ALKBH1 Is a Histone H2A Dioxygenase Involved in Neural Differentiation

RUNE OUGLAND,a DAVID LANDO,b IDA JONSON,a JOHN A. DAHL,a MARIVI NABONG MOEN,a LINE M. NORDSTRAND,a TONIJA RONGES,a JEANNIE T. LEE,c,d ARNE KLUNGLAND,a TONY KOZARIDES,b ELISABETH LARSENa

aCentre for Molecular Biology and Neuroscience, Institute of Medical Microbiology, Oslo University Hospital, Rikshospitalet and University of Oslo, Oslo, Norway; bGurdon Institute and Department of Pathology, University of Cambridge, Cambridge, United Kingdom; cDepartment of Molecular Biology, Massachusetts General Hospital, Howard Hughes Medical Institute, Boston, Massachusetts, USA; dDepartment of Genetics, Harvard Medical School, Boston, Massachusetts, USA

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ABSTRACT

AlkB homolog 1 (ALKBH1) is one of nine members of the family of mammalian AlkB homologs. Most Alkbh1+/− mice die during embryonic development, and survivors are characterized by defects in tissues originating from the ectodermal lineage. In this study, we show that deletion of Alkbh1 prolonged the expression of pluripotency markers in embryonic stem cells and delayed the induction of genes involved in early differentiation. In vitro differentiation to neural progenitor cells (NPCs) displayed an increased rate of apoptosis in the Alkbh1+/− NPCs when compared with wild-type cells. Whole-genome expression analysis and chromatin immunoprecipitation revealed that ALKBH1 regulates both directly and indirectly, a subset of genes required for neural development. Furthermore, our in vitro enzyme activity assays demonstrate that ALKBH1 is a histone dioxygenase that acts specifically on histone H2A. Mass spectrometric analysis demonstrated that histone H2A from Alkbh1−/− mice are improperly methylated. Our results suggest that ALKBH1 is involved in neural development by modifying the methylation status of histone H2A.

INTRODUCTION

Embryonic stem cells (ESCs) possess unique abilities to self-renew indefinitely and differentiate into all of the cell types of the three germ layers [1, 2]. The regulatory networks that control ESC pluripotency and self-renewal are key features in development.

The ability of ESCs to self-renew and differentiate requires a considerable degree of epigenetic plasticity. Genes crucial for pluripotency are rapidly silenced by histone modifications and DNA methylation during early differentiation, whereas genes that are required later in cellular differentiation are held in a transient repressed state by bivalent chromatin marks that are easily removed to activate transcription [3]. The maintenance of pluripotency is largely governed by the transcription factors OCT4, NANOG, and SOX2 [4–6]. Genome-wide studies have shown that these core factors occupy the promoters of actively transcribed genes encoding transcription factors and chromatin-modifying enzymes that promote ESC self-renewal. They also occupy the promoters of genes encoding regulators that are silent in ESCs but expressed during lineage commitment and subsequent differentiation [7, 8]. Although changes in DNA methylation and histone modification have been intensively explored during the earliest stages of differentiation of pluripotent cells, the mechanisms by which histone modifications influence pluripotency and development remain unknown [9].

Some years ago, the AlkB homolog 1 (ALKBH1) promoter was shown to be bound by NANOG and OCT4 in human ESCs (hESCs) [7], which implies that these key factors may regulate ALKBH1 expression. ALKBH1 is one of nine members of the newly discovered family of AlkB hydroxylases in mammals. The AlkB protein from *Escherichia coli* is a DNA repair enzyme that uses Fe(II) and 2-oxoglutarate (2OG) to hydroxylate the methyl groups associated with certain forms of DNA damage [10, 11]. The hydroxymethyl group is unstable and is spontaneously released as formaldehyde, resulting in the...
removal of the methyl group from DNA. The high degree of conservation of the AlkB sequence throughout evolution and across kingdoms suggests that AlkB homologs play an important biological role [12]. Of the nine mammalian homologs of AlkB, only four have been assigned enzymatic functions. Whereas ALKBH2 and ALKBH3 are DNA or RNA repair enzymes [13–15], ALKBH8 is involved in epigenetic regulation through its ability to modify tRNA [16, 17] and ALKBH9 (also called alpha-ketoglutarate-dependent dioxygenase (Fto)) demethylates 6-methyladenine in nuclear RNA to adenine [18]. The in vivo functions of the remaining ALKBH proteins remain elusive. In vitro, recombinant ALKBH1 can both demethylate 3-methylcytosine [19] and display DNA lyase activity [20]. It remains unclear, however, whether these two activities are physiologically relevant, and ALKBH1 is generally considered to be a nuclear enzyme with a role in epigenetic regulation [21–23]. Histone demethylases with JmjC domains use the same mechanism as AlkB to remove methyl groups from histones (Fig. 6A) [24]. Based on this shared mechanism and a protein binding motif in ALKBH1 potentially involved in binding of histones [25], it has been speculated that ALKBH1 might be a histone demethylase [11]. Recently, we showed that Alkbhl mutant mice display a complex phenotype with abnormalities in tissues originating from the ectodermal lineage, including neural tube defects such as exencephaly and spina bifida [26]. Approximately 10% of Alkbhl−/− mice appear relatively normal, whereas the most affected mice die during early embryogenesis. These findings indicate a key role for ALKBH1 in early development.

Here, we report that homozygous disruption of Alkbhl in mouse ESCs leads to the sustained expression of pluripotency markers upon differentiation and the delayed induction of neuroectodermal genes. In vitro differentiation to neural progenitor cells (NPCs) displayed an increased rate of apoptosis in the Alkbhl−/− NPCs when compared with wild-type (WT) cells. We identify genes bound and regulated by ALKBH1 using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). The majority of these genes are involved in early neural development. Furthermore, in vitro enzyme activity assays and mass spectrometric analysis of histones from Alkbhl−/− cells indicate that ALKBH1 is a histone dioxygenase that acts specifically on histone H2A. Our results suggest that ALKBH1 is involved in the epigenetic regulation of neural development by modifying the methylation status of histone H2A.

**Materials and Methods**

**Derivation, Culture, and Differentiation of Mouse ESC**

Mouse ESCs were established as described previously [51] and cultured in Knockout Dulbecco’s modified Eagle’s media (KO-DMEM) (Invitrogen 10829-018, Carlsbad, CA, USA, http:// www.stemcellstechnologies.com supplemented with 20% KO serum replacement (Invitrogen 10828-028), 100 U/ml Penicillin-Streptomycin (Invitrogen 15140-122), 0.1 mM nonessential amino acids (Invitrogen 11140-035), 2 mM GlutaMAX (Invitrogen 35050-038), 0.1 mM 2-mercaptopetanol (Sigma M7522), St. Louis, MO, USA, http://www.sigmaaldrich.com), and 1,000 U/ml leukemia inhibitory factor (LIF) (Millipore ESG1107, Billerica, MA, USA, http://millipore.com). All ESC cultures were grown on a layer of irradiated CF-1 MEFs (Globalstem GSC-6001G, Rockville, MD, USA, http://www.globalstem.com) on gelatin-coated plates. Differentiation was induced by adding 1 μM of all-trans retinoic acid (Stemgent 04-0021, Cambridge, MA, USA, https:// www.stemgent.com) and removing LIF. Proliferation and viability were assessed using the Countess Automated Cell Counter (Invitrogen C10227) with trypan blue. Cytotoxicity was induced sequentially; EBs were formed by aggregation in suspension culture for 4 days followed by treatment with all-trans retinoic acid for 4 days. EBs were then plated onto gelatin-coated plates and propagated in ITSFn medium (DMEM/F12 [Invitrogen 31330-095] containing 1× Insulin-Transferrin-Selenium-G Supplement [Invitrogen 41400-045], and fibronectin at 2.5 μg/ml [Invitrogen 33010-018]). After 5 days, the cells were dissociated into single cells and replated onto polyornithine-coated plates in B27/N2 medium (Neurobasal-A Medium [Invitrogen 10888-022]) containing 1× B27 (Invitrogen 17504-044), 0.5× N2 (Invitrogen 17502-048), 1 μg/ml laminin (Sigma L2052), and 10 ng/ml fibroblast growth factor 2 (FGF2) (Milenyi Biotec 130-093-841, Bergisch Gladbach, Germany, http://www.milenyibiotech.com). Cells were further induced toward the neural lineage by withdrawal of FGF2.

**CO2 Capture Assay**

Hydroxylation activity was determined radiochemically by measuring hydroxylation-dependent release of [14C]CO2, as described previously [52]. Standard assay conditions comprised 40 μl reactions containing 30 mM Hepes (pH = 7.5), 90 μM ZYG, 10 μM [1-14C]2OG (PerkinElmer Life Sciences, Waltham, MA, USA, http://www.perkinelmer.com), 4 mM ascorbate, 250 μM (NH4)2Fe(SO4)2, purified ALKBH1 or ALKBH1 H228A, and substrate. For each set of assays, two stocks were made. The first, with a total volume of 20 μl, contained substrate. The second contained purified enzyme and all other reagents. Assays were started by the addition of 20 μl freshly prepared enzyme stock to the substrate stock. To recover [14C]CO2, a strip of Whatman 3MM filter paper, which had been presoaked in 30 mM calcium hydroxide, was immediately inserted into the neck of the tube, and the tube was sealed. The assays were then incubated at 37°C for 60 minutes. Upon reaction completion, filter strips were removed, air-dried, treated with scintillant, and then counted for radioactivity in a scintillation counter. We then incubated 1 μg of purified ALKBH1 or ALKBH1 H228A with 10 μg of either purified HeLa core histones or core histones immunodepleted of H2A or H3. The ALKBH1 H228A variant is an inactive protein harboring a mutation in the iron-binding domain. Mutation of histidine 228 in ALKBH1 to alanine was carried out using the QuickChange site-directed mutagenesis kit (Stratagene 200518, Santa Clara, CA, USA, http://www.genomics.agilent.com). To prepare histone H2A and H3 immunodepleted samples, we incubated 100 μg of HeLa core histones overnight with 25 μg of H2A antibody (Abcam ab13923, Cambridge, United Kingdom, http://www. abcam.com) or H3 antibody (Abcam ab1791). The next day, antibody-bound histone was removed using protein-A Sepharose.

**Immunofluorescent Staining of ESCs**

Cultures of hESCs were trypsined, and the single-cell suspension was cytospun onto Superfrost Plus slides (Thermo Scientific 4951PLUS, Waltham, MA, http://www.thermofisher.com). Mouse ESCs were cultured on coverslips dispersed in 10 cm Petri dishes or cytospun onto Superfrost Plus slides. For hESCs, the following antibodies were used: rabbit anti-ALKBH1 (Abcam ab18525; 1:500), mouse anti-CTC4 (BD Biosciences 611202; 1:200, San Jose, CA, USA, http://www.bdbiosciences.com), and mouse antimitochondria [MTC02] (Abcam ab3298; 1:500). For mouse ESCs, the following antibodies were used: rabbit anti-NUCLEOPHOSMIN (Abcam ab15440; 1:100–1:1,000), rabbit anti-PML (Progressive Multifocal Leukoencephalopathy) (Millipore AB1370; 1:200–1:500), and mouse anti-NESTIN (Millipore MAB335; 1:200). Secondary antibodies used are Invitrogen Alexa Fluor 488 goat anti-rabbit A-11034 and/or Invitrogen Alexa Fluor 555 goat anti-mouse A-21242. All microscopy were done using an Axio Observer.Z1 microscope (Carl Zeiss, Thornwood, NY, USA, http://www.zeiss.com).
Apoptosis Assays
The TUNEL assay (Roche Applied Science 12156792910, Indianapolis, IN, https://www.roche-applied-science.com) was performed according to manufacturer’s instructions. For flow cytometric analysis, mESCs were stained with the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 and propidium iodide (PI) (Invitrogen V13241) according to manufacturer’s instructions. Analyses were performed on a BD Accuri C6 Flow Cytometer using the BD Accuri CFlow software. A minimum of 10,000 cells were analyzed per data point.

RT-PCR Analysis and TaqMan Low-Density Arrays
Total RNA was purified using the mirNeasy mini kit (Qiagen 217004, Valencia, CA, USA, http://www.qiagen.com) according to the manufacturer’s instructions. Any DNA remnants were removed using amplification-grade DNase 1 (Invitrogen 18068-015), and cDNA was made using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814, Carlsbad, CA, USA, http://www.appliedbiosystems.com). TaqMan Mouse Stem Cell Pluripotency Array v2 (Applied Biosystems 4385363) analysis was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System. Genes with a Ct value >32 were removed and considered not expressed. The RT-PCR reactions were carried out using a StepOnePlus instrument. For overexpression of ALKBH1 in Alkbh1−/− cells, mouse Alkbh1 was cloned into pC1-Neo (Promega E1841, Fitchburg, WI, http://www.promega.com/) and the construct was transfected into mESCs using FuGene (Roche 815 091 001) at a FuGene/DNA ratio of 6:1 (μl/μg). The transfected cells were cultured for 8 days in media supplemented with 500 μg/ml of G-418 (Invitrogen 10131) prior to RNA purification and real-time PCR analysis.

ChIP-Seq and Microarray Analysis
Total RNA was extracted from mESCs using a mirNeasy mini kit (Qiagen 217004) according to the manufacturer’s protocol. Quality was verified using an Agilent Bioanalyzer 2100 instrument (RIN value (RNA integrity number) between 9.5 and 10.0). The whole-genome gene expression profiling was performed using an Affymetrix GeneChip Mouse Genome 430 2.0 Array (Cleveland, OH, http://www.affymetrix.com). Affymetrix raw data were generated using GCOS 1.4 (GeneChip Operating Software, Affymetrix), and the signal intensities of each probe set were normalized using the robust microarray analysis algorithm. We used a t test with randomized variance (5% FDR correction) to identify differentially expressed genes. Class comparison analysis was used to identify genes for which (a) the signal in one group was always (i.e., for each the triplicate assays) higher or lower than in the other group, and (b) the fold difference in expression levels was ≥1.5-fold (log2). GO classification was done using TopGO (Bioconductor). The ChIP experiments were performed on hESCs (H9) using a mouse monoclonal anti-ALKBH1 (Sigma A8103). According to standard ChIP procedures, cells were exposed to formaldehyde to covalently cross-link protein to DNA and protein to protein to conserve interactions that are within 2 Å. Sequencing was done using an Illumina/Solexa Genome Analyzer Ix platform. Analysis was done as described previously [53]. All microarray and ChIP-seq data from this study have been submitted to the Gene Expression Omnibus (GEO) database (GSE30561).

Mass Spectrometry
Histones were purified and separated as described in Shechter et al. [54]. The H2A band was excised, digested with chymotrypsin, trypsin, and/or Asp-N, and analyzed by mass spectrometry using an LTQ-Orbitrap from Thermo Electron (Taplin MS Facility, Harvard University, Boston, MA [https://taplin.med.harvard.edu/home]).

RESULTS

ALKBH1 Is Located in the Nucleus
The cellular localization of ALKBH1 has been a matter of debate [19, 22]. Whereas Pan and colleagues reported in 2007 that mouse ALKBH1 is a nuclear protein that localizes to euchromatin, a year later, Westby and colleagues found human ALKBH1 in mitochondria and suggested that ALKBH1 was involved in the repair of mitochondrial DNA. We used a human cell line (H9) cultured under conditions favoring pluripotency and self-renewal, and performed a double immunofluorescence analysis using an anti-ALKBH1 antibody and an antibody that recognizes a 60-kDa nonglycosylated protein component of mitochondria found in human cells. In the hESC line, ALKBH1 localized predominantly to the nucleus, which is consistent with its proposed role in epigenetic regulation (Fig. 1). Only a very small fraction of ALKBH1 was found outside the nucleus, and these scarce sites of ALKBH1 accumulation did not colocalize with mitochondria.

Alkbh1 Is Dispensable for mouse ESC Self-Renewal, but Alkbh1-Deficiency Delays Differentiation
Approximately 80% of Alkbh1−/− embryos died during early development and was resorbed shortly after conception. We have previously shown that ALKBH1 is predominantly expressed during embryonic development and in adult testis, brain, and eye [26]. To further dissect the role of ALKBH1 upon differentiation, we derived Alkbh1−/− mouse ESCs (ESCs) from mouse embryos 3.5 days after gestation (E3.5) (supporting information Fig. S1). The WT and Alkbh1−/− mESCs in this study are derived from littermate E3.5 embryos after mating of heterozygous mice. The ability to either propagate ESCs in vitro in a pluripotent and self-renewing state or induce them to differentiate into any of the three germ layers [27, 28] makes them suitable for investigating lineage commitment and early differentiation. Over the course of >15 passages, mutant cells maintained a normal undifferentiated ESC morphology and stained positive for alkaline phosphatase (Fig. 2A). Data from quantitative real-time PCR (qRT-PCR) showed that the Alkbh1 mRNA level in WT mESCs grown under self-renewing conditions equaled the expression of representative pluripotency markers (Oct4 and Nanog) (Fig. 2B). Intriguingly, the transcript levels of Oct4, Nanog, and Sox2 were higher in the Alkbh1−/− mESCs than in WT mESCs (Fig. 2C). Level of Nanog transcripts was as much as fourfold higher in Alkbh1−/− mESCs than in WT mESCs, suggesting that Alkbh1−/− mESCs are refractory to differentiation. To test whether Alkbh1 expression is required for normal levels of Nanog expression, we expressed Alkbh1 in Alkbh1−/− mESCs. The mouse Alkbh1 gene was cloned in a
Alkbh1 deficiency was further confirmed by demonstrating that lineage-specific genes from all three germ layers were expressed in WT mESCs after 1 day of differentiation, whereas the neuroectodermal lineage was induced less extensively in Alkbh1−/− cells. Specifically, the induction of the SOX4 transcription factor and paired box protein 6 (PAX6) (Fig. 2G) was impaired in Alkbh1−/− mESCs. The SOX4 protein plays a central role during neuronal maturation and in ensuring the survival of neuronal cells. The PAX6 protein is crucial for the development of eyes and tissues derived from the ectodermal lineage. Representative transcript markers of mesodermal and endodermal tissues were not affected by Alkbh1 targeting (Fig. 2H, I). These data suggest that ALKBH1 is involved in the early differentiation along the neuroectodermal lineage. This notion is underscored by the phenotype of Alkbh1−/− mice.
Loss of Alkbh1 Leads to Increased Apoptosis

To further investigate the differentiation defect in the Alkbh1−/− ESCs, we characterized different aspects of differentiation (proliferation, cell death, and differentiation phenotype). Proliferation rate was analyzed by counting cells over several days (Fig. 3A). Under self-renewal conditions, there was no difference in proliferation rate and viability between the WT and Alkbh1−/−. Upon induction of differentiation, the culture of Alkbh1−/− mouse ESCs had a slower growth rate and increased cell death from differentiation day 3. (B): Timeline showing the steps of the neural differentiation protocol of mouse ESCs. (C): Alkbh1−/− mouse ESCs form embryoid bodies that are fewer and smaller than their WT counterpart. (D): After 24 days of differentiation, the mouse ESCs formed nestin-positive neural progenitor cells (NPCs). (E): TUNEL staining reveals an increased rate of apoptosis in the NPCs derived from Alkbh1−/− mESCs. (F): Ten percentage of E10.5-E11.5 Alkbh−/− embryos exhibits neural tube defects (white arrowheads) when compared with WT littermates [26]. Abbreviations: ALKBH1, AlkB homolog 1; ESC, embryonic stem cell; FGF2, fibroblast growth factor 2; LIF, leukemia inhibitory factor; RA, retinolic acid; WT, wild type.

ALKBH1 Regulates Neural Development

To better understand the dysregulation caused by Alkbh1 deficiency, we performed genome-wide expression microarray analysis on undifferentiated mESCs. Whereas 157 genes were upregulated in Alkbh1−/− mESCs relative to WT mESCs, 91 genes were downregulated (Fig. 4A). These cells were based
A 1.5-fold difference in abundance and \( p < .05 \). Hierarchical clustering of the differentially expressed genes revealed five discrete clusters of transcripts commonly regulated during the differentiation of mESCs. Two of these clusters contain genes expressed at a higher level in \( \text{Alkbh1}^{-/-} \) mESCs than in WT mESCs (upregulated genes), whereas three encode transcripts less abundant in \( \text{Alkbh1}^{-/-} \) mESCs than in WT mESCs (downregulated genes). Gene ontology (GO) analysis indicated that upregulated genes in cluster one are involved in differentiation along the neural lineage, in addition to several key markers of pluripotency. Again we identified upregulation of several key markers of undifferentiated mESCs, including \( \text{Nanog} \), \( \text{Oct4} \), and \( \text{Sox2} \) (Fig. 4B). The majority of downregulated genes in cluster two are involved in the development and differentiation of the ectodermal lineage (Fig. 4C), whereas upregulated genes in cluster three are involved in the establishment of polarity and Wnt receptor signaling. The remaining two clusters contain too few transcripts for GO analysis. We confirmed the data generated by the microarray analysis using a TaqMan Mouse Stem Cell Pluripotency Array v.2, containing probes specific for 96 transcripts (Fig. 4D). In accordance with previous results, genes involved in pluripotency and self-renewal were upregulated in the \( \text{Alkbh1}^{-/-} \) mESCs relative to WT mESCs. Additionally, compared with WT mESCs, gene expression in the ectodermal lineage was repressed in \( \text{Alkbh1}^{-/-} \) mESCs, whereas genes implicated in differentiation along mesodermal, endodermal, and trophectodermal lineages were unaffected. Consistent with previous results, we found elevated expression of ESC-specific genes, including \( \text{Oct4} \), \( \text{Nanog} \), and \( \text{Sox2} \), in \( \text{Alkbh1}^{-/-} \) mESCs differentiated for 4 days (Fig. 4E).

**Identification of ALKBH1 Target Genes**

To identify genes that are bound by ALKBH1, we performed ChIP-seq using a human ALKBH1-specific antibody (Fig. 5A). In our hands, the mouse ALKBH1-specific antibody did not work. The ChIP-seq experiments revealed 1,175 ALKBH1-occupied genomic regions in hESCs. Representative ChIP-seq data, shown for sequencing reads within \( \text{SIN3A} \), are shown in Figure 5B. The ChIP-seq results were confirmed by ChIP-qPCR (supporting information Fig. S3). Notably, no ALKBH1 sequencing reads were localized to mitochondrial DNA. This supports the observed nuclear localization of ALKBH1 (Fig. 1). Roughly 70% of all sequencing reads was
Figure 5. ChIP-seq analysis reveals that ALKBH1 occupies genes that encode developmental regulators in human embryonic stem cells. (A): The specificity of the antibody used in the ChIP-seq assay was confirmed by Western blotting of recombinant human ALKBH1 protein. (B): Image from the UCSC Genome Browser (http://genome.ucsc.edu/). This view of the paired amphipathic helix protein (SIN3A) illustrates the read distribution over one ALKBH1-binding region designated as a read. The locations of the amplexons detected by ChIP-qPCR are indicated at the bottom. Green bars represent reads from the control sample lacking antibody, whereas red bars represent reads from the ALKBH1 immunoprecipitated sample. (C): Analysis of the distribution of ALKBH1 reads over various genomic features revealed that the majority of reads reside in the gene body (70%), whereas 13% and 17% are associated with intergenic and promoter regions, respectively. (D): Gene ontology analysis of genes occupied by ALKBH1 revealed a significant enrichment for regulators of neural development. (E): Analysis by quantitative real-time PCR of a selection of the occupied genes confirmed that ALKBH1 regulates their expression. Vertical lines represent the 1 ± SEM. Abbreviation: ALKBH1, AlkB homolog 1.

Figure 5B and Figure 5C show the ChIP-seq analysis results. Figure 5D illustrates the gene ontology (GO) analysis of genes occupied by ALKBH1, highlighting the enrichment for regulators of neural development. Figure 5E confirms the regulation of selected genes by ALKBH1 through quantitative real-time PCR (qPCR).

**Histone H2A from Alkbh1<sup>−/−</sup> Cells Contains a Methylation Group Not Present in WT Histones**

The E. coli AlkB and related mammalian dioxygenases oxidize their preferred substrates by decarboxylating 2OG to form succinate and CO₂ (Fig. 6A). ALKBH1 is the mammalian AlkB homolog most similar in sequence to E. coli AlkB [39, 40]. Both E. coli AlkB and ALKBH1 are closely related to the JmjC domain-containing family proteins, which also contain a domain that binds both 2OG and Fe(II) [24]. The JmjC proteins are histone demethylases, and the JmjC domain of JmjD2 and E. coli AlkB is similar, particularly in the core domain that binds 2OG and Fe(II) (Fig. 6B). This similarity indicates that ALKBH1 may also function as a histone dioxygenase. While this article was in preparation, two studies suggested histone H2A dioxygenase activity of the Schizosaccharomyces pombe protein Ofd2 [41, 42]. Ofd2 is closely related to the human ALKBH1 protein.

To determine whether ALKBH1 has dioxygenase activity toward histones, we purified core histones from HeLa cells and incubated them with ALKBH1 in a CO₂ capture assay (Fig. 6C). We identified a substantial increase in CO₂ release when ALKBH1 was incubated with histones from HeLa cells relative to a control without substrate. The residues involved in binding to Fe(II) are well conserved in the AlkB homologs and are essential for catalytic activity [43]. To test whether the dioxygenase domain of ALKBH1 is required for activity, we mutated one of the iron-binding histidine residues to alanine and incubated the ALKBH1 H228A protein with HeLa histones. The release of CO₂ was completely abolished, thereby confirming that the dioxygenase domain is required for the activity observed on histones from HeLa cells. To determine whether H2A was the preferred substrate, we immunodepleted H2A or H3 from HeLa core histones, whereas histone H3 depletion did not affect CO₂ capture. These results confirmed the dioxygenase screen that showed H2A is a substrate for ALKBH1. A final experiment using purified histones from WT or Alkbh1<sup>−/−</sup> mESCs and CREBBB, intermediates in signaling pathways such as Wnt signaling (frizzled homolog 3 [FZD3]), and regulators of the cytoskeleton (DYNC2H1) and apoptosis (KDM2B and APAF1). To elucidate the role of ALKBH1 in regulation of these genes, we studied four genes in further detail, two associated with early development (rho-interacting, serine/threonine kinase 21 [CIT] and KDM2B lysine [K]-specific demethylase 2B [KDM2B]) [35, 36] and two at a later developmental stage (FZD3 and roundabout homolog 1 [ROBO1]) [37, 38]. WT and Alkbh1<sup>−/−</sup> mESCs, differentiated in the presence of retinoic acid for 12 days, revealed a delayed induction of the genes involved in late development, FZD3 and ROBO1. On the contrary, CIT and KDM2B, which are involved in early development, were expressed at a higher level in undifferentiated cells and maintained an increased expression upon differentiation (Fig. 5E). The former pattern of expression resembles the expression of differentiation markers (Fig. 2G) while the latter expression pattern is similar to genes involved in pluripotency (Fig. 2F). Together our results suggest that a subset of transcripts is repressed by ALKBH1, while another set is induced. The repressed genes are involved in pluripotency and early differentiation, while the induced genes are required later during development.
revealed that Alkbh1<sup>−/−</sup> histones stimulated ALKBH1 activity approximately 80% more than histones purified from WT mESCs (Fig. 6D). Along with the phenotype of Alkbh1<sup>−/−</sup> mice, our in vitro data support a role for ALKBH1 in epigenetic regulation and suggest that histone H2A is the substrate of ALKBH1 in vivo. To further substantiate this proposal, we used dot-blotting to demonstrate physical interaction between H2A and human ALKBH1 in vitro (Fig. 6E).
were 6.68 ground signal intensity) was present in the results using methylated histone peptides, likely because the on K118 or K119. We have not been able to confirm these specific for histone H2A. data suggest that ALKBH1 possesses dioxygenase activity demethylases [44]. Taken together, our in vitro and in vivo enzyme might require for function as seen for other histone peptides lack the intrinsic structure of nucleosomes that this firm confirmation of whether the dimethyl group is present to remove this methyl group. The amount of the methyl group (IAQGGVLPNIQAVLLPKKTESHH). The signal intensities for the unmethylated peptides (Fig. 6F; upper chromatograms for the WT and Alkbh1+/−, respectively) were 6.68 × 10^5 for WT and 4.30 × 10^5 for Alkbh1+/−. The lower chromatograms represent the same peptide with the addition of 28.0314 Da (the mass of a dimethyl group or two monomethyl groups). In the WT sample, the methylated peptide was undetectable, whereas a signal of 1.87 × 10^5 (approximately three to four times greater than background signal intensity) was present in the Alkbh1+/− sample. The same pattern was observed in multiple experiments. We conclude that Alkbh1+/− cells lack the enzyme to remove this methyl group. The amount of the methyl group is so low that mass spectrometry does not allow a firm confirmation of whether the dimethyl group is present on K118 or K119. We have not been able to confirm these results using methylated histone peptides, likely because the peptides lack the intrinsic structure of nucleosomes that this enzyme might require for function as seen for other histone demethylases [44]. Taken together, our in vitro and in vivo data suggest that ALKBH1 possesses dioxygenase activity specific for histone H2A.

**DISCUSSION**

Here, we have shown that ALKBH1 binds to genes encoding developmental regulators, and we provide evidence that ALKBH1 is a histone dioxygenase that acts specifically on histone H2A. Moreover, we show that ALKBH1 is involved in the differentiation of mESCs along the neural lineage, and that lack of ALKBH1 increase the rate of apoptosis in differentiating mESCs. This correlates well with the lack of silencing of key pluripotency markers and a delayed induction of early differentiation genes in Alkbh1+/− mESCs.

**Alkbh1 Deficiency Delays Differentiation**

The shutdown of the self-renewal machinery is a prerequisite for proper differentiation of ESCs. The protein NANOG is a central part of the regulatory transcriptional machinery in ESCs, where it sustains pluripotency. Forced expression of NANOG allows for autonomous self-renewal of cultured ESCs [4, 45]. Alkbh1+/− mESCs have elevated levels of Nanog transcripts relative to WT mESCs. Considering this, one would expect Alkbh1+/− mESCs to be refractory to differentiation. Upon exposure to retinoic acid, Alkbh1+/− mESCs differentiate and downregulate key markers of pluripotency, albeit later than in WT mESCs. At the same time, genes involved later in development revealed a delayed induction. The finely tuned balance between self-renewal and differentiation is governed by numerous interacting signaling pathways, and the exact mechanism(s) by which Alkbh1 deficiency induces increased Nanog expression awaits further investigation. Previously, it was shown that NANOG and OCT4 co-occupy the promoter of human ALKBH1 [7], which suggests that ALKBH1 is required for pluripotency of mESCs and/or during early differentiation. Signaling pathways often take advantage of regulatory feedback loops. However, our ChIP-seq study did not identify NANO or OCT4 as genes directly bound and regulated by ALKBH1. The effect of Alkbh1-deficiency on the pluripotency markers Nanog and Oct4 in mESCs is thus likely to be a result of downstream targets of ALKBH1 rather than a direct effect of ALKBH1 on Nanog and Oct4. Intriguingly, of the genes bound and regulated by ALKBH1 is the Jmjc domain-containing histone demethylase KDM2B which is a histone H3K36 dimethyl-specific demethylase regulating cell proliferation, cell cycle, and apoptosis [46]. KDM2B cooperates with OCT4 and is involved in cell fate determination [47]. Moreover, it was recently shown that KDM2B promotes the generation of induced pluripotent stem cells through demethylation of gene promoters, thereby enhancing the activation of early responsive genes in reprogramming [48]. This latter finding leads to the intriguing speculation that inhibition of ALKBH1 activity may contribute to increased reprogramming efficiency as well. This, however, requires further investigation.

**ALKBH1 Is a Histone H2A Dioxygenase**

Bioinformatics analysis reveals a substantial degree of homology (23% identity and 59% similarity) between human ALKBH1 and *E. coli* AlkB [49, 50]. Yet the enzymatic function and preferred substrate of ALKBH1 have remained enigmatic and diverse enzymatic functions of the ALKBH1 protein have been proposed. Partial rescue of an *E. coli alkb* mutant overexpressing ALKBH1 upon exposure to SN2-type alkylating agents (such as methyl methanesulfonate) suggested a role in repair [40], although this result has not been reproducible by others. Whereas Aas et al. failed to reveal ALKBH1 activity on methylated substrates [49], ALKBH1 has been reported to possess an apurinic/apyrimidinic (AP) lyase activity [20] that is independent of the cofactors 2OG and Fe(II) and it is unaffected by mutation of the putative metal-binding motif. The authors suggest that ALKBH1 might have dual functions that enable it to contribute to both regulation and repair. Our data show that histones stimulate the activity of recombinant human ALKBH1, and that this activity depends on histone H2A. To further substantiate this finding, we purified total histones from fibroblasts derived from WT and Alkbh1−/− mice. Mass spectrometric analysis of these histones showed the presence of a dimethyl group in the Alkbh1−/− cells that was lacking in the WT cells. We speculate that this specificity could possibly be supplied by a histone methyl transferase that methylates these novel sites in histone H2A. We, and others, have been unable to confirm these finding in vitro using synthetic histone peptides. This suggests that the observed ALKBH1 activity may require additional cofactors or interacting protein partners. It may also be that ALKBH1 activity requires correct three-dimensional folding of histone H2A.

**CONCLUSION**

Global mapping of human ALKBH1 binding sites in the ESC genome indicates that ALKBH1 is required for the...
differntiation of tissues originating from the ectodermal lineage. Differentially expressed genes in Alkbh1 and WT mESCs provide further evidence for the role of ALKBH1 in the development of the nervous system. These observations correlate well with the severe developmental abnormalities observed in Alkbh1 mice. Although aspects of pluripotency and differentiation differ between human and mouse ESCs, our data suggest that the ALKBH1-mediated regulation of the histone H2A methylation status is involved during the early stages of development in both mice and humans. This distribution is suggestive of a gene-related function for ALKBH1, presumably at the level of chromatin regulation through the epigenetic status of histone H2A K118 and/or K119.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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