[Cell Cycle 7:22, 3539-3547; 15 November 2008]; ©2008 Landes Bioscience

Report

KDM2A represses transcription of centromeric satellite repeats and maintains the heterochromatic state

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Key words: KDM2A; histone demethylation; non-coding RNAs, transcription; heterochromatin

Heterochromatin plays an essential role in the preservation of epigenetic information, the transcriptional repression of repetitive DNA elements and inactive genes, and the proper segregation of chromosomes during mitosis. Here we identify KDM2A, a JmjC-domain containing histone demethylase, as a heterochromatin-associated and HP1-interacting protein that promotes HP1 localization to chromatin. We show that KDM2A is required to maintain the heterochromatic state, as determined using a candidate-based approach coupled to an in vivo epigenetic reporter system. Remarkably, a parallel and independent siRNA screen also detected a role for KDM2A in epigenetic silencing. Moreover, we demonstrate that KDM2A associates with centromeres and represses transcription of small non-coding RNAs that are encoded by the clusters of satellite repeats at the centromere. Dissecting the relationship between heterochromatin and centromeric RNA transcription is the basis of ongoing studies. We demonstrate that forced expression of these satellite RNA transcripts compromise the heterochromatic state and HP1 localization to chromatin. Finally, we show that KDM2A is required to sustain centromeric integrity and genomic stability, particularly during mitosis. Since the disruption of epigenetic control mechanisms contributes to cellular transformation, these results, together with the low levels of *KDM2A* found in prostate carcinomas, suggest a role for KDM2A in cancer development.

Introduction

Heterochromatin is composed of highly repetitive satellite DNA and repressed genes, and this highly compacted and repressive environment is mediated by the presence of heterochromatin-associated proteins and specific epigenetic markers.[1,2] Centromeric heterochromatin contains sequences that produce small non-coding RNA molecules required for maintaining the heterochromatin state and centromeric integrity.[3-7]

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Submitted: 09/10/08; Revised: 09/23/08; Accepted: 09/24/08

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/article/7062

KDM2A is an evolutionarily conserved and ubiquitously expressed member of the KDM2 Lys histone demethylase family.[8] Because it contains a JmjC-domain and an F-box motif, this protein is also known as JHDM1A and FBXL11, respectively.[9,10] KDM2A was previously identified as a dimethyl Lys36 histone H3 (H3K36me2) histone demethylase.[10] In this study, we characterize KDM2A as an integral heterochromatin-associated protein, and we show that KDM2A plays a crucial role in silencing centromeric satellite repeats and maintaining the heterochromatic state.

Results

To begin assessing the biological function of KDM2A, we investigated its cellular localization in interphase cells by using indirect immunofluorescence analysis. KDM2A localized to the nucleus of cells and showed a punctate staining pattern throughout the nucleoplasm and perinucleolar enrichment (Fig. 1A), as compared to the human paralogue KDM2B/JHDM1B, which was previously identified as a nucleolar-resident protein.[11] Epe1, the fission yeast orthologue of KDM2, interacts with Swi6 [the yeast heterochromatin protein 1 (HP1) orthologue] and is distributed across all major heterochromatic domains, including pericentric heterochromatin.[12] To determine if the punctate staining pattern of KDM2A reflected heterochromatin association, we performed colocalization experiments with KDM2A and the HP1 variants (HP1-α, HP1-β and HP1-γ). Indirect immunofluorescence demonstrated that KDM2A partially colocalized with heterochromatin foci associated with endogenous HP1 variants (Fig. 1B). Partial colocalization of KDM2A and HP1 proteins was calculated by the Pearson (r_p) and Spearman correlation coefficients (r_s)[13] (Fig. 1B and C). Notably, KDM2A was not observed to preferentially colocalize with any of the HP1 variants, which are known to differ slightly in their heterochromatic localization.[1] Moreover, immunoprecipitation of exogenous HP1 variants showed association with endogenous KDM2A, but not KDM2B (Fig. 1D). Accordingly, an independent TAP-tagged immunopurification of KDM2A expressed in HEK-293T cells followed by mass spectrometry analysis of copurified endogenous proteins revealed the presence of peptides corresponding to HP1-α (NLD CPE LIS EFMK, SNF SNS ADDIK, LTW HAY PED AENK and WKD TDE ADL VLAK), HP1-β (GFS DED NTW EPE ENL

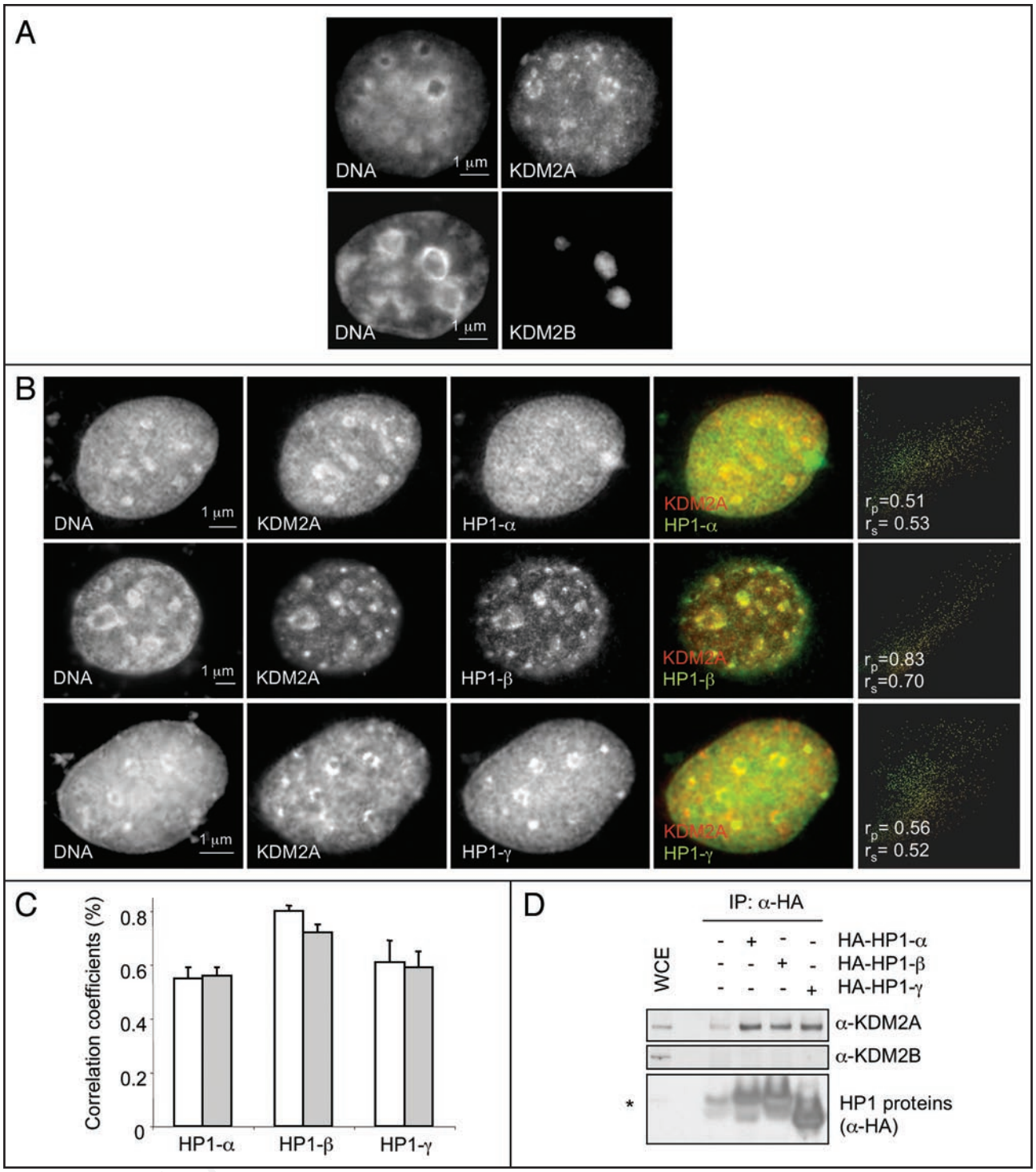

Figure 1. KDM2A localizes to heterochromatin and associates to HP1. (A) Indirect immunofluorescence analysis of HeLa cells transfected with constructs encoding FLAG-tagged KDM2A or FLAG-tagged KDM2B, as indicated. Cells were stained with an anti-FLAG antibody and DAPI. (B) Indirect immunofluorescence analysis of U2OS cells transfected with a construct encoding FLAG-tagged KDM2A. Cells were stained with an anti-FLAG antibody, antibodies to heterochromatin protein 1 variants (HP1-α, HP1-β and HP1-γ), as indicated, and DAPI. Scatter-plots used to determine Pearson (r_p) and Spearman correlation coefficients (r_s) are shown. (C) Graph shows the average colocalization coefficients between KDM2A and HP1 proteins (n = 10, +/- SD), as determined by quantification of Pearson (r_p) (open columns) and Spearman correlation coefficients (r_s) (filled columns). The value given for 100% was set as 1. (D) Endogenous KDM2A, but not endogenous KDM2B, associates with HP1 variants. The indicated HA-tagged HP1 proteins were expressed in HEK-293T cells. Whole cell extracts (WCE) were subjected to immunoprecipitation with anti-HA resin followed by immunoblotting with antibodies to the indicated proteins. The asterisk indicates the IgG light chain.

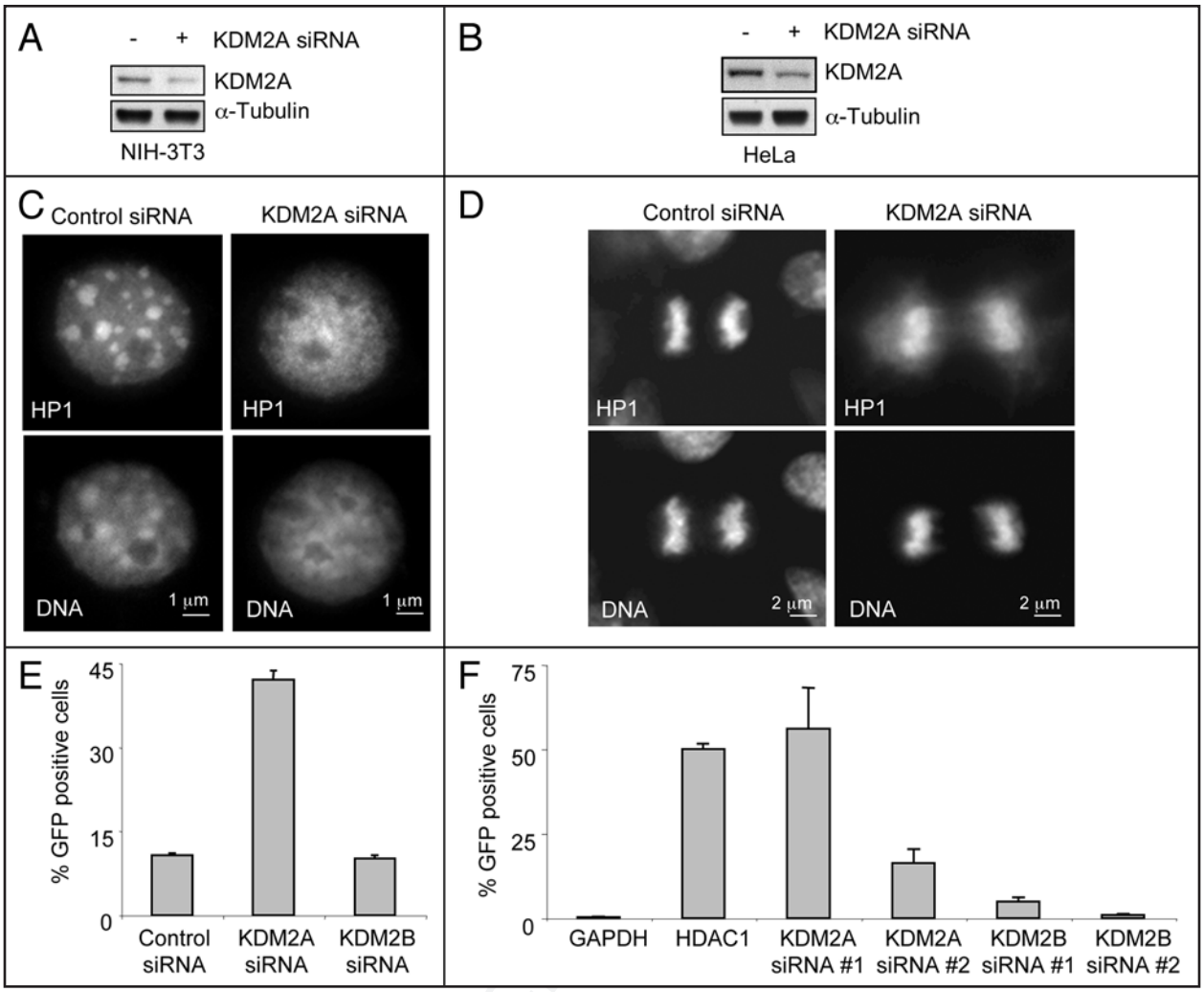

Figure 2. KDM2A maintains the heterochromatic state. (A) Representative example showing knockdown efficiency for KDM2A siRNA oligos by Western blot. Immunoblot of cell extracts from NIH-3T3 cells transfected with control oligos or KDM2A siRNA oligos. Efficacy of siRNA was assessed by immunoblotting to detect KDM2A, and α-Tubulin levels were used as loading controls. (B) Immunoblot of cell extracts from HCT-116 cells transfected with control oligos or KDM2A siRNA oligos. Efficacy of siRNA was assessed by immunoblotting as in (A). (C) Representative indirect immunofluorescence of HP1-γ in NIH-3T3 cells transfected with control oligos or KDM2A siRNA oligos. Cells were pre-extracted with TritonX-100 detergent prior to fixation and permeabilization with 100% methanol to visualize loss of HP1-γ. (D) Representative indirect immunofluorescence of HP1-γ in mitotic HeLa cells transfected with control oligos or KDM2A siRNA oligos. (E) Reactivation of a GFP reporter gene in GFP(-) HeLa cells transfected with control or KDM2A-siRNA oligos as measured by FACS. (F) Reactivation of a GFP reporter gene in GFP(-) HeLa cells transfected with control, HDAC1, KDM2A or KDM2B siRNA oligos as measured by FACS.

DCP DLI AEF LQS QK, KKV EEV LEE EEE EYV VEK, NSD EAD LVP AK and CPQ VVI SFY EER) and HP1-γ (VEE AEP EEF VVEK and LTW HSC PED EAQ). In addition, KDM2A was also independently identified by mass spectrometry analysis as an HP1-α interacting protein in a pool-down experiment using a GST-HP1-α fusion protein incubated with a HeLa nuclear extract (Kato H and Pagano M, unpublished results). Together, these data demonstrate that KDM2A is a heterochromatin-associated protein that interacts with HP1.

Heterochromatin-associated proteins have been shown to regulate HP1 localization and contribute to heterochromatin formation and maintenance in mammalian cells.[1,2,14-16] Since KDM2A was found to localize to heterochromatin and associate with HP1, we examined the status of HP1 on chromatin following KDM2A knockdown in mouse NIH-3T3 and human HeLa cells (Fig. 2A and B). Indirect immunofluorescence of HP1-γ in KDM2A knockdown mouse cells displayed a significant loss of HP1 heterochromatin-associated nuclear foci compared to control cells (Fig. 2C). Moreover, we observed a decrease in the amount of HP1-γ associated with chromatin in KDM2A siRNA-treated mitotic cells during anaphase (Fig. 2D and data not shown), at a time when HP1-γ is found to be exclusively on DNA.[17] These data suggest KDM2A knockdown results in the delocalization of HP1 from chromatin.

Because heterochromatin maintenance requires the presence of HP1 on chromatin, we sought to determine if the silencing of KDM2A compromises the overall heterochromatic state. To this end, we employed the use of epigenetically silent GFP (green fluorescent protein) reporter genes that have been integrated into the genome of HeLa cells.[18,19] In these GFP(-) HeLa reporter cells, reactivation of GFP expression occurs following heterochromatin disruption, such as after HP1-γ knockdown or via inhibition of histone deacetylases by histone deacetylase inhibitors like trichostatin A.[18,19] Using

these reporter cells, we found that knockdown of KDM2A, but not KDM2B, resulted in the robust reactivation of GFP compared to cells transfected with a control siRNA (Fig. 2E). These results suggest KDM2A is involved in heterochromatin maintenance similar to Epe1.[7,12,20,21] Strikingly, an independent siRNA library screen utilizing these GFP(-) HeLa reporter cells (performed by Poleshko A and Katz R, *manuscript in preparation*) identified *KDM2A*, but not *KDM2B*, as a gene involved in the maintenance of epigenetic silencing (Fig. 3F). Thus, these observations are consistent with a role of KDM2A as a regulator of the heterochromatic state.

All HP1 variants associate with centromeric heterochromatin.[1] Thus, we investigated whether KDM2A was associated with centromeric heterochromatin by examining the localization of KDM2A in comparison with the centromeric protein CENP-A (centromere protein A). In interphase cells, we observed partial KDM2A colocalization with CENP-A, including within the heterochromatin that surrounds the nucleolus (Fig. S1). In areas where colocalization between KDM2A and CENP-A was not observed, we detected KDM2A foci immediately adjacent to centromeric regions (Fig. S1). Partial colocalization of KDM2A and CENPA was calculated by the Pearson (r_p) and Spearman correlation coefficients (r_s)[13] (Fig. S1). Moreover, we investigated whether KDM2A was associated with centromeric heterochromatin by performing chromatin immunoprecipitation (ChIP) assays followed by quantitative real-time polymerase chain reaction (PCR) using primers spanning the pericentric region of a human chromosome (chromosome 4). KDM2A was found to bind to this region of chromosome 4 (Fig. 3A), and the specificity of this binding was confirmed by the fact that a DNA-binding mutant [KDM2A(CXXC)] and KDM2B failed to bind to this locus. The lack of significant enrichment of KDM2A on a housekeeping gene promoter (*GAPDH*) (data not shown), further validated specificity for pericentric loci. Treatment of these DNA complexes with the methylation-sensitive *Hpa*II (5'-CCGG-3') enzyme abolished the binding of KDM2A to the pericentric region of human chromosome 4 (Fig. 3A), suggesting recognition by KDM2A to unmethylated CpG sequences at this locus. Thus, these data show that KDM2A is enriched at pericentric heterochromatin and binds to CpG islands, as predicted based on the presence of the CXXC DNA-binding motif.[22]

In fission yeast and in higher eukaryotic cells, small RNA molecules transcribed by RNA polymerase II (Pol II) from within centromeric and pericentromeric regions appear to be necessary to initiate and sustain repressive chromatin modifications.[3-7] In human cells, the primary repetitive DNA at centromeres is α-satellite DNA, which consists of a 171-bp monomer that is tandemly arranged into higher-order arrays that extend for 100-kb to several megabases. Given that KDM2B was characterized as a transcriptional repressor of ribosomal RNA genes,[11] which are also encoded by tandem DNA repeats within the nucleolus, we investigated whether KDM2A contributed to the transcriptional regulation of repetitive α-satellite DNA sequences within centromeric heterochromatin in human HCT-116 cells. Using previously reported primers for analyzing transcription of these repeats, we conducted semi-quantitative RT-PCR on total RNA from KDM2A-depleted HCT-116 cells (Fig. 3B). We observed an increase in the transcription of the α-satellite transcripts of chromosomes 2, 4 and 13/21 in KDM2A-depeleted cells compared to control cells (Fig. 3C). H3K36me2 is a histone modification that is associated with positive regulation of Pol II-mediated transcription[23] and is negatively regulated by KDM2A.[8] In agreement with an increase in α-satellite transcription, ChIP analysis at the pericentric region of human chromosome 4 in KDM2A siRNA-treated cells displayed a significant increase in the levels of H3K36me2, but not H3K9me3, compared to control (Figs. 3D and S2), while the levels of H3K36me2 and H3K9me3 at the *GAPDH* promoter were unaffected (Figs. 3D and S2).

Since KDM2A repressed α-satellite transcription in human cells, we also investigated whether KDM2A contributed to the transcriptional regulation of repetitive DNA sequences within the centromeric heterochromatin of murine cells by using previously characterized primers for analyzing minor and major satellite repeats.[3,16] Major satellite repeats surround the minor satellites, and these two sets of repeats are separated by short stretches of CG-rich sequences[24] that may provide an ideal platform for the DNA-binding capabilities of KDM2A.[22] Semi-quantitative RT-PCR on total RNA in KDM2A-depleted NIH-3T3 cells displayed a substantial increase in the transcription of the major, but not the minor, satellite repeats compared to control cells (Fig. 3E). Taken together, these data demonstrate that KDM2A is a transcriptional repressor of pericentric α-satellite and major satellite repeats in human and mouse cells, respectively.

KDM2A specifically demethylates H3K36me2,[10] and since we observed an increase in H3K36me2 at pericentric chromosome 4 following KDM2A knockdown (Fig. 3D), we determined whether the JmjC-domain of KDM2A is required for satellite silencing in mammalian cells. Constructs encoding human KDM2A and a JmjC-domain mutant [KDM2A(ΔJmjC)] were introduced into NIH-3T3 cells that were previously treated with siRNA oligos against murine KDM2A. KDM2A siRNA-treated NIH-3T3 cells exogenously expressing human (and therefore siRNA insensitive) KDM2A displayed a significant decrease in major satellite RNA transcription compared to KDM2A siRNA-treated NIH-3T3 cells transfected with an empty vector (Fig. 3F). In contrast, KDM2A(ΔJmjC) was unable to rescue this effect due to KDM2A downregulation (Fig. 3F), suggesting a role for histone demethylation in the inhibition of transcription by KDM2A.

Dissecting the relationship between heterochromatin and centromeric RNA transcription is the basis of ongoing studies.[25-27] Given the reactivation of GFP expression in GFP(-) HeLa reporter cells detected in KDM2A siRNA treated cells (Fig. 2E and F) and the increase in α-satellite transcription observed following KDM2A knockdown (Fig. 3C), we sought to determine if forced accumulation of satellite RNA transcripts compromise the heterochromatic state. To that end, we transfected GFP(-) HeLa reporter cells with an expression vector driving transcription of one α-satellite repeat (171-bp monomer) from a human chromosome (chromosome 4) and monitored reactivation of GFP expression (Fig. 3G). Remarkably, we found that cells exogenously expressing these centromeric RNAs exhibited reactivation of GFP compared to cells transfected with an empty vector (Fig. 3G). Similarly, indirect immunofluorescence of HP1-γ in NIH-3T3 cells exogenously expressing major satellite RNA transcripts displayed a significant loss of HP1 heterochromatin-associated nuclear foci compared to cells transfected with an empty vector (Fig. 3H). These data suggest that the accumulation of satellite transcripts observed in KDM2A depleted cells (Fig. 3C and E) may

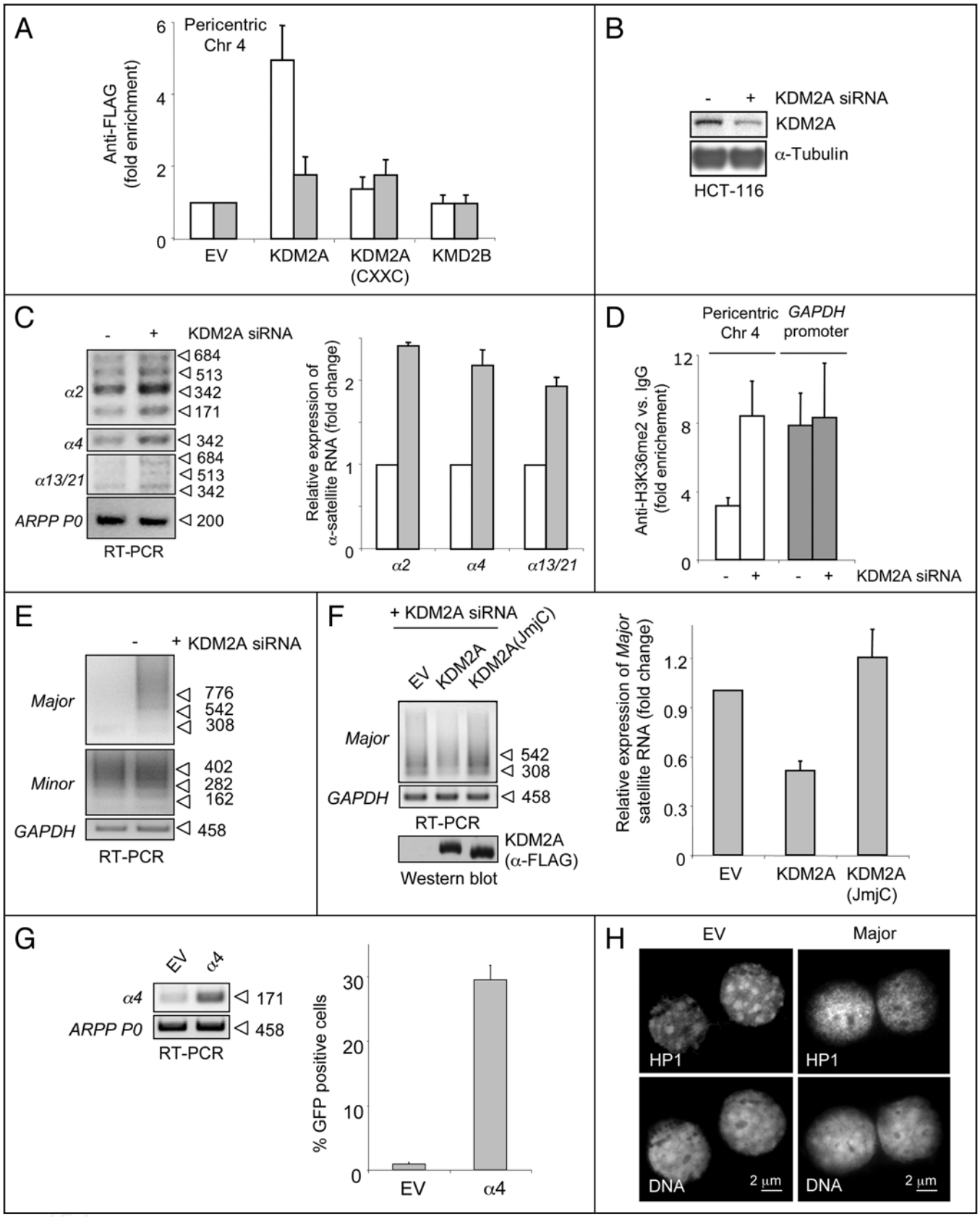

Figure 3. For figure legend, see page 6.

Figure 3. KDM2A represses transcription of pericentric satellite repeats in a JmjC-dependent manner. (A) KDM2A binds to the pericentric region of a human chromosome. Enrichment at the pericentric region of human chromosome 4 was obtained with anti-FLAG antibody as determined by ChIP analysis using chromatin prepared from HEK-293T cells transfected with FLAG-EV (empty vector), FLAG-KDM2A, FLAG-KDM2A(CXXC) and FLAG-KDM2B (open columns). Enrichment at the pericentric region of human chromosome 4 was additionally obtained after DNA complexes were treated with the methylation-sensitive *Hpa*II (5'-CCGG-3') enzyme that only cleaves unmethylated CpG sequences (closed columns). DNA-binding was quantified by real-time PCR. The value given for the amount of PCR product in EV-transfected cells was set at 1 (n = 3, +/- SD). (B) Representative example showing knockdown efficiency for KDM2A siRNA oligos by Western blot. Immunoblot of cell extracts from HCT-116 cells transfected with control oligos or KDM2A siRNA oligos. Efficacy of siRNA was assessed by immunoblotting to detect KDM2A, and α-Tubulin levels were used as loading controls. (C) RT-PCR analysis with oligo-dT-primed, reverse-transcribed (RT) cDNA derived from total RNA that was prepared from HeLa cells transfected with control or KDM2A siRNA oligos. PCR amplification using 25 cycles was conducted using locus-specific primers for the indicated chromosome satellites and *ARPP P0* (as control). Panel on the right shows the quantification of α-satellite RNA expression in (C) averaged with one additional, independent experiment. Levels of *ARPP P0* were used for normalization. The value given for the amount of α-satellite RNAs in control siRNA cells was set as 1 (n = 2, +/- SD). (D) Increase of H3K36me2 at the pericentric region of human chromosome 4 after downregulation of KDM2A. Analysis of H3K36me2 at the pericentric region of human chromosome 4 (open columns) and the *GAPDH* promoter (closed columns). ChIP was performed as in (A) with samples from cells treated with control (open columns) or KDM2A (filled columns) siRNA. The value given for the amount of PCR product present in IgG control samples was set at 1 (n = 3, +/- SD). (E) RT-PCR was conducted as in (C) using NIH-3T3 cells transfected with control or KDM2A siRNA oligos specific for mouse cells. Locus-specific primers for major and minor satellite repeats and levels of *GAPDH* (as control) were used. (F) RT-PCR analysis of major satellite repeats and *GAPDH* in KDM2A siRNA-treated NIH-3T3 cells, as performed as in (E), following transfection with vectors encoding FLAG-tagged KDM2A or FLAG-tagged KDM2A(JmjC). KDM2A expression levels were analyzed by immunoblotting using anti-FLAG antibody. Panel on the right shows the quantification of *Major* satellite RNA expression in (E) averaged with one additional, independent experiment. Levels of *GAPDH* were used for normalization. The value given for the amount of *Major* satellite RNAs in control cells (EV) was set as 1 (n = 2, +/- SD). (G) Forced expression of an α-satellite repeat results in disruption of heterochromatin. RT-PCR was conducted as in (C) using HeLa cells transfected with an empty expression vector (EV) or an expression vector driving transcription of one α-satellite repeat (171-bp) from a human chromosome (chromosome 4). Locus-specific primers for RNAs encoded by α4 and *GAPDH* (as control) were used. Panel on the right shows quantification of the expression of a GFP reporter gene in GFP(-) HeLa cells transfected with an empty expression vector (EV) or an expression vector driving transcription of one α-satellite repeat (171-bp) from human chromosome 4 (n = 300, +/- SD). (H) Forced expression of major satellite repeats results in HP1 delocalization from chromatin. Representative indirect immunofluorescence of HP1-γ in NIH-3T3 cells transfected with an empty expression vector (EV) or an expression vector driving transcription of one major satellite repeat. Cells were pre-extracted with TritonX-100 detergent prior to fixation and permeabilization with 100% methanol to visualize loss of HP1-γ.

be responsible for the disruption of heterochromatin and HP1 localization following the downregulation of KDM2A (Fig. 2).

RNA transcripts generated from within pericentric heterochromatin are thought to assist in the maintenance of centromere structure,[26] and interactions with single-stranded RNA molecules are required for integrity of the kinetochore structure in mitosis.[28] Importantly, while RNA molecules are required for centromeric integrity, the accumulation of satellite transcripts may lead to defects in chromosome segregation and mislocalization of centromere-associated proteins essential for centromere function.[6] Based on these reports, we determined whether silencing of KDM2A impacted centromere integrity and chromosome segregation in mitosis. We analyzed HCT-116 cells, which have a relatively stable karyotype,[29] transfected with control or KDM2A siRNA oligos and conducted indirect immunofluorescence analysis of CENP-A and α-tubulin to determine the state of centromeres and the mitotic spindle, respectively. While the mitotic spindle appeared unaffected, knockdown of KDM2A resulted in an increase in the misalignment of centromeres along the metaphase plate (36%, 45/125), as visualized with CENP-A, compared to control (12%, 15/125) (Fig. 4A). This phenotype was accompanied by segregation defects that were revealed by the detection of chromosome breaks (Fig. 4A) and a dramatic increase in the amount of chromosome bridges in KDM2A-siRNA treated cells (51%, 89/174) compared to control cells (16%, 38/228) (Fig. 4B). Together, these data suggest that KDM2A-mediated regulation of satellite RNAs and maintenance of heterochromatin may result in the loss of mitotic fidelity.

The loss of centromeric integrity, minor changes in the expression of non-coding RNAs and the deregulation of heterochromatin contribute to cellular transformation and genomic instability.[6,30,31] As such, a recent study has demonstrated that HP1 variants display decreased expression levels in prostate cancer cell lines and prostate cancer tissues compared to normal tissues.[32] In addition, an elevated frequency of chromosomal rearrangements is observed in the centromeric regions of prostate cancer patients,[33] which is consistent with the loss of centromeric integrity associated with decreased levels of HP1 and heterochromatin stability. To investigate a potential involvement of KDM2A in prostate cancer, we searched the Oncomine on-line databases for differential *KDM2A* expression in normal versus tumorigenic prostate tissues. Interestingly, in 4 out of 4 studies, the expression of *KDM2A*, but not *KDM2B*, was found significantly decreased in prostate carcinomas compared to normal prostate tissue (Figs. 4C; S3 and S4). Furthermore, a fifth study showed that the decrease in *KDM2A* expression was correlated with prostate tumor grade (Fig. S2). Taken together, these results suggest that the decrease in *KDM2A* levels may contribute to the high incidences of centromeric rearrangements and mitotic aberrations observed in prostate carcinomas.

Discussion

Abnormal chromosome segregation is a common characteristic of human tumors, and many of the molecular origins of chromosome missegregation derive from defective centromere and kinetochore assembly and function.[31] While mutations and/or the altered expression of genes that impinge on kinetochore function may impact chromosome segregation, recent studies have focused on characterizing the function of non-coding RNAs derived from centromeric repeats in the formation and maintenance of heterochromatin in supporting proper centromere function.

Here we show that KDM2A localizes to heterochromatin and binds HP1 proteins. As HP1 interactors provide various possibilities for HP1-binding to sites that may not have intrinsically strong affinity for HP1, the association of KDM2A and HP1 may contribute to the stability of HP1 on chromatin that displays

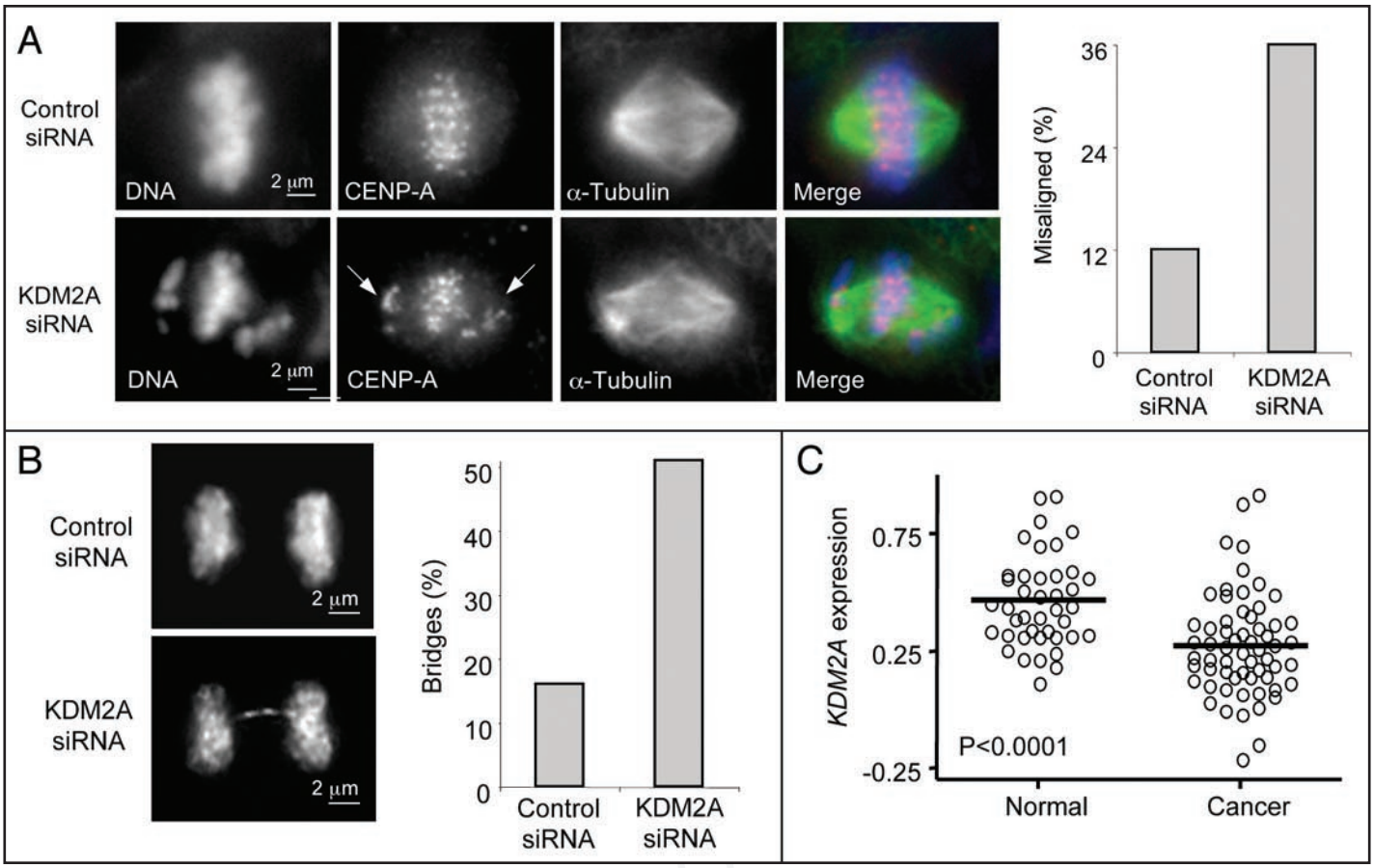

Figure 4. Silencing of KDM2A compromises mitotic fidelity and low levels of KDM2A are found in prostate cancer. (A) Indirect immunofluorescence analysis of mitotic HCT-116 cells transfected with control or KDM2A siRNA oligos. Cells were stained with antibodies to CENP-A (centromere protein A) and α-Tubulin, as indicated, and DAPI. Panel on the right shows the percentage of mitotic cells with misaligned centromeres in HCT-116 cells transfected with control or KDM2A siRNA oligos. (B) Representative chromosome bridges observed in HCT-116 cells transfected with control or KDM2A siRNA oligos. Chromosome bridges were visualized by phosphorylated histone H3 immunostaining. Panel on the right shows the percentage of mitotic cells with chromosome bridges in HCT-116 cells transfected with control or KDM2A siRNA oligos. (C) KDM2A expression data from Dhanasekaran et al., 2001. Normal indicates normal prostate from adult tissues, and cancer indicates prostate carcinoma.

specific epigenetic marks on DNA or nucleosomes. Moreover, we show KDM2A is essential for maintaining the heterochromatic state. We also show that KDM2A provides a fundamental function for silencing the transcription of satellite RNAs that are embedded within centromeric repeats in a JmjC-domain dependent manner. Implicating the regulation of satellite RNA expression in heterochromatin maintenance, we demonstrate that forced accumulation of RNA transcripts encoded by centromeric satellite repeats compromises heterochromatin stability and HP1 localization.

Intriguingly, the molecular function of KDM2A uniquely parallels that of the human paralogue KDM2B, which localizes to a particular cellular compartment (the nucleolus) and binds DNA repeats (ribosomal) to modulate expression of ribosomal RNAs.[11] Equally noteworthy is that the fission yeast orthologue of KDM2A (Epe1) interacts with the *S. pombe* HP1 orthologue (Swi6) and plays a central role in maintaining heterochromatin and protecting centromere function and chromosome segregation fidelity.[7,12,20,21] Since a natural mutation in the catalytic region of the JmjC-domain of Epe1 is considered to render the protein a catalytically inactive histone demethylase,[34] the requirement for the JmjC-domain in Epe1 function is currently not entirely understood. As the disruption of epigenetic control mechanisms contribute to cellular transformation, our study provides critical insight into the role of aberrant transcription of centromeric satellite repeats in heterochromatin maintenance for fidelity in mitosis.

Materials and Methods

Cell culture. HeLa, HeLa(-GFP), HEK-293T, HCT-116 and NIH-3T3 cells were cultured as described previously.[11,18,29,35]

Transcription analysis by RT-PCR. Total RNA was extracted from NIH-3T3 the use of the RNeasy kit (Qiagen), and reverse transcriptions were performed as described previously.[11] RT-PCR procedures and the primers used for α-satellites and major and minor satellites were as described previously.[3,28]

RNA interference. Cells were transfected by using HiPerFect transfection reagent (Qiagen), in accordance with the manufacturer's protocol, with pool of four siRNA oligonucleotides (5'-CAA CAG CGA UCC CAA GUUA-3', 5'-GGA CAC GGG UUC CAA AUAA-3', 5'-CAA CAU CCC UAU GCA GUUA-3' and 5'-GCA AGC AGA UCA CUC GAAA-3') targeting human KDM2A (Dharmacon) and with pool of four siRNA oligonucleotides (5'-CAA GAA UUC UGA UGG ACUU-3', 5'-GAG AUU GCG UGG UAC

CAUG-3', 5'-GAG GUA CUU CUG UUU GGUA-3' and 5'-GCA CAA UGG ACA CGA UACU-3') targeting mouse KDM2A (Dharmacon). siRNA oligonucleotides used for KDM2B were previously described.[11] The siRNA oligonucleotide sequences used in the GFP(-) screen were as follows: HDAC1 (5'-CUA AUG AGC UUC CAU ACA AUU-3'), KDM2A siRNA #1 (5'-ACC CAU UUC GUU GCU ACC CAA-3'), KDM2A siRNA #2 (5'-CUC ACU GGA GUU CCU AUA GUA-3), KDM2B siRNA #1 (5'UAG GAG UGG ACU AGA AGU UUA-3'), KDM2B siRNA #2 (5'-AGG AGT GGA CTA GAA GTT TAA-3'). A 21-nucleotide siRNA duplex corresponding to a non-relevant gene (*lacZ*) was used as control.

Antibodies. In collaboration with Invitrogen, a polyclonal antibody against KDM2A was generated by immunizing rabbits with a peptide containing amino-acid residues 747–760 of human KDM2A. A polyclonal antibody against KDM2B was generated and previously characterized.[11] Rabbit polyclonal antibodies were as follows: anti-FLAG (F7425; Sigma), anti-di-methyl H3K36 (07–274; Upstate), anti-tri-methyl H3K9 (07–523; Upstate), anti-Alexa Fluor 568 (A11036; Molecular Probes) and anti-phospho-H3 (Ser10) (06–570; Upstate). Mouse monoclonal antibodies were as follows: anti-M2 FLAG (F3165; Sigma), anti-HA (Covance), anti-HP1-α (MAB3584, Chemicon), anti-HP1-β (07–333, Upstate), anti-HP1-γ (MAB3450, Chemicon), anti-α-tubulin (32–2500; Zymed), anti-KDM2B (H00084678-M09; Abnova) and anti-Alexa Fluor 488 (A21121; Molecular Probes).

Oncomine data. Gene expression data was collected and processed from the Oncomine website (www.oncomine.org) as previously described[11] for five studies profiling gene expression of *KDM2A* or *KDM2B* in prostate cancer.[36-40]

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was conducted as previously described.[11] Primers for the pericentric region of human chromosome 4 were previously described.[41] *Hpa*II treatment was conducted as previously described.[42]

Biochemical methods. Extract preparation, immunoprecipitation, immunoblotting and purification and analysis of KDM2A interactors were as described previously.[29,35]

Vectors. KDM2A mutants (KDM2A(CXXC); (C571A,C574A,C577A) and KDM2A(JmjC); (Δ148-316)) were generated using the QuickChange Site-directed Mutagenesis kit (Stratagene). To exogenously express α-satellite transcripts, an α-satellite repeat (171-bp monomer) from human chromosome 4,[43] was subcloned into the multiple cloning site (MCS) of pECFP-N1 (Clontech) with previously used primers specific for that locus.[28] To exogenously express major satellite transcripts, a major satellite repeat (308-bp monomer) was subcloned into the multiple cloning site (MCS) of pECFP-N1 (Clontech) with previously used primers specific for that locus.[3] A stop codon was added immediately following these inserts to prevent the generation of a pECFP-fusion protein.

Indirect immunofluorescence. Indirect immunofluorescence procedures were as described previously.[11] Images were acquired with a Nikon Eclipse E800 fluorescence deconvolution microscope.

Image processing. The PSC colocalization plug-in (http://www.cpib.ac.uk/~afrench/coloc.html) for ImageJ software (NIH) was used to determine Pearson (r_p) and Spearman correlation coefficients (r_s) as described previously.[13] Images were prepared in Adobe Photoshop CS3 and ImageJ.

FACS analysis. FACS analysis to detect GFP was conducted as previously described.[18,19]

Acknowledgements

We thank N. Tenese for suggestions and critically reading the manuscript; F. Bassermann for helping with FACS analysis; and N. Tenese and L. Wong for reagents. D.F. is grateful to A. Nans. M.P. is grateful to T.M. Thor for continuous support. This work was supported by a grant from the National Institutes of Health to K.S.E-J. (R33-CA112061). This work was supported by grants from the National Institutes of Health to M.P. (R01-GM57587 and R37-CA76584) and an award from the Multiple Myeloma Research Foundation. M.P. is an Investigator with the Howard Hughes Medical Institute. The authors declare no competing financial interests.

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/FrescasCC7-22-Sup.pdf

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