A demethylation deficient isoform of the lysine demethylase KDM2A interacts with pericentromeric heterochromatin in an HP1a-dependent manner

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ABSTRACT
Histone modifications have a profound impact on the chromatin structure and gene expression and their correct establishment and recognition is essential for correct cell functioning. Malfunction of histone modifying proteins is associated with developmental defects and diseases and detailed characterization of these proteins is therefore very important. The lysine specific demethylase KDM2A is a CpG island binding protein that has been studied predominantly for its ability to regulate CpG island-associated gene promoters by demethylating their H3K36me2. However, very little attention has been paid to the alternative KDM2A isoform that lacks the N-terminal demethylation domain, KDM2A-SF. Here we characterized KDM2A-SF more in detail and we found that, unlike the canonical full length KDM2A-LF isoform, KDM2A-SF forms distinct nuclear heterochromatic bodies in an HP1a dependent manner. Our chromatin immunoprecipitation experiments further showed that KDM2A binds to transcriptionally silent pericentromeric regions that exhibit high levels of H3K36me2. H3K36me2 is the substrate of the KDM2A demethylation activity and the high levels of this histone modification in the KDM2A-bound pericentromeric regions imply that these regions are occupied by the demethylation deficient KDM2A-SF isoform.

KEYWORDS
alternative isoforms; HP1a; H3K36me2; KDM2A; pericentromeric heterochromatin

Introduction
Histone modifications are involved in various nuclear processes such as transcription, DNA repair, DNA replication, recombination or pre-mRNA splicing. Malfunction of histone modifying proteins is frequently associated with developmental defects and human diseases including cancer. Therefore, the enzymes bearing the ability to establish a histone modification (writers), to remove a histone modification (erasers), or to recognize these histone modifications (readers) have been intensively studied. However, very little attention has been paid to alternative protein isoforms of chromatin modifying proteins that arise through the action of alternative intronic promoters and thus lack some important functional domains encoded by the exons lying upstream of such intronic promoters.

In this study we focused on characterization of an alternative isoform of the lysine demethylase KDM2A. KDM2A, also known as FBXL11 or JHDM1A, is a DNA binding protein that binds directly to CpG islands in gene promoters and represses the activity of these promoters by demethylating their H3K36me2 through its N-terminal demethylase JmjC domain. Although methylation of H3K36 is known to repress spurious transcription initiation in bodies of transcriptionally active genes and it has been detected on promoters of transcriptionally repressed genes, H3K36me2 has been also shown to be associated with transcriptionally active promoters. Demethylation of promoter-associated H3K36me2 by KDM2A has been shown to result in transcriptional repression of these promoters, whereas a loss of KDM2A leads to increased levels of promoter-associated H3K36me2 and transcriptional de-repression of these promoters. KDM2A also demethylates lysine residues of non-histone proteins such as the NF-kB p65 subunit or β-catenin. KDM2A has been found to be misregulated in various cancers and its loss-of-function mouse mutants are embryonically lethal. As opposed to the full length “long form” KDM2A protein (KDM2A-LF), which contains all the
functional domains, the “short form” KDM2A protein (KDM2A-SF), lacks the N-terminal JmjC demethyla-
tion domain and therefore it is unable to function as a
demethylase.15,23,24 KDM2A is known to directly
interact with the heterochromatin protein HP1a and
the KDM2A amino acid motif necessary for this inter-
action is also present in KDM2A-SF.25–27 HP1a plays
an important role in transcriptional repression of peri-
centromeric heterochromatin, where it directly inter-
acts with H3K9me3 through its chromodomain.28–32
Although both KDM2A-LF and KDM2A-SF are
known to directly interact with HP1a, only KDM2A-
LF has been studied with regards to regulation of peri-
centromeric heterochromatin.33 Moreover, KDM2A-
SF has been found to be overexpressed in cancer cells
and to promote their proliferation most likely through
its ability to induce rDNA transcription.23,24 In this
study, we characterize KDM2A-SF more in detail and
we show that KDM2A-SF, unlike its full length can-
onical KDM2A-LF counterpart, forms distinct nuclear
heterochromatic bodies in an HP1a-dependent
manner.

Results

The KDM2A mRNA isoforms

To analyze the level of the KDM2A-SF mRNA seper-
ately from that of the the full length KDM2A-LF
mRNA, we designed an isofrm specific quantitative
RT-PCR (Q-RTPCR) assay: Based on the sequence
of the human KDM2A-LF mRNA (RefSeq: NM_012308.2) we designed a pair of primers in
KDM2A exons 12 and 13 that detect specifically just the
KDM2A-LF mRNA (Fig. 1A, black arrows, supplen-
tary table 1 primers F3 and R3). We confirmed the se-
quence of KDM2A alternative first exon 1b (RefSeq:
NM_001256405.1) by 5RACE with primers specific for
KDM2A exon 13 (supplementary table 1 primers R7
and R7b). Based on the sequence of the human
KDM2A-SF mRNA (RefSeq: NM_001256405.1) we
designed another pair of primers in KDM2A alternative
first exon 1b and exon 13 that detect specifically just the
KDM2A-SF mRNA (Fig. 1A, gray arrows, supplen-
tary table 1 primers F3b and R3).

Using the KDM2A mRNA isofrm specific Q-
RTPCR assay we determined the level of the KDM2A-
LF and KDM2A-SF mRNA isoforms in 4 human cul-
tured cell lines (HEK293T, HeLa, MCF-7, U2OS)
(Fig. 1B). This experiment revealed that the breast
carcinoma MCF-7 cells exhibit the highest level of the
KDM2A-LF and KDM2A-SF mRNAs (Fig. 1B), which
 corresponds to amplification and very high KDM2A
expression levels in various breast cancer cells.24 We
further confirmed the KDM2A-LF and KDM2A-SF
mRNAs by a northern analysis of the mRNA from the
MCF-7 cells using 3 different antisense RNA probes
that detect: a. both KDM2A mRNA isoforms, b. just the
KDM2A-LF mRNA, c. just the KDM2A-SF mRNA
(Fig. 1C). This northern and our 5RACE experiment
confirm that the KDM2A-SF mRNA is not an alterna-
tive splice variant, but that it is an alternative mRNA
that originates in KDM2A intron 12 through the
action of an alternative intronic promoter. The pres-
ence of this yet uncharacterized intronic promoter is
supported by the publicly available ChIP-seq data that
can be visualized in the UCSC genome browser.34 The
UCSC genome browser ChIP-seq tracks show that in
various human cells (e.g. MCF7, hESCs) the region
around the KDM2A exon 1b is enriched for the his-
tone modifications that are known to be associated
with transcriptionally active promoters (e.g.,
H3K27Ac and H3K4me3).1,9

We also characterized the Kdm2a exon structure
in the mouse: a 5RACE experiment with primers
specific for mouse Kdm2a exon 13 (RefSeq: NM_001001984.2, supplementary table 1 primers R8
and R8b) confirmed that an alternative Kdm2a
mRNA isoform is present also in the mouse and
originates in Kdm2a intron 12. The exon structure
of the mouse Kdm2a-SF mRNA is similar to that of the human KDM2A-SF mRNA shown in Fig. 1A.
Based on the sequence of the mouse Kdm2a-LF
mRNA (RefSeq: NM_001001984.2) we then designed
a pair of Q-RTPCR primers in exons 12 and 13 to
specifically analyze the level of the mouse Kdm2a-LF
mRNA (supplementary table 1 primers F6 and R6).
Further, we designed a pair of Q-RTPCR primers in
mouse Kdm2a exons 1b and 13 to specifically ana-
lyze the level of the mouse Kdm2a-SF mRNA (sup-
plementary table 1 primers F6b and R6). Using the
Kdm2a mRNA isofrm specific Q-RTPCR assay we
determined the level of the Kdm2a-LF and Kdm2a-
SF mRNA isoforms in various mouse adult and
embryonic tissues including mouse embryonic stem
(ES) cells (Fig. 1D). Our Q-RT-PCR experiments
showed that except for the brain front lobe, in which
the level of the KDM2A-SF mRNAs was barely
detectable, both Kdm2a mRNA isoforms were
detected in all the tested mouse adult and embryonic tissues including the mouse ES cells (Fig. 1D).

Although we tested the expression of the KDM2A-SF mRNA in human cancer cells (Fig. 1B, C), the considerable levels of the Kdm2a-SF mRNA in various mouse wild type tissues (Fig. 1D) and the fact that several human ESTs from wild type human tissues (e.g., human ES cells, GenBank CN299030.1) contain the alternative KDM2A exon 1b imply that KDM2A-SF is a naturally occurring and evolutionarily conserved isoform.

**The KDM2A protein isoforms**

To further analyze KDM2A-SF and KDM2A-LF separately, we designed an siRNA against KDM2A exon 2 (siRNA #3, supplementary table 1) to knock down the KDM2A-LF mRNA and an siRNA against KDM2A exon 1b (siRNA #5, supplementary table 1) to knock down the KDM2A-SF mRNA. Using these siRNAs we knocked down the KDM2A mRNA isoforms in the MCF-7 cells and we confirmed down-regulation of the mRNAs by Q-RTPCR (Fig. 2A).
and downregulation of the proteins by western blot (Fig. 2B).

Our in silico analysis of the sequence of the KDM2A-SF mRNA (RefSeq: NM_001256405.1) revealed 2 potential start codons that are in-frame with the downstream amino acid sequence and whose usage would create a protein with an intact DNA binding domain, one in exon 1b and one in exon 14. We in vitro translated 2 different proteins: one starting with the start codon in KDM2A exon 1b and the other one starting with the start codon in KDM2A exon 14, and we analyzed them together with the nuclear extract from the MCF-7 cells by western blot using the anti-pan-KDM2A antibody. This western blot analysis revealed that the size of the protein translated from the start codon in exon 14 corresponds to that of the endogenous approximatelly 75 kDa protein (Fig. 2C). This 75 kDa protein is strongly downregulated in the MCF-7 cells treated with the siRNA against exon 1b (Fig. 2B), which further confirms that this protein represents KDM2A-SF.

The human KDM2A alternative first exon 1b is annotated as coding (RefSeq: NM_001256405.1). However, our analysis showed that exon 1b is not coding and that the translation of KDM2A-SF starts in the third exon of the KDM2A-SF mRNA, which corresponds to KDM2A exon 14.

KDM2A-SF forms distinct heterochromatic structures in an HP1α dependent manner

Using the anti-pan-KDM2A antibody we analyzed the nuclear pattern of KDM2A by immunofluorescence. This experiment revealed that KDM2A forms distinct foci in both MCF-7 (Fig. 3A, arrows) and U2OS cells (not shown). To further characterize the KDM2A structures, we performed a series of immunofluorescence experiments, which revealed that: 1. the KDM2A nuclear bodies partially co-localize with H3K9me3 (Fig. 3B), a histone modification associated with transcriptionally silent heterochromatin1,9,35 and also with HP1α (Fig. 3C), a protein associated with pericentromeric and telomeric heterochromatin,28-32 2. the KDM2A structures localize next to CENP-A (Fig. 3D), a histone variant associated with centromeric heterochromatin.36 These results imply that the KDM2A structures are formed on pericentromeric heterochromatin. To discriminate whether the distinct
KDM2A “bodies” are formed by KDM2A-LF or by KDM2A-SF, we performed similar immunofluorescence experiments, but with the MCF-7 cells, in which we knocked down either KDM2A-LF or KDM2A-SF using siRNA #3 or siRNA #5, respectively. The KDM2A bodies were not formed when we knocked down KDM2A-SF with siRNA #5 (Fig. 3E), but they remained present after knocking down KDM2A-LF with siRNA #3 (Fig. 3F, arrows). These results show that the KDM2A structures are formed by KDM2A-SF and not by KDM2A-LF.

Since KDM2A is a CpG binding protein and the pericentromeric heterochromatin is gene poor and scarce of CpG islands, we hypothesized that
KDM2A interacts with pericentromeric heterochromatin indirectly through some additional protein. Based on the following facts we further hypothesized that this additional protein is HP1a: 1. HP1a is known to directly interact with the pericentromeric H3K9me3 modification and by doing so to participate on repressing pericentromeric heterochromatin. 28-32 2. KDM2A-LF and KDM2A-SF are known to directly interact with HP1a, through which they complex with H3K9me3. 25,27 3. KDM2A-SF partially co-localizes with both H3K9me3 and HP1a (Fig. 3B and C). To determine whether HP1a is necessary for the pericentromeric KDM2A-SF foci to form, we knocked down HP1a in the MCF-7 cells using an siRNA against HP1a exon 3 (siRNA #9, supplementary table 1) and we then analyzed the nuclear pattern of KDM2A and HP1a by immunofluorescence. This immunofluorescence experiment revealed that the KDM2A-SF bodies do not form in the absence of HP1a (Fig. 3G). Down-regulation of HP1a was confirmed at the mRNA level by Q-RTPCR and at the protein level by western blot (not shown). Taken together, our immunofluorescence data show that KDM2A-SF accumulates on pericentromeric heterochromatin in an HP1a-dependent manner.

**KDM2A-bound pericentromeric heterochromatin exhibits high levels of H3K36me2**

To complement our immunofluorescence data, we analyzed the levels of KDM2A on selected pericentromeric regions using chromatin immunoprecipitation (ChIP). These ChIP experiments revealed that the tested pericentromeric regions exhibit approximately the same KDM2A levels as those that we detected on the control CpG island-containing promoters (Fig. 4A). Using ChIP we further found that the pericentromeric regions bound by KDM2A exhibit high levels of H3K36me2 (Fig. 4B). H3K36me2 is the substrate of the demethylation activity of KDM2A-LF and its high levels on the tested KDM2A-bound pericentromeric regions imply that these regions are bound the

![Figure 4](image_url)
KDM2A-SF isoform that lacks the demethylation activity.

**Discussion**

KDM2A is a DNA binding protein that binds directly to CpG islands in gene promoters and demethylates H3K36me2 in these regions. Although H3K36me3 and H3K36me2 are associated mainly with active gene bodies, where they prevent spurious transcription initiations, H3K36me2 has been also found to be associated with transcriptionally active gene promoters and its removal by KDM2A leads to transcriptional repression of such promoters. The short demethylation-deficient KDM2A isoform KDM2A-SF lacks the N-terminal demethylation domain, but it retains the ability to bind to CpG islands. KDM2A-SF is therefore likely to compete with KDM2A-LF for the same CpG islands. By binding to a CpG island-containing promoter, KDM2A-SF is thus likely to prevent KDM2A-LF from demethylating H3K36me2 in this region and from repressing the associated promoter. KDM2A-SF would thus function as transcriptional activator and it has been indeed recently shown to induce transcription of rDNA. Moreover, both KDM2A-LF and KDM2A-SF have been shown to directly interact with HP1a. HP1a is known to be involved in transcriptional silencing of gene poor pericentromeric heterochromatin by directly interacting with H3K9me3 present in these chromatin regions. Although KDM2A has been studied with regards to regulation of pericentromeric heterochromatin, it is not clear from the published data whether the observed derepression of the pericentromeric repeats is caused by the absence of KDM2A-LF or by the absence of KDM2A-SF. Our results show that KDM2A-SF accumulates at pericentromeric regions in an HP1a-dependent manner (Fig. 3) and that the KDM2A-bound pericentromeric regions exhibit high levels H3K36me2 (Fig. 4). Although it is not possible to distinguish between KDM2A-LF and KDM2A-SF in ChIP using the anti-pan KDM2A antibody, the high levels of H3K36me2 on the KDM2A-bound pericentromeric regions indirectly imply that these regions are bound by KDM2A-SF and not by KDM2A-LF. These results suggest that KDM2A-SF is involved in transcriptional silencing of pericentromeric heterochromatin. We hypothesize that H3K36me2 is important for keeping the pericentromeric heterochromatin transcriptionally silent and that KDM2A-SF is drawn to these regions instead of KDM2A-LF to prevent H3K36me2 from being demethylated. However, we did not detect any transcriptional activity of the KDM2A-bound pericentromeric regions both after knocking down the KDM2A isoforms (data not shown).

KDM2A-SF is a naturally occurring demethylation deficient isoform of KDM2A that is expressed in various adult mouse tissues, in developing mouse embryo and in mouse ES cells (Fig. 1D). Based on the publicly available GenBank EST and RNA-seq data, KDM2A-SF is likely to be expressed also in healthy human tissues including human ES cells and it is therefore likely to play an important role during development similarly to its parologue, the short isoform of the lysing demethylase KDM2B (KDM2B-SF). A recent study shows that the knockout mice deficient for KDM2B-SF exhibit various lethal phenotypes different from those seen in the KDM2B-LF knockouts. It will be important to establish a similar knockout mouse line also for KDM2A-SF and compare its phenotype to the mice deficient for KDM2A-LF.

**Materials and methods**

**Cells**

Human cell lines (HEK293T, U2OS, HeLa, MCF-7) were grown in DMEM (ThermoFisher 31966047) with fetal bovine serum (ThermoFisher 10270106) and antibiotics (ThermoFisher 15140122) in 5% CO2 at 37°C. Mouse embryonic stem cells were grown in DMEM (Sigma D6429) with fetal bovine serum (ThermoFisher 16141079), LIF (Millipore ESG1107), MEM (Sigma M7145) and 1-Thioglycerol (Sigma M6145).

**RNA analysis**

Total RNA was prepared with TRIzol (ThermoFisher 15596026) and reverse transcribed with the SuperScript III kit system (ThermoFisher 18080051). mRNA was isolated from total RNA using the GenElute mRNA Miniprep Kit (Sigma MRN10–1KT) and analyzed by northern using the NorthernMax kit (ThermoFisher AM1940) and the Chemiluminescent Detection Module (ThermoFisher 89880). The anti-sense RNA northern probes were prepared using the
T7 RNA Polymerase (Sigma 10881767001) and the Biotin RNA labeling mix (Sigma 11685597910), and were purified with the RNeasy MinElute Cleanup Kit (QIAGEN 74204). The templates for the in vitro transcription reaction were created by RTPCR with the primers listed in supplementary table 1.

**Quantitative PCR**

Q-RT-PCR and Q-ChIP-PCR was performed using the CFX96 Touch Real-Time PCR Detection System (BIO-RAD) and iQ SYBR Green Supermix (BIO-RAD 1708880). The Q-PCR primers used are listed in supplementary table 1.

**Recombinant proteins**

The KDM2A-SF coding sequences were amplified by RT-PCR, cloned into the pCS2(+)-Flag expression construct and FLAG-tagged proteins were prepared in vitro using the TNT SP6 Quick Coupled Transcription Translation system (Promega L2080).

**Gene knockdown**

Gene specific and control Silencer Select siRNAs (Life Technologies) were transfected into various cell lines using Lipofectamine 3000 (Life Technologies L3000008) and after 48 hrs the cells were harvested and processed for the downstream applications. The siRNAs used are listed in supplementary table 1.

**Western analysis**

Protein extracts were prepared as described and proteins were resolved by SDS-PAGE and immunodetected by western blotting. The following antibodies were used: anti-KDM2A (Bethyl A301–475A) and anti-FLAG (Sigma F1804).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation was done from MCF-7 cells using the Magnify ChIP system (Life Technologies 492024) and as described previously. The following antibodies were used: anti-pan-KDM2A (anti-JHDM1A, Bethyl A301–475A), anti-H3K36me2 (Abcam ab77256), anti-H3K9me3 (Millipore 05–1250), anti-CENP-A (Abcam ab13939). The images and intensity plots were processed using the Fiji software.

**Microscopy**

Immunofluorescence experiments were done using confocal microscopy. Cells were fixed, permeabilized, immunolabelled and fluorophores were visualized on the Leica TCS SP5 confocal microscope. The following primary antibodies were used: anti-pan-KDM2A (anti-JHDM1A, Bethyl A301–475A), anti-HP1a (Abcam ab77256), anti-H3K9me3 (Millipore 05–1250), anti-CENP-A (Abcam ab13939). The images and intensity plots were processed using the Fiji software.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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