deep sequencing identified 3,668 high-confidence Kdm5c-binding sites for ESCs and 6,656 for NPCs (false discovery rate [FDR] < 0.05). A total of 1,224 binding sites were common to both ESCs and NPCs. The accuracy of the ChIP sequencing (ChIP-seq) signals was verified for a random selection of Kdm5c peaks by ChIP-PCR (Figure S2B).

We also profiled H3K4me1 and H3K4me3 modifications in ESCs, which indicated colocalization of Kdm5c with some, but not all, of the peaks for H3K4me1 or H3K4me3 (Figure 1A). Clustering analysis using our data sets and published data sets for ESCs (Creyghton et al., 2010; Min et al., 2011; Peng et al., 2009; Schnetz et al., 2010) indicated that Kdm5c binding overlaps within regions of high, intermediate, or low levels of H3K4me3 (Figure 1B). As expected, high H3K4me3 regions (~1/3 of the total) correspond to active promoters and these regions also display with high signals for H3K27ac and for global run-on sequencing (GRO-seq), which measures ongoing transcription (Min et al., 2011). Indeed, the vast majority of these regions localize close to a TSS (Figure 1C). In contrast, regions with intermediate or low H3K4me3 levels contain lower but significant H3K27ac or GRO-seq signals and are clearly positive for H3K4me1. In addition, H3K27me3 is absent from these regions (data not shown). This pattern corresponds to putative intergenic enhancers (Buecker and Wysocka, 2012; Creyghton et al., 2010; Ernst et al., 2011; Heintzman et al., 2009; Pekowska et al., 2011; Zentner et al., 2011) and these Kdm5c peaks localize mostly >5 kb from a TSS (Figure 1C). Enhancer proteins such as p300 and Chd7 (Buecker and Wysocka, 2012; Creyghton et al., 2010; Schnetz et al., 2010) are enriched at all Kdm5c binding sites (Figures 1B and S3). Differentiation into NPCs mainly results in loss of Kdm5c binding for regions with enhancer-like characteristics (Figure 1B). Interestingly, binding of the Kdm5a paralog (Peng et al., 2009) overlaps with Kdm5c at high H3K4me3 promoter regions, but not at the putative enhancers (Figure 1B). In conclusion, Kdm5c localizes to both promoter- and enhancer-like genomic elements in ESCs.

Kdm5c Can Interact with the Gene-Specific Transcription Factors c-MYC and ELK1

Previous investigations of several Kdm5 demethylases revealed interactions with gene-specific transcription factors, such as REST and MYC, that could mediate their recruitment to specific genomic loci (Blair et al., 2011; Secombe et al., 2007; Tahiliani et al., 2007). To investigate chromatin localization for Kdm5c in ESCs, we examined the ChIP-seq peaks for enrichment of specific DNA sequences using the GREAT algorithm (McLean et al., 2010). Unexpectedly, we observed no enrichment of REST motifs in ESCs, and we also could not confirm previous interactions of Kdm5c with the CoREST complex (Tahiliani et al., 2007) in this cell system (data not shown). Instead, Kdm5c peaks for high H3K4me3 regions are enriched for DNA motifs of gene-specific transcription factors binding to proximal promoters such as SP1, MYC, and ELK1 (Figure 2A). In contrast, regions with intermediate H3K4me3 are enriched for binding motifs for the GATA transcription factors (Figure 2A), which are typical enhancer pioneering factors (Zaret and Carroll, 2011). Next, we compared the enrichment of these sequence motifs with the Kdm5c binding strength. This showed that the fraction of regions bearing binding sites for MYC and ELK1 increases with the Kdm5c ChIP-seq signal

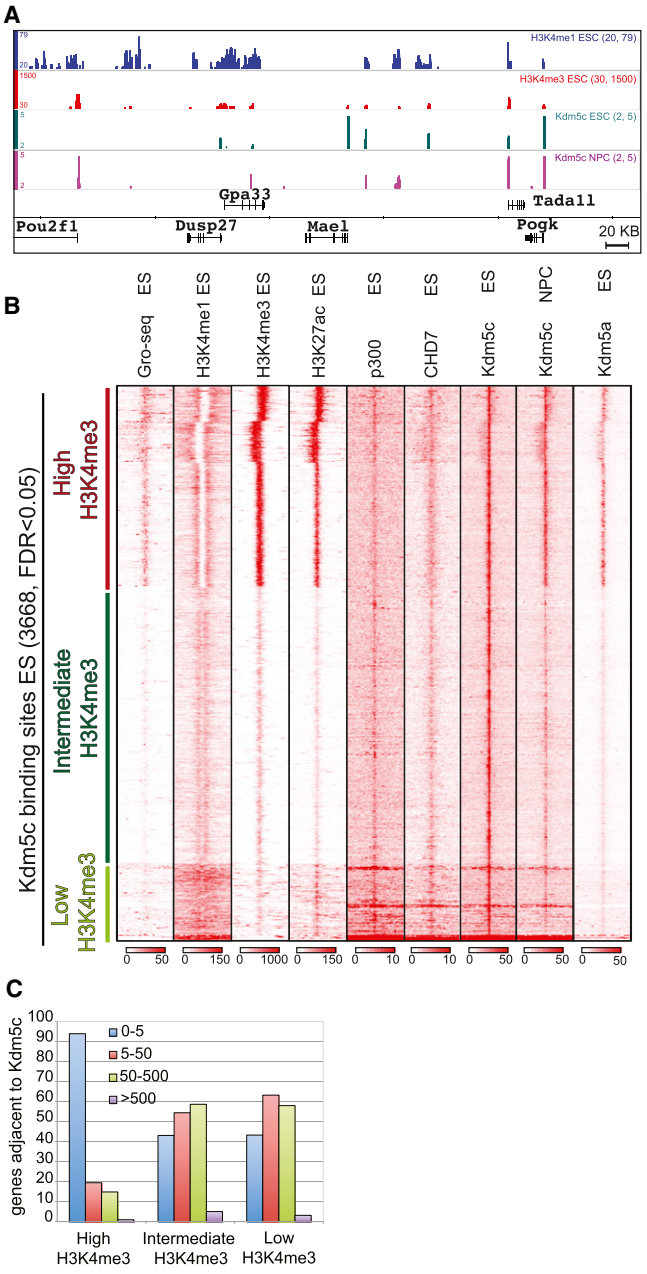

Figure 1. Kdm5c Binds to Promoters and Enhancers in Mouse ESCs

(A) Genome browser representation of the Kdm5c binding sites in ESCs and NPCs with H3K4me1 and H3K4me3 profiles of ESCs.

(B) Heat maps of ChIP-seq signals (as indicated above) surrounding the 3,668 high-confidence (FDR < 0.05) Kdm5c binding sites (±5 kb) in ESCs. Regions were classified into high-, intermediate-, or low-H3K4me3 categories. Color scales are indicated below the columns.

(C) Genes linked to the genomic regions in the three categories are shown as percentage of binding sites and ranked on the distance (in kb) from the gene nearest to the binding site. Please note that in some instances, more than one gene associates with a Kdm5c binding, resulting in a total percentage of more than 100%.

See also Figures S1, S2, and S3.

(Figures 2B and 2C). Please note that only 25 of the 6,656 Kdm5c peaks in NPC have a ChIP-seq score > 7; nine of them have the MYC motif and five contain an ELK1 motif. The correlation between GATA sites and Kdm5c binding is difficult to determine because these sites occur frequently in the mammalian genome.

Next, we examined the published c-MYC ChIP-seq data set for ESCs (Chen et al., 2008) using the same criteria as for Kdm5c. Of the 3,668 Kdm5c binding sites and 1,077 c-MYC binding sites, 356 are overlapping. Other analyses (Figures S3B and S3C) also showed a strong correlation between Kdm5c and c-MYC binding in terms of ChIP-seq scores and locations. Overlapping binding may result from a direct interaction between Kdm5c and c-MYC protein, as indicated by coimmunoprecipitation of these proteins after cotransfection in 293T cells (Figure 2D). As expected (Secombe et al., 2007), Kdm5a (and to a much lesser extent Kdm5b) also interacts with c-MYC protein. Overrepresentation of the DNA-binding motifs for ELK1 in Kdm5c regions (Figures 2A and 2C) may also result from direct protein-protein interactions. Kdm5c and Kdm5b (and to a lesser extent Kdm5a) coimmunoprecipitate with transfected ELK1 protein (Figure 2E). In contrast to c-MYC and ELK1, we could not detect Kdm5c interactions with GATA1, GATA2, GATA4, or GATA6 in coimmunoprecipitation experiments (data not shown). Recently, it was reported that recruitment of Kdm5a and Kdm5b demethylases to transcribed regions involves interaction with the chromo-domain protein MRG15 binding to H3K36me3 (Hayakawa et al., 2007; Xie et al., 2011). In contrast to its family members, Kdm5c does not interact with MRG15 (Figure S2C), and no Kdm5c peaks in the transcribed regions are apparent in the ChIP-seq data set. Together, our data indicate that c-MYC is a major recruiter for Kdm5c, and that different members of the Kdm5 demethylase family display distinct interaction profiles with several chromatin- or DNA-binding proteins.

Knockdown of Kdm5c Results in Local Increases of H3K4me3 and an Altered mRNA Expression Pattern

Recently, it was found that knockdown of Kdm5b expression in mouse ESCs results in a strong increase in global H3K4me3 with surprisingly little effect on mRNA expression (Schmitz et al., 2011). To investigate the contribution of Kdm5c to H3K4 methylation levels, we created knockdown ESC lines using two different small hairpin RNAs (shRNAs) against Kdm5c expressed from lentiviruses (Figure 3A). Global H3K4me3 levels were not affected by Kdm5c knockdown (Figure 3B), indicating that Kdm5b, and not Kdm5c, is the predominant demethylase in this cell system (Schmitz et al., 2011). This suggested a localized function for Kdm5c, which we investigated by determining ChIP-seq profiles for H3K4me3 and H3K4me1 after Kdm5c knockdown. Indeed, analyses of these profiles revealed localized increases in H3K4me3 and reductions in H3K4me1 modifications, both of which correlated with Kdm5c binding strength (Figures 3C, 3D, S4, S5A, and S5B).

Parallel mRNA profiling by DNA microarrays revealed that Kdm5c knockdown results in both activation and downregulation of gene expression (Figure 4A). We investigated whether this is correlated to the location of Kdm5c binding sites (Figures

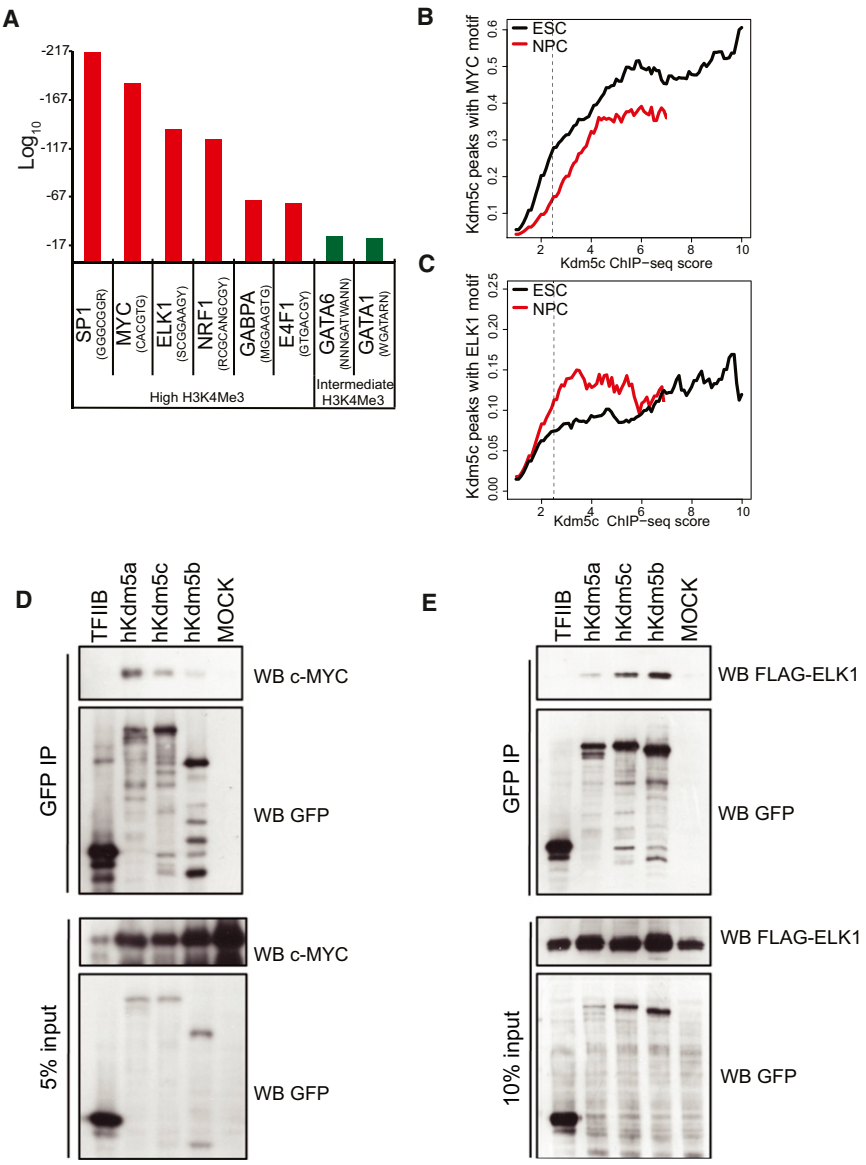

Figure 2. Direct Protein Interactions of Kdm5c with c-MYC and ELK1

(A) DNA motif enrichment analyses for the regions of different H3K4me3 categories in ESCs. Values on the y axis correspond to the $\log_{10}$ values of the binominal uncorrected p values. No motifs were enriched in regions of low H3K4me3.

(B) Proportion of Kdm5c peaks with an E-box (CACGTG) binding site for c-MYC. The dashed line indicates the approximate threshold for significant Kdm5c-binding sites (FDR < 0.05; see Extended Experimental Procedures).

(C) Proportion of Kdm5c peaks with the ELK1 motif ([GC]CGGAAG[CT] in both orientations) shown on the y axis.

(D) Interaction of GFP-fused Kdm5a, Kdm5b, and Kdm5c with c-MYC protein in HEK293T extracts. GFP fusions were precipitated using anti-GFP beads and analyzed with the indicated antibodies.

(E) Interaction of GFP fusions with ELK1.

See also Figures S2 and S3.

4B and 4C). Interestingly, genes associated with Kdm5c binding sites in their promoters that contain high H3K4me3 (as determined in Figure 1B) are predominantly upregulated. In sharp contrast, genes with distal enhancer-like binding sites (intermediate- and low-H3K4me3 regions in Figure 1B are combined) are predominantly downregulated. This effect was consistent for both Kdm5c knockdown cell lines.

Regulatory regions such as promoters and enhancers are marked by an increased accessibility for DNase1 (Buecker and Wysocka, 2012; Bulger and Groudine, 2011; Thurman et al., 2012). We compared our Kdm5c ChIP-seq data with a public ENCODE data set for DNase1 hypersensitive sites in mouse ESCs (Figure 4D). Strikingly, binding of Kdm5c in both the high and intermediate H3K4me3 regions strongly correlated with DNase1 hypersensitivity. This supports the notion that the intermediate H3K4me3 regions represent enhancers. Together with the Kdm5c knockdown, these results suggest a model in which this demethylase acts as a transcriptional repressor at promoter sites to restrict mRNA output and as an activator to maintain the activity from enhancer elements.

Kdm5c Acts As an Activator of Enhancer Function and a Repressor of Promoter Activity

We decided to test this model directly in transient assays for promoter and enhancer activity in ESCs. To this end, selected Kdm5c binding regions with an average size of ~450 bp (270–693 bp; Table S1; Figures 5A and S5C) were cloned in two luciferase reporters: pGL3-basic for promoter activity and pPou5F1 for enhancer activity (with a minimal Oct4-promoter active in ESCs). One of the selected loci corresponded to Kdm5c binding at two previously documented enhancers at the *Gata2* locus (Grass et al., 2006) and is shown in Figure 5A. Genomic screenshots of the other tested regions are shown in Figure S5C. In agreement with our hypothesis, Kdm5c knockdown reduced the enhancer activity of these regions in the pPou5F1-derived constructs (Figure 5B), and in most cases their activity was increased as a promoter element in pGL3 (Figure 5C). TFIID binding is a hallmark of active promoters and can bind directly to the H3K4me3 mark via its TAF3 subunit (Vermeulen et al., 2007). Indeed, Kdm5c knockdown increased the ChIP signal of TAF1, the largest subunit of TFIID, at most of the tested enhancer and promoter regions (Figure 5D). Together, our data show that by removing H3K4me3, the Kdm5c demethylase restricts TFIID binding, thereby regulating transcription complex assembly at both promoter and enhancer sites.

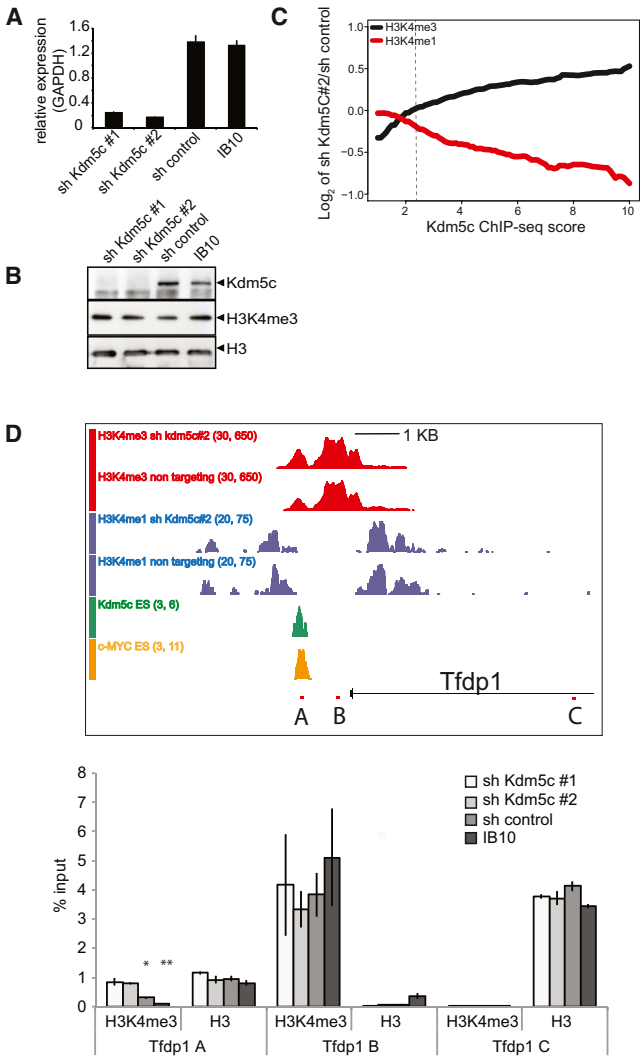

Figure 3. Knockdown of Kdm5c Affects H3K4me1 and H3K4me3 Levels around the Kdm5c Binding Sites

(A) qRT-PCR analysis of Kdm5c mRNA levels upon lentiviral knockdown.

(B) Immunoblot analysis of Kdm5c protein levels and global levels of the H3K4me3 upon stable knockdown. Two independent Kdm5c-targeting shRNAs (shKdm5c#1: 3812, and shKdm5c#2: 3813) and a nontargeting control shRNA were used. The parental ESC line (IB10) served as an additional control.

(C) The levels of H3K4me1 and H3K4me3 as defined by a ChIP-seq analysis within 500 bp of the Kdm5c peaks change upon Kdm5c knockdown. The fold change is dependent on the strength of Kdm5c binding as indicated by the Kdm5c ChIP-seq score. Values on the y axis represent the average ratios of H3K4me3 methylation in Kdm5c knockdown over nontargeting shRNA control cells. The dashed line indicates the threshold for significant Kdm5c binding sites (FDR < 0.05).

(D) Upper panel: Genome browser screenshot of the *Tfdp1* locus where a highly localized increase of the H3K4me3 levels in region A is observed in the ChIP-seq experiment. Lower panel: The data obtained from the ChIP-seq experiment were confirmed in an independent ChIP-qPCR experiment with three primers sets, indicated as A, B, and C. Data are presented as mean ± SE. The significance of differences in H3K4me3 levels was determined by an unpaired t test of the Kdm5c knockdown cell lines against the control shRNA cell line (*$p < 0.05$, **$p < 0.001$).

See also Figures S4, S5, and Table S2.

DISCUSSION

Distinct methylation states of histone H3K4 correlate with the transcriptional roles of underlying DNA elements. Our study reveals that the H3K4 demethylase Kdm5c is involved in specifying the functional identity of transcription regulatory DNA elements in the genome. Although Kdm5c can be recruited to both promoter and enhancer elements via gene-specific transcription factors (e.g., ELK1 and c-MYC), the transcriptional responses upon knockdown of Kdm5c expression are predominantly dependent on the initial H3K4 methylation state. In places with a high H3K4me3 level (i.e., gene promoters), Kdm5c binding acts to limit levels of H3K4me3, which leads to a reduced binding of activator complexes, such as TFIID and possibly others (Ruthenburg et al., 2007; Shilatifard, 2008; Vermeulen et al., 2007; Wysocka et al., 2006), and hence restricts transcription. Thus, Kdm5c has a repressive role at promoter sites similar to that of its paralogs, Kdm5a and Kdm5b (Blair et al., 2011). In contrast, at sites with relatively high levels of H3K4me1 (gene enhancers), Kdm5c maintains this modification state by removing spurious H3K4me3/2 modifications. This would prevent inappropriate loading of transcription complexes, which would compete with their binding to the nearby promoter. It is interesting to note that of the small set of genes (185) with Kdm5c peaks at both a promoter-proximal and a promoter-distal position, 11 are downregulated upon Kdmc5c knockdown and none are upregulated (data not shown). Taken together, our findings indicate that Kdm5c activity supports enhancer function, which would be consistent with the previous classification of its *Drosophila* paralog, *Lid*, as a Trithorax-group gene (Eissenberg et al., 2007; Gildea et al., 2000). This indicates that failure to remove H3K4 methylation affects developmental mRNA expression programs.

Whyte et al. (2012) recently found that Lsd1 is required for silencing of enhancers during differentiation of ESCs and is essential for complete shutdown of ESC-specific genes. Possibly, Kdm5 family members such as Kdm5b (Dey et al., 2008; Schmitz et al., 2011) and Kdm5c (this work) collaborate with Lsd1 to erase H3K4 methylation during ESC differentiation, allowing switching of gene expression programs and transition to new cell states. Together, these findings raise questions about the stability of H3K4 methylation. Although global turnover seems to be low (Zee et al., 2010), localized H3K4 methylation may be much more dynamic. Analogously to histone deacetylases (Wang et al., 2009) and in support of a dynamic turnover, the Kdm5a (Peng et al., 2009), Kdm5b (Schmitz et al., 2011), and Kdm5c (Figures 1B and 1C) demethylases colocalize with their substrates at active promoters. To determine local turnover of H3K4me3/2 states and their direct effects on transcription methylation, inhibitors targeting the Kdm5 demethylases and/or the SET1/MLL methyltransferases are needed.

The proposal that histone H3K4 methylation controls developmental switches is further supported by the observation that loss-of-function mutations in the human *KDM5C* gene are causative of X-linked mental retardation (Blair et al., 2011). In addition, somatic mutations of *KDM5C* in humans are linked to renal cancers (Dalgliesh et al., 2010). We propose that some of

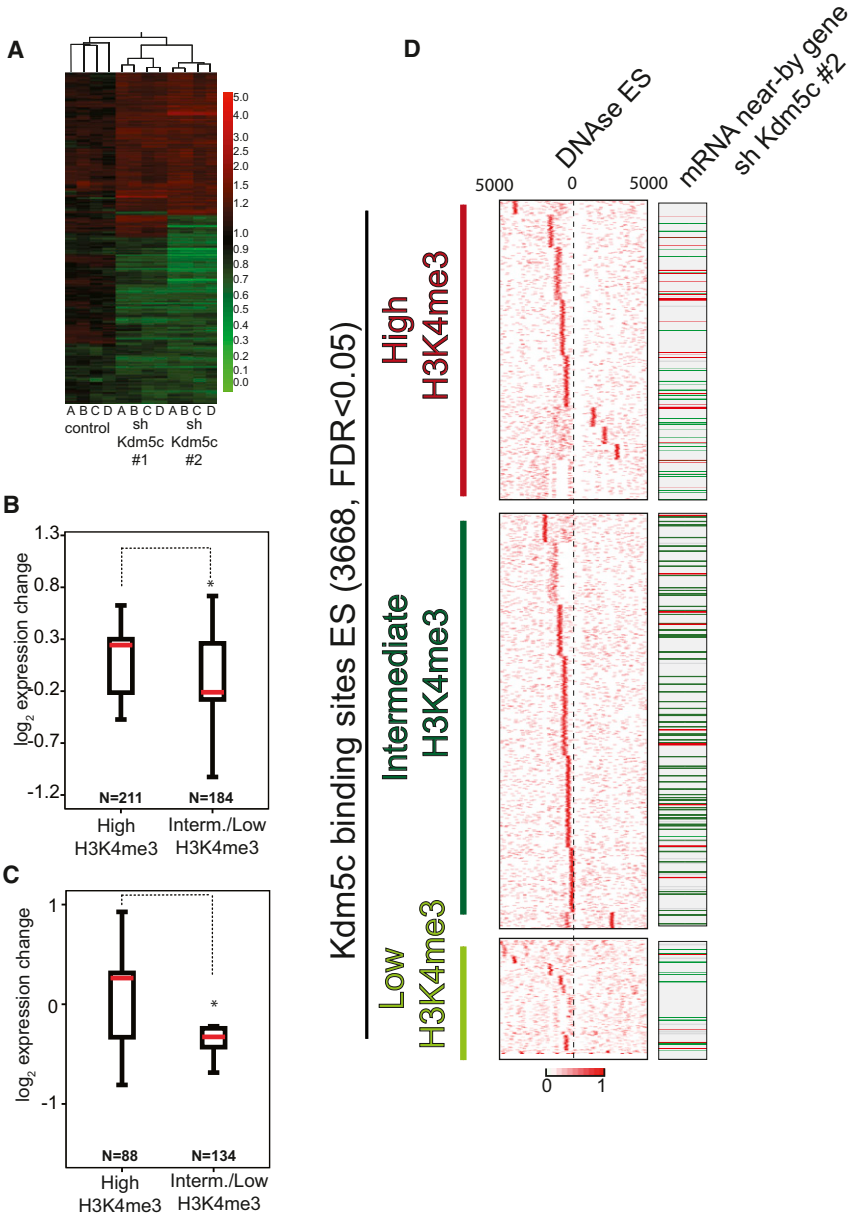

Figure 4. Transcriptional Responses upon Kdm5c Knockdown Depend on the Initial Chromatin State

(A) Heat map of the significantly changing genes that overlap in both Kdm5c knockdown cell lines, analyzed for each cell line in quadruple.

(B) Significantly changing genes (microarray, $p < 0.05$) associated to the Kdm5c binding regions with high and intermediate/low H3K4me3 levels are shown for the shKdm5c#1(3812) knockdown cell line. Significance levels of $p < 0.05$ between the gene expression changes of the high-H3K4me3 and intermediate/low-H3K4me3-region-associated genes are indicated with a star. The red lines indicate the mean values. Duplicates of multiple regions associated with the same gene were removed.

(C) Same as in (B) but for the shKdm5c#2(3813) knockdown cell line.

(D) Clustering of the DNase1 sensitivity peaks from mouse ESCs (ES-E14 ENCODE data set NCBI GEO: GSM1014154) around the Kdm5c binding sites with high, intermediate, and low H3K4me3 levels. The right lane compares transcriptional responses to Kdm5c knockdown of the nearest gene as defined in the Extended Experimental Procedures. Color code: gray, genes did not change significantly ($p > 0.05$); red, genes were significantly upregulated ($p < 0.05$); and green, genes were significantly downregulated ($p < 0.05$) genes. White indicates that no gene could be associated with this region.

the effects of these *KDM5C* mutations relate to a disrupted enhancer function. Enhancers were identified more than three decades ago (Banerji et al., 1981), but the chromatin modifications that distinguish these elements from promoters were recognized only recently (Buecker and Wysocka, 2012; Heintzman et al., 2009). This distinction may not be as clear-cut, since active enhancers in primary lymphocytes and neurons are also associated with H3K4me3 and pol II (Kim et al., 2010; Pekowska et al., 2011). Moreover, enhancer-associated pol II may not be a by-product of chromatin loops (Bulger and Groudine, 2011; Ong and Corces, 2011), as enhancer-derived RNAs resulting from bidirectional initiation events have been detected (Core et al., 2008; Kim et al., 2010; Koch and Andrau, 2011; Kowalczyk et al., 2012). Such enhancers can act as alternative tissue-specific promoters (Kowalczyk et al., 2012), which may be controlled by the H3K4 methylation status. Accordingly, we find that loss of Kdm5c can activate a latent promoter activity in enhancers.

An implication of enhancer control by Kdm5c is that this demethylase would act in concert with the H3K4 methylation enzymes to functionally catalog genomic regions destined for enhancer or promoter function. The highly localized effects of Kdm5c reducing the H3K4me3 state to H3K4me1 demonstrate that Kdm5c is not primarily responsible for establishing H3K4me1 states, but dynamically modulates H3K4me3 levels in regions that are targeted by the H3K4 methyltransferases. Recent results in *Drosophila* suggest that the MLL3/4 complexes are involved in establishing the H3K4me1 state in mammals (Herz et al., 2012). Interestingly, systematic sequencing in renal carcinoma, characterized by inactivating mutations mostly in the *VHL* gene, identified additional gene mutations in *KDM5C*, *MLL2*, and the *KDM6A/UTX* H3K27me3 demethylase gene, whose protein product is part of MLL3 and MLL4 complexes (Dalgliesh et al., 2010; Lee et al., 2007).

Overall, our work provides insight into the modulation and establishment of the different H3K4 methylation states

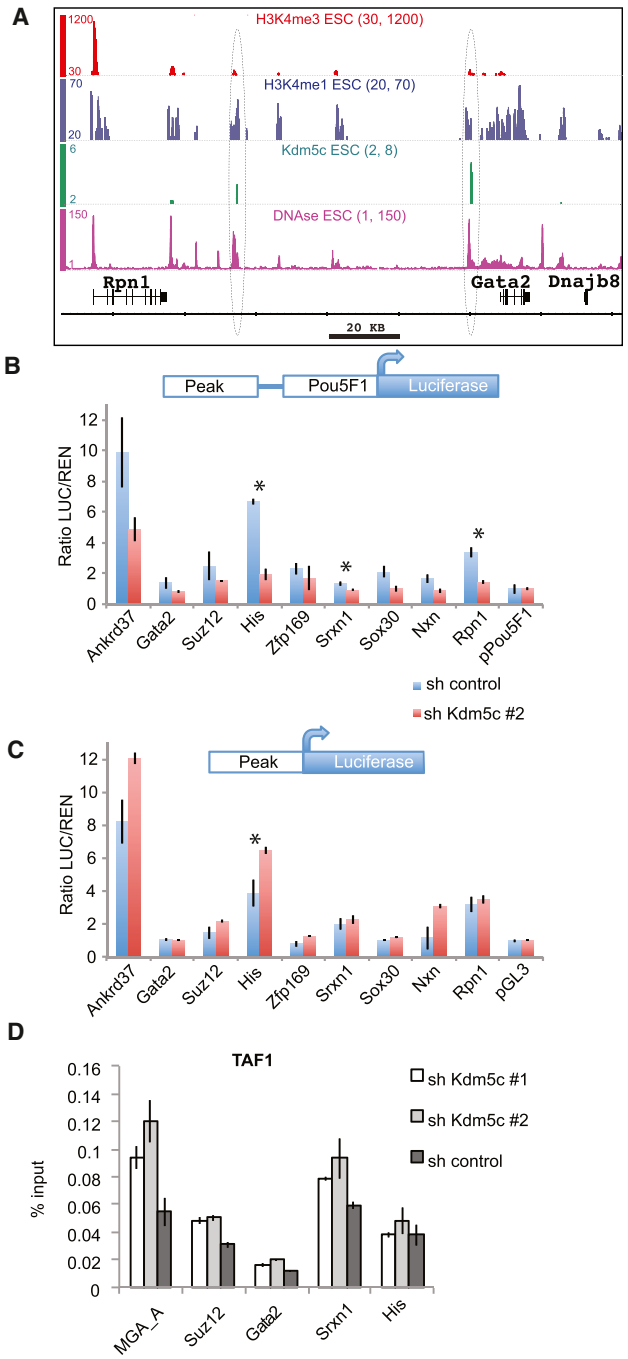

Figure 5. Kdm5c Is a Negative Regulator of Promoter Activity and a Positive Regulator of Enhancer Activity

(A) Genome browser screenshot of the Kdm5c binding sites in the *Gata2* locus together with H3K4me1, H3K4me3, and DNase1 hypersensitivity profiles. The regions analyzed in the luciferase experiments are encircled by a dotted ellipse.

(B) Normalized firefly luciferase activity in ESCs transfected with enhancer constructs containing the Kdm5c binding sites in front of the Oct4 minimal promoter (pPou5F1) in both the control and Kdm5c knockdown cell lines.

(C) Promoter activity of constructs of the same Kdm5c binding sites, driving expression of the firefly luciferase construct lacking promoter sequences (pGL3-basic).

(D) Knockdown of Kdm5c results in increased binding of TFIID. The presence of TAF1 protein on the selected loci Kdm5c binding loci was quantified by ChIP-qPCR. Data are presented as mean ± SD.

See also Figure S5 and Tables S1 and S2.

throughout the genome, the functional specification of enhancers and promoters, and the dynamics of H3K4 methylation states.

EXPERIMENTAL PROCEDURES

Cell Culture and BAC Recombineering

IB10 (E14-ESC) and its derivative BirA (Driegen et al., 2005) mouse ESCs were cultured on gelatin-coated dishes under standard conditions. A *Kdm5c* containing bacterial artificial chromosome (BAC: #RP23-391D18) was obtained from the BACPAC Resources Center (http://bacpac.chori.org). The triple-tag V5-FLAG-BIO cassette was inserted as a C-terminal in-frame fusion using BAC recombineering (Poser et al., 2008) BirA ESCs were transfected with the recombineered BAC using Lipofectamine 2000 (Invitrogen) and selected on geneticin (G418 sulfate; GIBCO). BirA and transgenic derivatives containing the tagged *Kdm5c* gene were differentiated into NPCs using the procedure described in Conti et al. (2005). Cos7 and HEK293T were cultured using standard conditions.

ChIP-Seq Data analysis

All of the ChIP-seq experiments were performed in triplicate and analyzed in a single lane on Hiseq2000. Sequence reads were mapped to the unmasked *M. musculus* genome (NCBI37; ftp://ftp.ncbi.nih.gov/genomes/M_musculus) using the SOAPv2 program (Ruffalo et al., 2011), allowing a maximum of two mismatches. Read-enriched regions were detected using CSAR (Muiño et al., 2011). Essentially, uniquely mapped reads were extended directionally 200 bp. Low-coverage regions in the control with fewer than a minimum of eight extended reads were set to this number for both Kdm5c ESCs and NPCs. Enrichment of sample to control was calculated using a ratio score. FDR thresholds were estimated by permutation of reads between the IP sample and control. Based on an FDR < 0.05, the thresholds were 2.45 for the ESC and 2.24 for the NPC line. Primers for ChIP-PCR are listed in Table S2.

Antibodies

H3K4me3 (05-745R [Millipore] for ChIP-seq or ab8580 [Abcam] for ChIP-quantitative PCR [ChIP-qPCR]), H3K4me1 (pAb-037-050; Diagenode) or H3 (ab1791; Abcam), Nanog (A300-397A-I; Bethyl), Nestin (611658; BD Biosciences), Kdm5c (A301-034A; Bethyl), GFP (11814460001; Roche), V5 (combined clones 5C5 and 10D11, a gift from Dr. Dies Meijer, Rotterdam), TAF1 (ab51540; Abcam), Lin28 (ab460200; Abcam), and MYC (05-724, clone 4A6; Millipore) antibodies were used in this study.

Data analysis

Significantly enriched Kdm5c-binding regions (FDR < 0.05) were compared with published data sets (see Results section) using seqMINER (Ye et al., 2011). Genes associated to Kdm5c binding sites (FDR < 0.05) were identified with GREAT (McLean et al., 2010) using standard basal plus extension search settings (proximal 5 kb upstream, 1 kb downstream, plus distal up to 1,000 kb). Motif enrichments within 1 kb of a Kdm5c-binding site were determined using GREAT.

Further detailed information can be found in the Extended Experimental Procedures.

ACCESSION NUMBERS

Raw data and .wig files can be found in the Gene Expression Omnibus (GEO) under accession number GSE34975. Microarray data have been deposited in ArrayExpress under accession number GSE38862.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.030.

LICENSING INFORMATION

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