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ORIGINAL RESEARCH

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.

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Introduction

Histone modifications are involved in various nuclear processes such as transcription, DNA repair, DNA replication, recombination or pre-mRNA splicing.[1] Malfunction of histone modifying proteins is frequently associated with developmental defects and human diseases including cancer.[2] 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.[3] 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.[4,5]

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.[2,6-8] 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.[2,9-15] 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.[10,14,16-19] KDM2A also demethylates lysine residues of non-histone proteins such as the NF-kB p65 subunit or β-catenin.[20,21] KDM2A has been found to be misregulated in various cancers and its loss-of-function mouse mutants are embryonically lethal.[16,17,22]

As opposed to the full length "long form" KDM2A protein (KDM2A-LF), which contains all the

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functional domains, the “short form” KDM2A protein (KDM2A-SF), lacks the N-terminal JmjC demethylation 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 interaction is also present in KDM2A-SF.[25-27] HP1a plays an important role in transcriptional repression of pericentromeric heterochromatin, where it directly interacts 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 pericentromeric 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 canonical 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 seperately from that of the the full length *KDM2A-LF* mRNA, we designed an isoform 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, supplementary table 1 primers F3 and R3). We confirmed the sequence of *KDM2A* alternative first exon 1b (RefSeq: NM_001256405.1) by 5'RACE 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, supplementary table 1 primers F3b and R3).

Using the *KDM2A* mRNA isoform specific Q-RTPCR assay we determined the level of the *KDM2A-LF* and *KDM2A-SF* mRNA isoforms in 4 human cultured 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 5'RACE experiment confirm that the *KDM2A-SF* mRNA is not an alternative splice variant, but that it is an alternative mRNA that originates in *KDM2A* intron 12 through the action of an alternative intronic promoter. The presence 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 histone 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 5'RACE 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 analyze the level of the mouse *Kdm2a-SF* mRNA (supplementary table 1 primers F6b and R6). Using the *Kdm2a* mRNA isoform 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

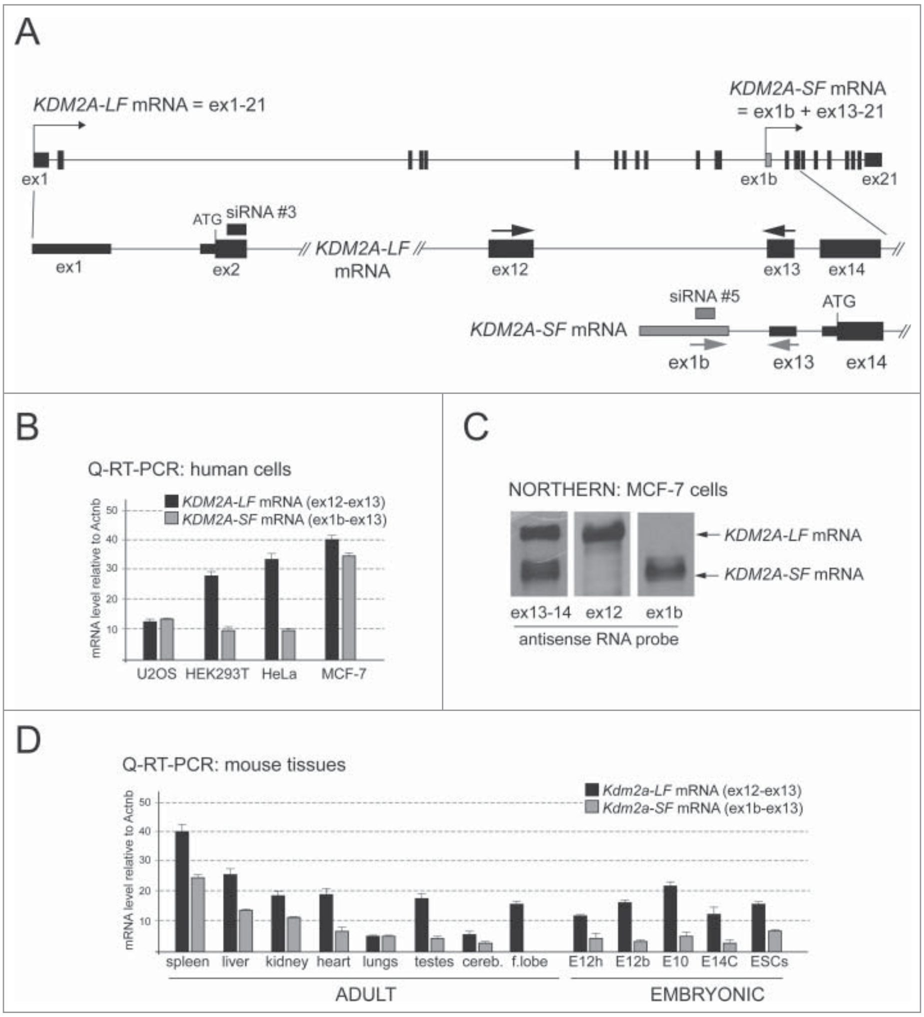

Figure 1. The KDM2A mRNA isoforms: (A) KDM2A-SF is encoded by an mRNA (RefSeq: NM_001256405.1) that originates in *KDM2A* intron 12 with the alternative first exon 1b (the gray rectangle). The rectangles represent exons and the positions of the Q-RT-PCR primers (arrows) and of the siRNAs are shown. (B) The levels of the human *KDM2A-SF* (gray bars) and *KDM2A-LF* (black bars) mRNAs were determined by Q-RT-PCR in 3 independent experiments and were related to the levels of the β-*actin* mRNA (Actnb). Similar results were obtained when the mRNA levels were related to the levels of the *GAPDH* mRNA (not shown). (C) The human *KDM2A* mRNA isoforms were detected in the MCF-7 cells by northern using a probe against *KDM2A* exons 13–14 that detects both mRNA isoforms, a probe against *KDM2A* exon 12 that detects just the *KDM2A-LF* mRNA and a probe against *KDM2A* exon 1b that detects just the *KDM2A-SF* mRNA. (D) The levels of the mouse *Kdm2a-SF* (gray bars) and *Kdm2a-LF* (black bars) mRNAs were analyzed as in B. Similar results were obtained when the mRNA levels were related to the levels of the *Hprt* mRNA (not shown). cereb.: cerebellum, f.lobe: front lobe, E12h: embryonic day 12 head, E12b: embryonic day 12 body, E10: embryonic day 10 whole embryo, E14c: embryonic day 14 cortex, ESCs: ebmryonic stem cells.

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 evolutionarilly 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)

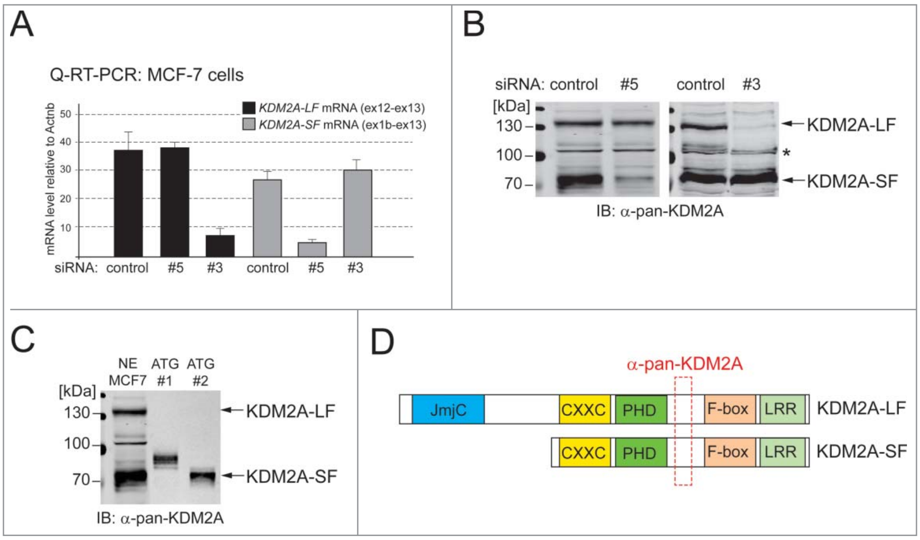

Figure 2. KDM2A isoforms and their knockdown: (A) The *KDM2A-SF* (gray bars) and *KDM2A-LF* (black bars) mRNAs were knocked down with siRNA #5 and siRNA #3, respectively. The mRNA levels, relative to the level of the β-*actin* mRNA, were determined by Q-RT-PCR in 3 independent experiments. Similar results were obtained when the mRNA levels were related to the level of the *GAPDH* mRNA (not shown) (B) Downregulation of KDM2A-SF and KDM2A-LF by siRNA #5 and siRNA #3, respectively, was confirmed by a western blot analysis of the nuclear extract from the treated MCF-7 cells using the anti-pan-KDM2A antibody. The asterisk marks a crossreactive band recognized by the KDM2A antibody. (C) *In vitro* translation from an ATG in *KDM2A* exon 14 (ATG #2) yields a protein of the same size as that of the endogenous KDM2A-SF protein detected by the anti-pan-KDM2A antibody in the nuclear extracts from the MCF-7 cells (NE MCF-7). (D) The full length KDM2A protein (KDM2A-LF, AA1–1162) contains all the functional domains, while the shorter KDM2A-SF (KDM2A AA543–1162) form lacks the N-terminal demethylation JmjC jumonji domain (in blue). The DNA binding domain is in yellow and the plant homeodomain (PHD) is in green. The epitope of the anti-pan-KDM2A antibody (Bethyl A301–475A) is shown with the red dashed rectangle.

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 HP1a dependent manner

Using the anti-pan-KDM2A antibody we analyzed the nuclear pattern of KDM2A by immunofluorescence. This experiment revealed that KDM2A forms disctinct 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 heterochromatin[1,9,35] and also with HP1a (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

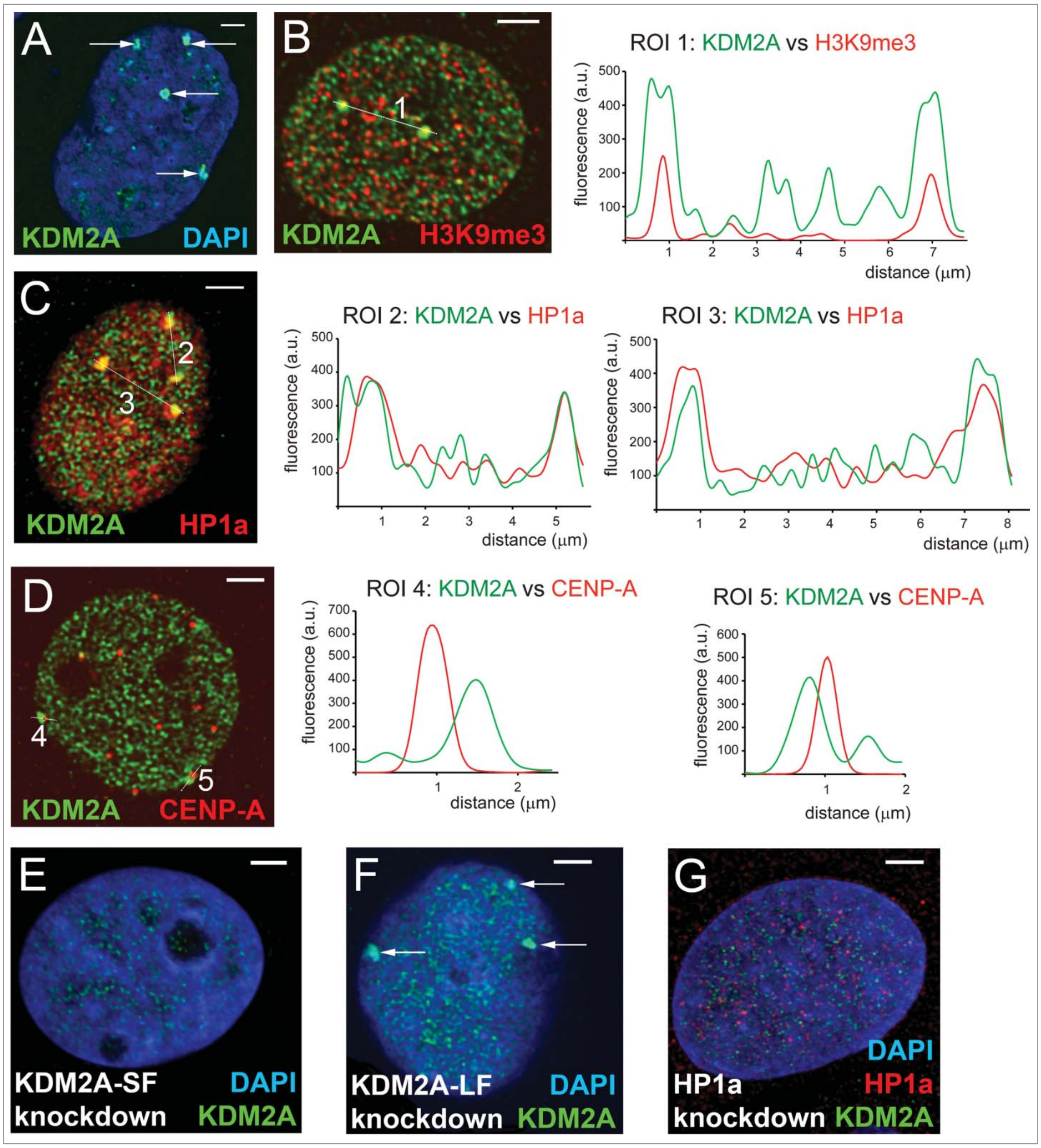

Figure 3. KDM2A-SF forms distinct heterochromatic bodies: (A) The disctinct KDM2A nuclear bodies (arrows) were detected by the anti-pan-KDM2A antibody on a confocal slice through a cell nucleus. (B) KDM2A foci partially co-localize with H3K9me3 and (C) with HP1a and are formed just next to the centromeric heterochromatin marked by CENP-A (D). (E) A maximal intensity projection of 52 z-stacks through a cell nucleus lacking KDM2A-SF shows that the KDM2A bodies are not formed in the absence of KDM2A-SF. (F) A single confocal z-stack shows that the KDM2A structures (arrows) remain intact in the MCF-7 cells lacking KDM2A-LF. (G) A maximal intensity projection of 46 z-stacks through a cell nucleus lacking HP1a shows that the KDM2A-SF bodies are not formed in the absence of HP1a. The shown images are representative images selected out of at least 5 analyzed nuclei for each condition. The images and intensity plots were processed using the Fiji software.[37] Scale bar = 3 μm.

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,[2,6-8,31,35] 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 neccessary 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-containg promoters (Fig. 4A). Using ChIP we further found that the 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

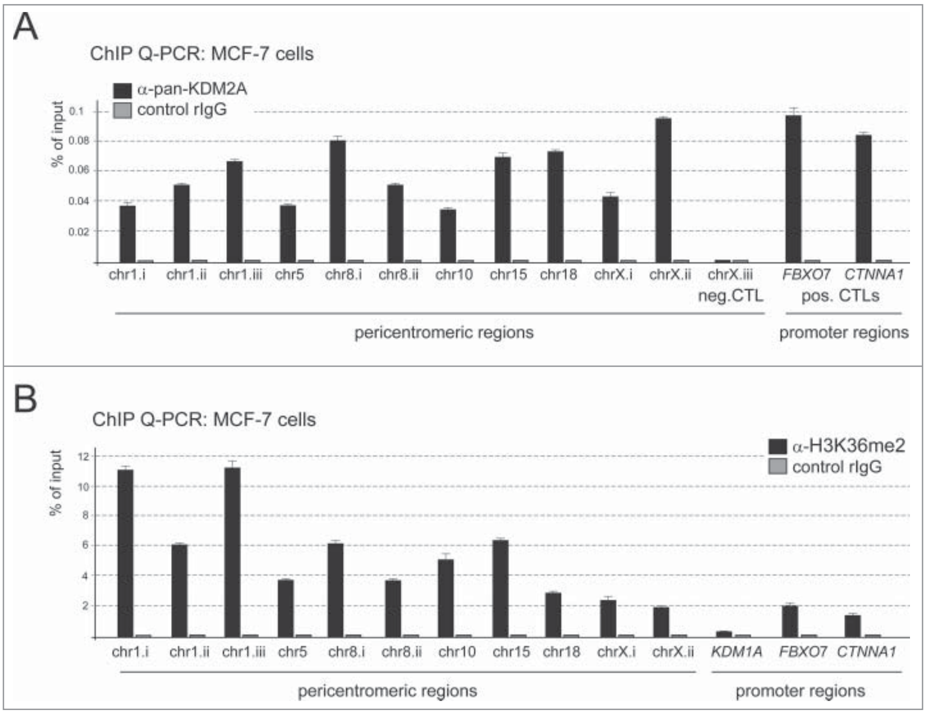

Figure 4. KDM2A bound regions and their H3K36me2 levels: (A) The elevated levels of KDM2A (black bars) on the tested pericentromeric regions were determined by 2 independent ChIP experiments and 2 independent Q-PCR reactions from each ChIP. ChIP with the control IgG did not yield any enrichment of the tested regions (gray bars). One of the regions on chromosome X (chrX.iii) does not exhibit any KDM2A binding and serves as the negative control, whereas the 2 promoter regions (FBXO7, CTNNA1) known to be bound by KDM2A serve as the positive controls. (B) The high levels of H3K36me2 (black bars) on the tested pericentromeric regions were quantified by ChIP Q-PCR as in A. No enrichment with the rabbit IgG (gray bars) confirms the specificity of the assay. In comparison to the extremely high H3K36me2 levels on the tested pericentromeric regions H3K36me2 is virtually absent on the *KDM1A* promoter, which thus serves as the negative comparison point.

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.[2,6-8] 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.[1,9-19] The short demethylation-deficient KDM2A isoform KDM2A-SF lacks the N-terminal demethylation domain, but it retains the ability to bind to CpG islands.[15,23,24] 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.[23] Moreover, both KDM2A-LF and KDM2A-SF have been shown to directly interact with HP1a.[25-27] HP1a is known to be involved in transcriptional silencing of gene poor pericentromeric heterochromatin by directly interacting with H3K9me3 present in these chromatin regions.[28-30] 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.[33] 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 paralogue, the short isoform of the lysing demethylase KDM2B (KDM2B-SF).[38] 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.[39] 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.[22]

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 antisense 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.[40] 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.[41] The following antibodies were used: anti-pan-KDM2A (anti-JHDM1A, Bethyl A301–475A), anti-H3K36me2 (Abcam ab9049), rabbit control IgG (Abcam ab46540).

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.[37]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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