SF-KDM2A binds to ribosomal RNA gene promoter, reduces H4K20me3 level, and elevates ribosomal RNA transcription in breast cancer cells

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Abstract. Regulation of rRNA transcription is an important factor for control of cell proliferation. We previously found that the JmjC domain-containing demethylase KDM2A reduces H3K36me2 in the rRNA gene promoter and rRNA transcription under starvation, which results in suppression of cell proliferation. The *KDM2A* gene also produces another protein product, SF-KDM2A, which lacks a JmjC domain and has no demethylase activity. As yet, the function of SF-KDM2A is not clear. Recently, it was reported that *KDM2A* was frequently amplified and that elevated expression of KDM2A was significantly associated with short survival of breast cancer patients. SF-KDM2A was more abundant than full-length KDM2A in a subset of breast cancers. In the present study, we report that SF-KDM2A localized in nucleoli and bound to the rRNA gene promoter in breast cancer cells. Overexpression of SF-KDM2A stimulated the transcription of rRNA. While the zf-CXXC domain was required for SF-KDM2A binding to the rRNA gene promoter, SF-KDM2A with mutations in the zf-CXXC domain lost the binding to the rRNA gene promoter and did not stimulate rRNA transcription. Knockdown of SF-KDM2A reduced rRNA transcription and cell proliferation. When SF-KDM2A was overexpressed, a transcriptionally repressive mark, H4K20me3, in the rRNA gene promoter was specifically reduced in a zf-CXXC domain-dependent manner, and knockdown of SF-KDM2A increased the H4K20me3 level. Taken together, these results demonstrate that SF-KDM2A binds to the rRNA gene promoter, reduces the H4K20me3 level, and activates rRNA transcription, suggesting that the stimulation of rRNA transcription by SF-KDM2A may contribute to tumorigenesis in breast cancer.

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Abbreviations: zf-CXXC, zinc finger CXXC; SF-KDM2A, short form-KDM2A; rDNA, rRNA gene; ChIP, chromatin immunoprecipitation

Key words: KDM2A, SF-KDM2A, rRNA transcription, zf-CXXC, H4K20 methylation, nucleolus

Introduction

Cell growth depends on new ribosome synthesis. RNA polymerase I (Pol I) transcribes ribosomal RNA (rRNA), and its activity plays a critical role in the regulation of ribosome biogenesis (1-3). In eukaryotic cells, a long precursor transcript called pre-ribosomal RNA (pre-rRNA) is initially transcribed from the rRNA gene (rDNA) by Pol I, and is processed to 18S, 5.8S and 28S rRNA, which are three of the four structured RNA molecules constituting the ribosome. Dysregulations of rRNA transcription are often observed in cancer cells (4). Some oncogene products, such as Myc, stimulate the transcription of rRNA, and tumor suppressors, such as PTEN, reduce the transcription of rRNA.

Many discoveries about the relationship between chromatin structures and transcription have been made during the past two decades. Several chemical modifications of chromatin components, including histone methylation, have been identified (5), and found to be involved in the regulation of transcription (6). Generally, while methylated Lys4 and Lys36 of histone H3 function as transcriptionally active signals, methylated Lys9 and Lys27 of histone H3 and methylated Lys20 of histone H4 function as transcriptionally repressive signals (7,8). Histone lysine methylation is catalyzed by histone methyl transferases (HMTs), and the demethylation of histones is carried out by histone demethylases (HDMs) (9,10). The existence of these enzymes highlights the dynamic nature of the regulation of histone methylation (6,11).

KDM2A, a member of the JmjC domain-containing enzymes, has demethylase activity on the mono- and dimethylated Lys36 of histone H3 (H3K36me1/2) (11,12). KDM2A contains several domains including the zinc finger CXXC (zf-CXXC) domain, the PHD finger, F-box and three leucine-rich repeats (11,12). Previously, we found that KDM2A localizes in nucleoli and binds to the rDNA promoter through the zf-CXXC domain, and that glucose starvation induces the demethylase activity of KDM2A in the rDNA promoter and reduces rRNA transcription (12-14). The *KDM2A* gene also

produces a short form of KDM2A (SF-KDM2A), which lacks the JmjC domain on the N-terminal side (12). However, the function of SF-KDM2A was not clear.

Recently, it was reported that *KDM2A* is frequently amplified and overexpressed, and that elevated expression of *KDM2A* is significantly associated with short survival of breast cancer patients (15). Detailed characterization of the *KDM2A* gene revealed that SF-KDM2A is more abundant than full-length KDM2A in a subset of breast cancers, and may have oncogenic potential (15). However, it is unclear how the elevated expression of SF-KDM2A contributes to tumorigenesis.

In the present study, we found that SF-KDM2A localized in nucleoli, bound to the rDNA promoter via zf-CXXC domain, reduced the level of histone H4K20me3 marks in rDNA promoter and stimulated rRNA transcription.

Materials and methods

Cells and cell culture. The human breast adenocarcinoma cell line MCF-7 was cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS) or in Dulbecco's modified Eagle's medium (DMEM; cat. no. D5796; Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% FCS. Cells were cultured at 37˚C in an atmosphere containing 5% CO_2 and 100% humidity. Mammalian expression plasmids were introduced into cells using FuGENE6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. MCF-7tet-on cells (parent) (13) were transfected with ptetFlag-SF-KDM2A, ptetSF-KDM2A, or ptetmCXXC-SF-KDM2A plus pAct-Hyg, which confers hygromycin resistance, and cultured in the presence of 150-250 μg/ml hygromycin and 200 μg/ml G418. The selected colonies were picked up and cultured for 24 h in the presence of 1 μg/ml doxycycline (Dox), and the expression of SF-KDM2A or its mutant proteins was detected by indirect immunofluorescence and western blotting using an anti-Flag antibody or an anti-Fbxl11 (KDM2A) antibody. The expression of SF-KDM2A and mCXXC-SF-KDM2A was induced by adding 1 μg/ml Dox, or induced by adding Dox at the concentrations indicated in the figure legends.

siRNA and transfection. Cells were transfected with stealth siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The siRNA oligonucleotide sequences for SF-KDM2A were 5'-CAGAAUAUUCAAGUAAAUCCGGAUU-3' (#1 oligo) and 5'-GGCAGAAUAUCUAACUCCUUCAGGA-3' (#2 oligo). The positions of the sequence are shown in Fig. 1A. The siRNA oligonucleotide sequence for KDM2A was 5'-GAACCCGAAGAAGAAAGGAUUCGUU-3', which was previously described (11,12). Cells were also transfected with control stealth RNA (Stealth RNAi Negative Control Medium GC Duplex; Thermo Fisher Scientific).

Antibodies. Mouse monoclonal anti-β-actin antibody (AC-15; Sigma-Aldrich), mouse monoclonal anti-nucleolin antibody, C23 (MS-3, sc-8031; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit monoclonal anti-flag antibody (SIG1-25; Sigma-Aldrich), goat anti-rabbit IgG-HRP (sc-2054; Santa Cruz Biotechnology), Alexa 488-conjugated goat anti-mouse IgG (H+L) (A11029; Thermo Fisher Scientific) and Alexa 568-conjugated goat anti-rabbit IgG (H+L) (A11011; Thermo Fisher Scientific) were purchased. The rabbit polyclonal anti-dimethylated histone H3 lys4 antibody (ab7766; Abcam, Cambridge, UK), rabbit polyclonal anti-trimethylated histone H3 lys4 antibody (ab8580; Abcam), mouse monoclonal anti-dimethylated histone H3 lys9 antibody (MABI 0307; Active Motif, Carlsbad, CA, USA), rabbit polyclonal anti-trimethylated histone H3 lys9 antibody (ab1186; Abcam), mouse monoclonal anti-dimethylated histone H3 lys27 antibody (MABI0324; Active Motif), mouse monoclonal anti-dimethylated histone H3 lys36 antibody (MABI0332; Active Motif), mouse monoclonal anti-trimethylated histone H3 lys36 antibody (MABI0333, GTX50908; GeneTex, Inc., Irvine, CA, USA), rabbit polyclonal anti-trimethylated histone H4 lys20 antibody (07-463; Merck Millipore, Darmstadt, Germany), rabbit polyclonal anti-acetylated histone H4 antibody (06-866; Merck Millipore), rabbit polyclonal anti-histone H3 antibody (ab1791; Abcam), and rabbit polyclonal anti-histone H4 antibody (ab10158; Abcam) were also purchased. The anti-Fbxl11 (KDM2A) antibody (ab99242; Abcam) was purchased and used as the anti-pan-KDM2A antibody. The anti-KDM2A antibody was previously described (12).

Western blotting and immunofluorescence staining. Cells were trypsinized and extracted in 3% SDS solution containing 100 mM Tris-HCl, pH 6.8, 0.1 M DTT and 20% glycerol. Cell extracts were separated on SDS-PAGE and transferred to a microporous PVDF membrane (Merck Millipore). After treatment with antibodies, bands were detected using an Immobilon Western System (WBKLS0100; Merck Millipore). For indirect immunofluorescence staining, cells grown on glass coverslips were fixed in methanol for 30 min at -20˚C and incubated in 1% skim milk in phosphate-buffered saline (PBS). The first antibodies (rabbit polyclonal antibody and/or mouse monoclonal antibody) were added and incubated for 60 min at 37˚C. After cells were washed three times in 0.1% skim milk in PBS, Alexa 488-conjugated anti-mouse IgG and/or Alexa 568-conjugated anti-rabbit IgG were added, incubated for 60 min at 37˚C and washed three times with 0.1% skim milk in PBS. Finally, cells were embedded in Immunon (Thermo Fisher Scientific) and observed via confocal fluorescence microscopy (LSM 5 Exciter; Carl-Zeiss, Oberkochen, Germany).

RNA preparation and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Total RNA was isolated from cells using a NucleoSpin RNA II kit (#U0955C; Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Synthesis of single-strand cDNA was performed on total RNA (1 μg) by a SuperScript III First-Strand Synthesis system (Thermo Fisher Scientific) using random hexamers according to the manufacturer's instructions. The cDNA products were diluted with distilled water and mixed with Thunderbird qPCR Mix (#QPS-201; Toyobo, Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. An Mx3000P QPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA) or CFX Connect (Bio-Rad Laboratories, Hercules, CA, USA) is used to quantify the amount of DNA. The values were normalized using the amounts for a control

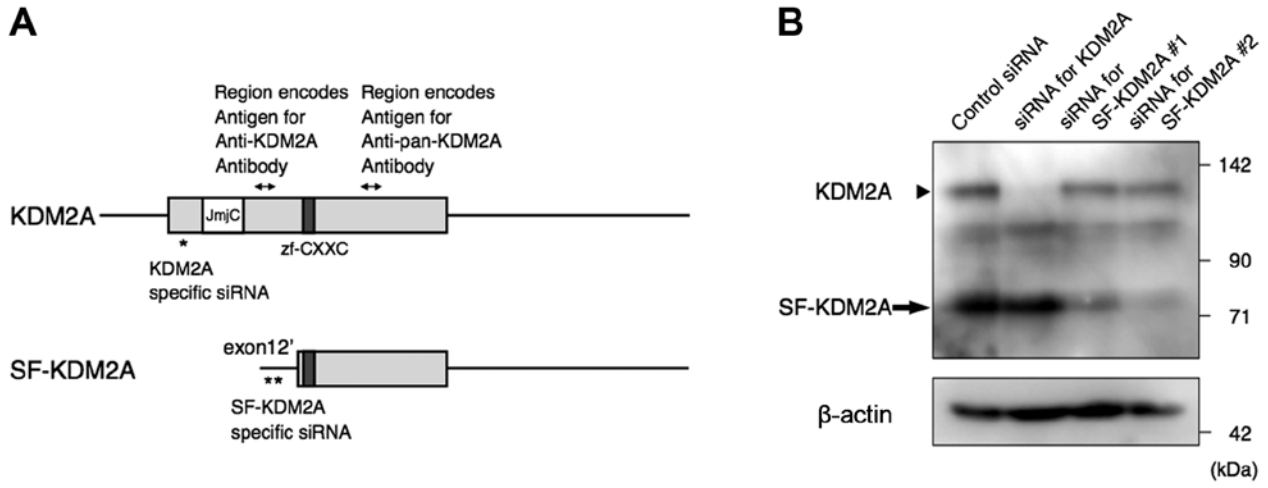

Figure 1. KDM2A and SF-KDM2A. (A) Diagrams of human KDM2A and SF-KDM2A mRNA. The *KDM2A* gene codes two transcripts, KDM2A mRNA (upper bar) and SF-KDM2A mRNA (lower bar). The bars show untranslated regions and the boxes translated regions. The white box shows the mRNA region encoding the JmjC domain, the dark grey box encoding the zf-CXXC domain. The positions for two siRNAs for SF-KDM2A and siRNA for KDM2A, which are targeted to sequences only for SF-KDM2A and KDM2A, respectively, are shown by asterisks. The anti-KDM2A antibody that recognized only KDM2A was produced against the polypeptide from Ser360 to Val451 of KDM2A. The anti-pan-KDM2A antibody that recognized both KDM2A and SF-KDM2A was produced against the synthetic peptide corresponding to a region between Ser825 and Ala875 of KDM2A (Ser283 and Ala333 of SF-KDM2A). The regions encoding the antigens are indicated by double-headed arrows. (B) Western blot analysis to detect KDM2A and SF-KDM2A proteins. Breast adenocarcinoma cell line MCF-7 cells were transfected with specific siRNAs for full-length KDM2A, SF-KDM2A (#1 and #2), or control siRNA. After 72-h culturing, cells were lysed, and the extracts were subjected to western blotting using anti-pan-KDM2A and anti-β-actin antibodies. The positions of KDM2A and SF-KDM2A are indicated by an arrowhead and an arrow, respectively. The positions of protein markers with defined molecular weights are indicated on the right side of the pictures.

mRNA, TATA-binding protein (TBP) mRNA. The sets of PCR primers used for amplification of the pre-rRNA (a sequence in the 5' region 1-155) were 5'-GCTGACACGCTG TCCTCTG-3' and 5'-TCGGACGCGCGAGAGAAC-3'; for SF-KDM2A, the primers used were 5'-GGATTTTCCCAGA GGCAGA-3' and 5'-TAACTTGGGATCGCTGTTGG-3'; for exogenously expressed SF-KDM2A, the primers used were 5'-ATTGCTGGGAATGTCCAAAG-3' and 5'-ATGGAAGTG GGTGAATGAGG-3'; for TBP, the primers used were 5'-TG CTGCGGTAATCATGAGGATA-3' and 5'-TGAAGTCCAA GAACTTAGCTGGAA-3'.

Statistical analysis. P-values were calculated by two-tailed paired Student's t-test.

Chromatin immunoprecipitation (ChIP). ChIP assay was performed as previously described (12,13). To detect specific binding, the values simultaneously obtained using the control antibody (normal rabbit IgG) were subtracted from those using specific antibodies. The values for specific binding were divided by total input (% of input).

Results

SF-KDM2A is localized in the nucleoli. *KDM2A* encodes two proteins, KDM2A and a short form of KDM2A, SF-KDM2A (12). While KDM2A contains the JmjC domain, which is critical for histone demethylase activity, SF-KDM2A lacks it (Fig. 1A). To investigate how SF-KDM2A is produced from the *KDM2A* gene, we designed siRNAs against the sequence of exon 12' in the *KDM2A* gene (12), which was found in SF-KDM2A mRNA, but not KDM2A mRNA (Fig. 1A). Western blot analysis using anti-pan-KDM2A antibody, which recognized both KDM2A and SF-KDM2A (Fig. 1A), showed that the siRNAs for exon 12' (both #1 and #2) reduced the band for SF-KDM2A (lower band) but not the band for KDM2A (upper band) (Fig. 1B), whereas KDM2A-specific siRNA reduced the band for KDM2A but not the band for SF-KDM2A (Fig. 1B). These results suggest that the majority of SF-KDM2A mRNA is not a product processed from the full-length KDM2A mRNA but is transcribed from the *KDM2A* gene separately from KDM2A mRNA. After this, siRNAs for exon 12' (both #1 and #2) are referred as SF-KDM2A siRNAs.

In order to investigate the subcellular localization of SF-KDM2A, we established a cell line in which Flag-tagged SF-KDM2A (Flag-SF-KDM2A) was expressed under the doxycycline (Dox)-inducible (tet-on) promoter (MCF-7tet-Flag-SF-KDM2A cells). The cDNA for Flag-SF-KDM2A under the tet-on promoter was introduced into parental cells, which were MCF-7 cells expressing the tet-on transcription factor (MCF-7tet-on cells) (13). Western blotting showed that Flag-SF-KDM2A was expressed by adding Dox (Fig. 2A). Immunostaining of MCF-7tet-Flag-SF-KDM2A cells with anti-Flag antibody showed that most of the signals for Flag-SF-KDM2A overlapped with those for nucleolin, suggesting that SF-KDM2A localized in the nucleoli (Fig. 2B). No signals were detected in MCF-7tet-on cells either with or without Dox treatment (Fig. 2B).

We previously showed that endogenous full-length KDM2A is localized in nucleoli (12). Immunostaining with anti-KDM2A antibody, which recognizes only full-length KDM2A, produced signals in nucleoli, and the siRNA for KDM2A reduced the nucleolar signals by anti-KDM2A antibody (Fig. 3A), confirming our previous results. SF-KDM2A siRNAs (#1 and #2) did not reduce the nucleolar signals

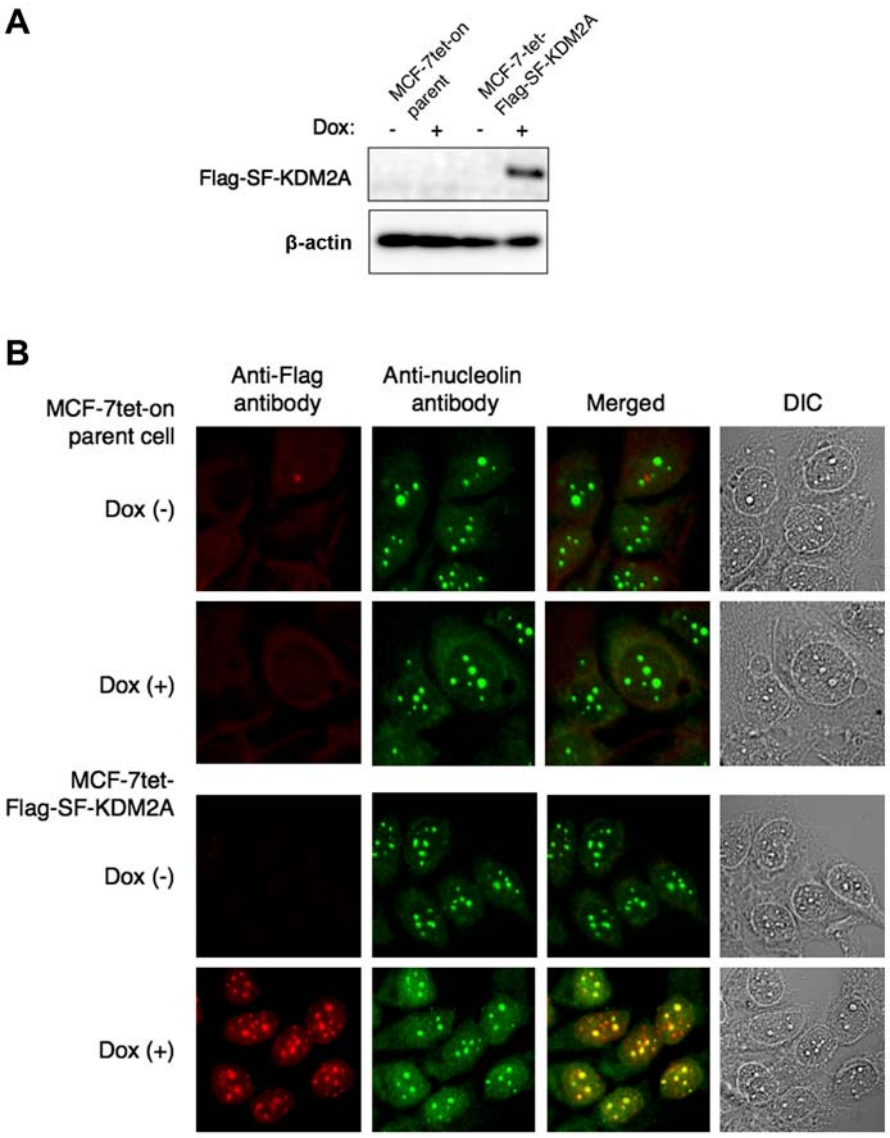

Figure 2. Flag-SF-KDM2A is present in the nucleoli. (A) Western blot analysis to detect Flag-SF-KDM2A proteins. MCF-7tet-Flag-SF-KDM2A and parental MCF-7tet-on cells were cultured in the presence or absence of doxycycline (Dox) for 48 h. Cells were collected and analyzed by western blotting using anti-Flag and anti-β-actin antibodies. (B) Subcellular localization of Flag-SF-KDM2A. Cells cultured as described in (A) were analyzed by indirect immunofluorescence using anti-Flag (red) and anti-nucleolin (green) antibodies. The specimens were observed through fluorescence and differential interference contrast (DIC) microscopes and representative images are shown. Most of the signals for SF-KDM2A were co-localized with those for nucleolin when Flag-SF-KDM2A was expressed (Merged).

by anti-KDM2A antibody (Fig. 3A and data not shown), supporting our conclusion that full-length KDM2A mRNA was distinct from SF-KDM2A mRNA. Next, we observed subcellular localization of endogenous SF-KDM2A. Because the C-terminal side of KDM2A contains the complete amino acid sequence of SF-KDM2A, it is impossible to produce an SF-KDM2A-specific antibody. Immunostaining with an anti-pan-KDM2A antibody, which recognized both SF-KDM2A and KDM2A, produced signals that overlapped with those of nucleolin (Fig. 3B). The siRNAs specific for KDM2A partially reduced the signals of the anti-pan-KDM2A antibody (Fig. 3B), confirming that KDM2A localized in the nucleoli (12). SF-KDM2A siRNAs (#1 and #2) also reduced the nucleolar signals by anti-pan-KDM2A antibody (Fig. 3B and data not shown). These results indicate that endogenous SF-KDM2A localizes in the nucleoli.

SF-KDM2A binds to the ribosomal RNA gene promoter via the zf-CXXC domain. The binding of SF-KDM2A to the rDNA promoter (Fig. 4A) was tested by chromatin immunoprecipitation (ChIP). The anti-Flag antibody collected the fragment of the rDNA promoter when the expression of Flag-SF-KDM2A (WT) was induced by Dox in MCF-7tet-Flag-SF-KDM2A cells (Fig. 4B), showing that Flag-SF-KDM2A binds to the rDNA promoter. To confirm the results, we established a cell line in which SF-KDM2A without the Flag-tag was induced by Dox (Fig. 4C). The amounts of rDNA promoter fragment collected by anti-pan KDM2A antibody were increased when the expression of SF-KDM2A was induced by Dox (Fig. 4D).

SF-KDM2A has the zf-CXXC domain (Fig. 1A), which binds to unmethylated CpG dinucleotides (13,16) and functions for full-length KDM2A binding to the rDNA promoter (13). We established a cell line in which SF-KDM2A with mutations in

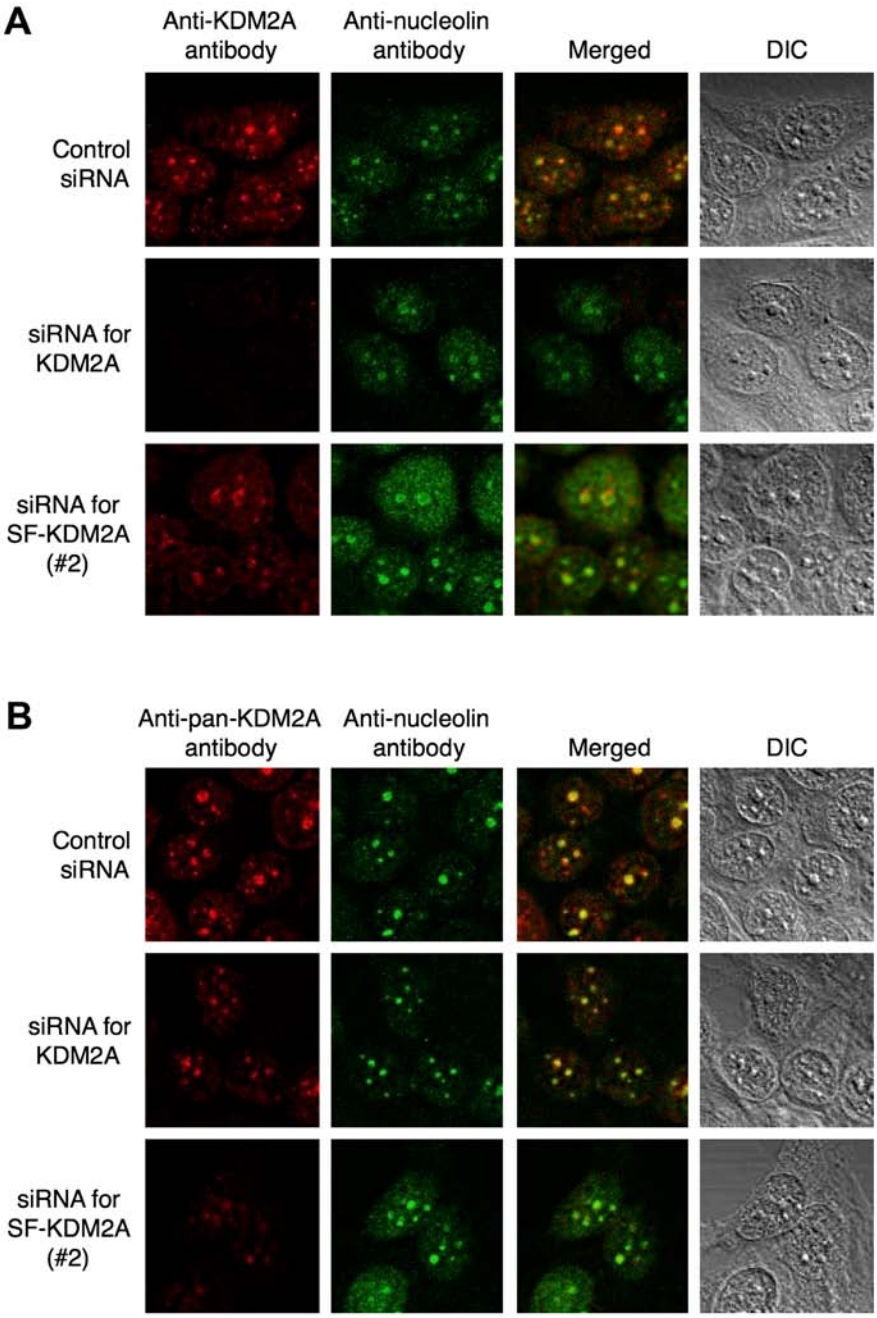

Figure 3. Subcellular localization of endogenous SF-KDM2A. Cells were transfected with KDM2A siRNA, SF-KDM2A siRNA (#2), or control siRNA. After 72-h culturing, cells were analyzed by indirect immunofluorescence technique using anti-KDM2A (red) and anti-nucleolin (green) antibodies in (A) or using anti-pan-KDM2A (red) and anti-nucleolin (green) antibodies in (B). Images captured by differential interference contrast (DIC) microscopy are also shown. The anti-pan-KDM2A antibody produced signals overlapped with those for nucleolin (Merged). siRNAs specific for either KDM2A or SF-KDM2A reduced them. The anti-KDM2A antibody produced signals localized in the nuclei (control siRNA), which were reduced by siRNA specific for KDM2A but not SF-KDM2A.

the zf-CXXC domain (mCXXC-SF-KDM2A) was expressed under the tet-on system (MCF-7tet-SF-KDM2A-mCXXC cells). In mCXXC-SF-KDM2A, Cys 29, Cys 32 and Cys 35 in the zf-CXXC domain were replaced with Ala. Western blotting showed that mCXXC-SF-KDM2A was expressed at a level comparable to wild-type SF-KDM2A in MCF-7tet-SF-KDM2A cells in the presence of Dox (Fig. 4C). ChIP analysis showed that the amount of rDNA promoter fragment collected by the anti-pan-KDM2A antibody was not increased by the induction of mCXXC-SF-KDM2A expression (Fig. 4D). The anti-histone H3 antibody collected the rDNA promoter at similar levels in all experimental conditions tested here (Fig. 4D). These results indicate that SF-KDM2A binds to the rDNA promoter through the zf-CXXC domain.

Next, we examined the binding of endogenous SF-KDM2A to the rDNA promoter in MCF-7 cells. ChIP analysis using the anti-pan-KDM2A antibody showed that the binding of endogenous SF-KDM2A to the rDNA promoter was reduced by the treatment with SF-KDM2A siRNA (Fig. 4E). The amounts of histone H3 binding to the rDNA promoter was not changed by SF-KDM2A siRNA (Fig. 4E). Together, these results demonstrate that SF-KDM2A binds to the rDNA promoter.

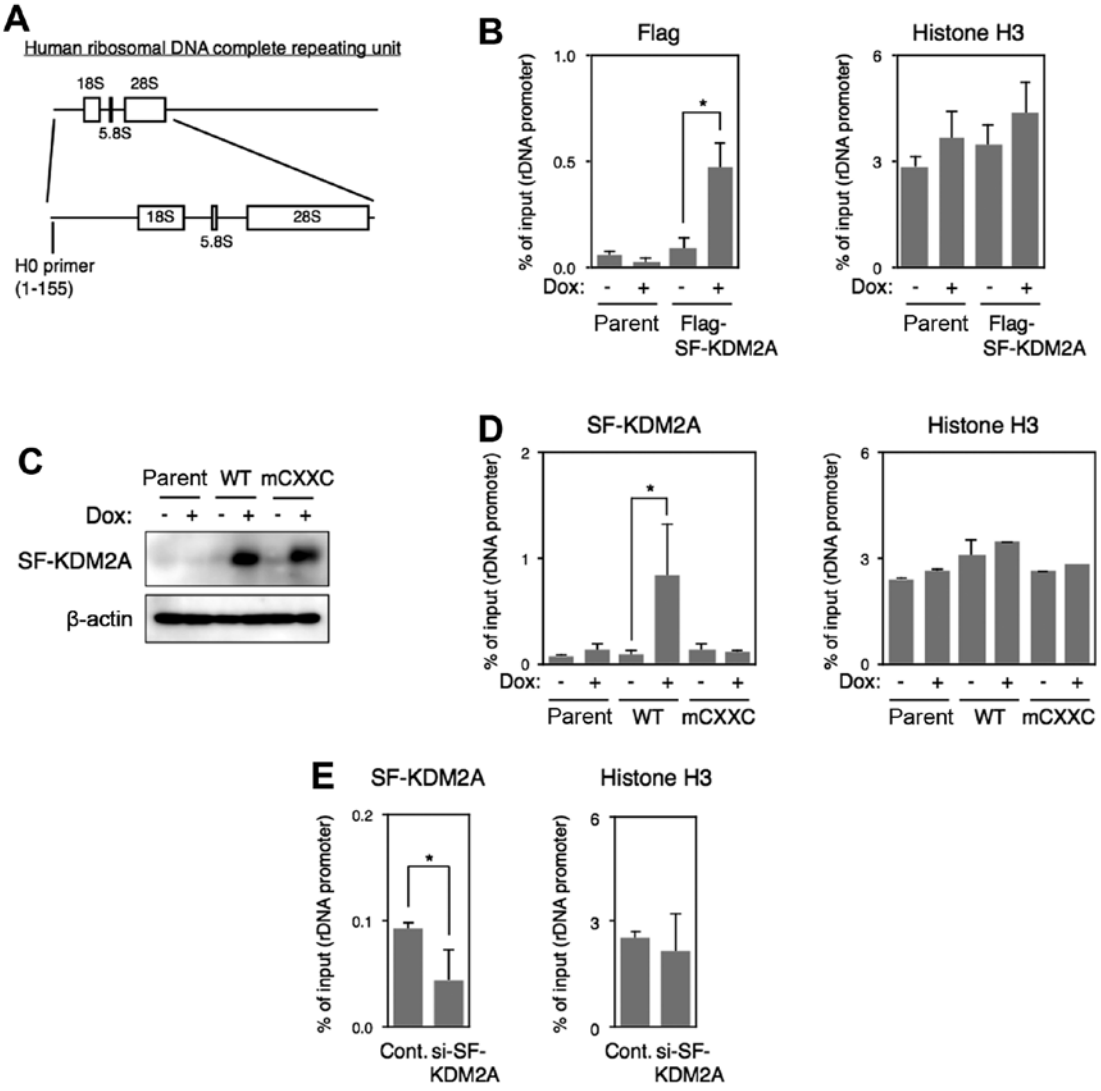

Figure 4. Binding of SF-KDM2A to rDNA promoter *in vivo*. (A) A diagram of human rDNA structure. The boxes show regions encoding rRNA that constitute ribosomes. The position of PCR primers used in the experiments is shown at the bottom (H0 primer). The numbers in parentheses show nucleotide numbers in human ribosomal DNA complete repeating unit (GenBank Accession no. U13369). (B) MCF-7tet-Flag-SF-KDM2A cells (Flag-SF-KDM2A) and MCF-7tet-on cells (parent) were cultured in the presence or absence of Dox (1 μg/ml) for 72 h, and chromatin immunoprecipitation (ChIP) analyses were performed using anti-Flag and histone H3 antibodies to detect Flag-SF-KDM2A and histone H3, respectively, in the rDNA promoter. The results are shown as the percentage of input. The experiments were performed three times, and mean values with standard deviations are indicated. *P<0.05. (C) Expression of wild-type and zf-CXXC domain-mutant SF-KDM2A proteins. MCF-7tet-SF-KDM2A cells (WT), MCF-7tet-mCXXC-SF-KDM2A cells (mCXXC), and MCF-7tet-on cells (parent) were cultured in the presence or absence of Dox for 72 h. Cells were lysed, and expression of SF-KDM2A was detected by western blotting using anti-pan-KDM2A antibody. (D) Cells were cultured as described in (C), and analyzed by ChIP analyses using pan-KDM2A and histone H3 antibodies. The results are shown as in (B). (E) MCF-7 cells were transfected with SF-KDM2A siRNAs (#2), or control siRNA. After 72-96 h of culture, cells were harvested and ChIP analyses were performed using pan-KDM2A and histone H3 antibodies. The results are shown as in (B).

SF-KDM2A stimulates rRNA transcription. Next, we investigated whether SF-KDM2A regulated rDNA transcription. When the expression of SF-KDM2A was induced by Dox in MCF-7tet-SF-KDM2A cells, the expression of SF-KDM2A mRNA and protein was increased in a Dox concentration-dependent manner (Fig. 5A and B). The amount of pre-rRNA was also increased in a Dox concentration-dependent manner (Fig. 5A). Similarly, in MCF-7tet-Flag-SF-KDM2A cells, the expression of Flag-SF-KDM2A increased the amount of pre-rRNA (Fig. 5C). In MCF-7tet-on cells (parent), the Dox treatment increased neither SF-KDM2A protein nor pre-rRNA levels (Fig. 5A and B). In MCF-7tet-SF-KDM2A-mCXXC cells, Dox treatment did not change the amount of pre-rRNA, in spite of the elevation of mCXXC-SF-KDM2A expression (Fig. 5D). When endogenous SF-KDM2A was reduced using SF-KDM2A siRNA in MCF-7 cells, the amount of rRNA transcription was decreased (Fig. 5E). Together, these results suggest that SF-KDM2A stimulates rRNA transcription and the binding of SF-KDM2A via the zf-CXXC domain to the rDNA promoter is required for the stimulation of rRNA transcription.

Level of a repressive histone mark H4K20me3 in the rDNA promoter is reduced by SF-KDM2A. To investigate how SF-KDM2A regulates rRNA transcription, histone modifications in the rDNA promoter were investigated by ChIP analyses. In MCF-7tet-SF-KDM2A cells, the levels of H3K4me2, H3K4me3, H3K9me2, H3K27me2, H3K36me2 and

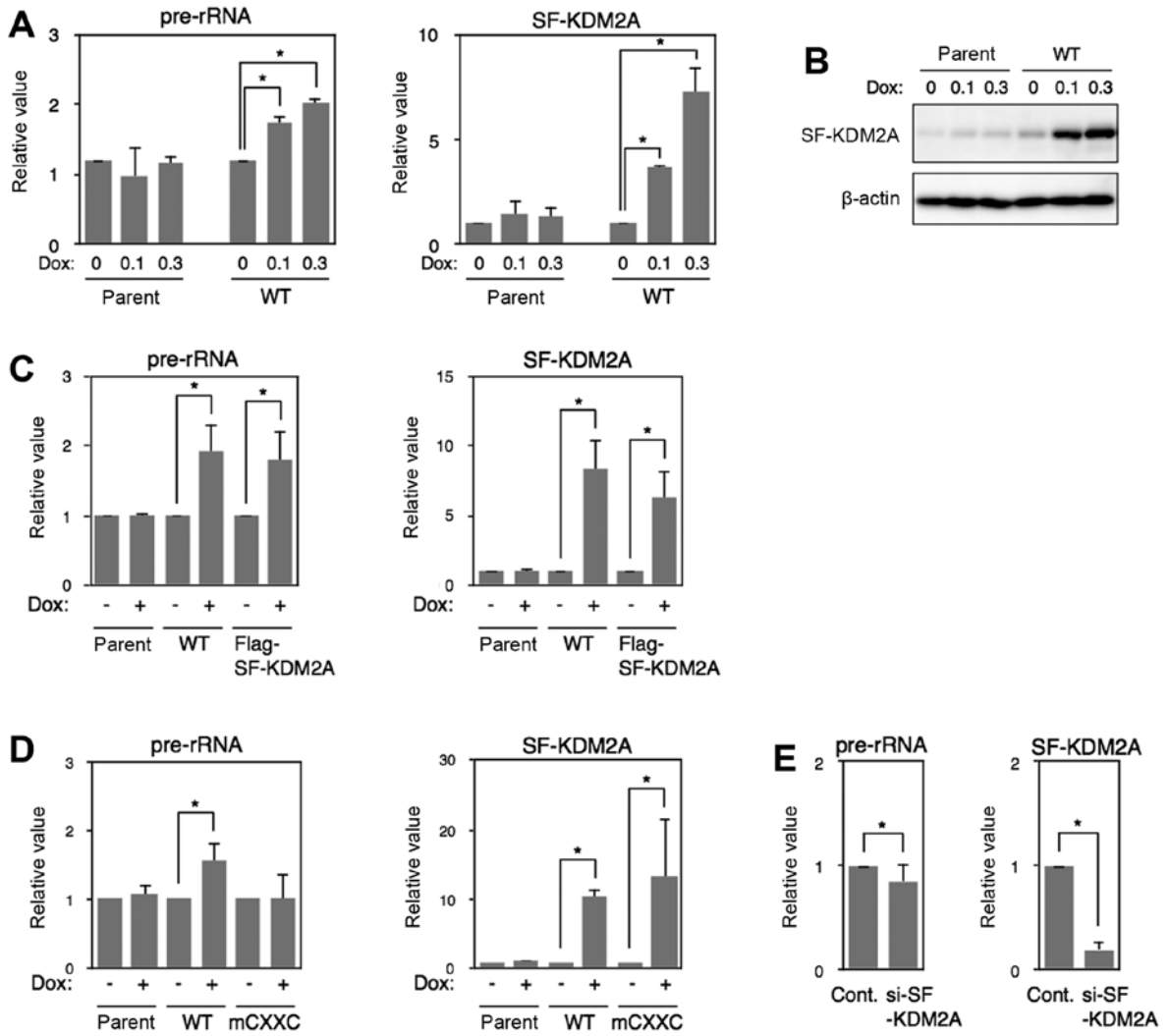

Figure 5. SF-KDM2A increases rRNA transcription. (A) MCF-7tet-on cells (parent) and MCF-7tet-SF-KDM2A cells (WT) were cultured in the presence of Dox at the indicated concentrations (0, 0.1 and 0.3 μg/ml) for 72 h. Total RNA was isolated, and the amounts of pre-rRNA and SF-KDM2A mRNA were measured by qRT-PCR. (B) Cells prepared as in (A) were lysed, and the expression of SF-KDM2A proteins was detected by western blotting. β-actin was also detected as a loading control. (C) MCF-7tet-on cells (parent), MCF-7tet-SF-KDM2A cells (SF-KDM2A), and MCF-7tet-Flag-SF-KDM2A cells (Flag-SF-KDM2A) were cultured in the presence or absence of Dox (1 μg/ml) for 72 h. Total RNA was isolated and analyzed by qRT-PCR to detect pre-rRNA, SF-KDM2A mRNA. (D) MCF-7tet-SF-KDM2A cells (WT), MCF-7tet-mCXXC-SF-KDM2A cells (mCXXC), and MCF-7tet-on cells (parent), were cultured in the presence or absence of Dox (1 μg/ml) for 72 h, and the amounts of pre-rRNA, SF-KDM2A mRNA, were measured by qRT-PCR. (E) MCF-7 cells were transfected with SF-KDM2A siRNA (#2), or control siRNA. After culturing for 72-96 h, the amounts of pre-rRNA and SF-KDM2A mRNA were measured by qRT-PCR. All experiments except (B) were performed at least three times, and mean values with standard deviations are indicated. *P<0.05.

H3K36me3 marks were hardly changed by Dox treatment, and the level of H3K9me3 and H4 acetylation marks showed a weak tendency of increase without statistical significance (Fig. 6A). On the contrary, Dox treatment significantly reduced a repressive histone mark, H4K20me3, while not in MCF-7tet-on or MCF-7tet-mCXXC-SF-KDM2A cells (Fig. 6A).

ChIP analyses of MCF-7 cells showed that the treatment of SF-KDM2A siRNA reduced the amounts of SF-KDM2A (Fig. 4E) and increased the levels of H4K20me3 marks in rDNA promoter (Fig. 6B). The amounts of histone H3 and H4 were changed by neither SF-KDM2A siRNA nor control siRNA treatment. These results suggest that SF-KDM2A reduces the level of H4K20me3 marks in the rDNA promoter.

To test whether full-length KDM2A also reduces H4K20me3 marks in the rDNA promoter, full-length KDM2A was overexpressed in MCF-7 cells (Fig. 6A). ChIP analyses using anti-pan-KDM2A antibody showed that KDM2A overexpression produced a level of signals similar to that of SF-KDM2A overexpression (compare Fig. 7A with 4D, both are indicated by % of input). However, KDM2A overexpression did not decrease the level of H4K20me3 marks. KDM2A overexpression did not affect the amounts of histone H3 and histone H4 in the rDNA promoter (Fig. 7B). These results suggest that SF-KDM2A has a distinct activity from KDM2A in the rDNA promoter.

SF-KDM2A is required to maintain cell proliferation. Whether the reduction of SF-KDM2A affected cell proliferation was investigated. After MCF-7 cells were transfected with SF-KDM2A siRNA, cell numbers were counted. After three days, cell numbers were reduced to about a half (Fig. 8). These results suggest that endogenous SF-KDM2A

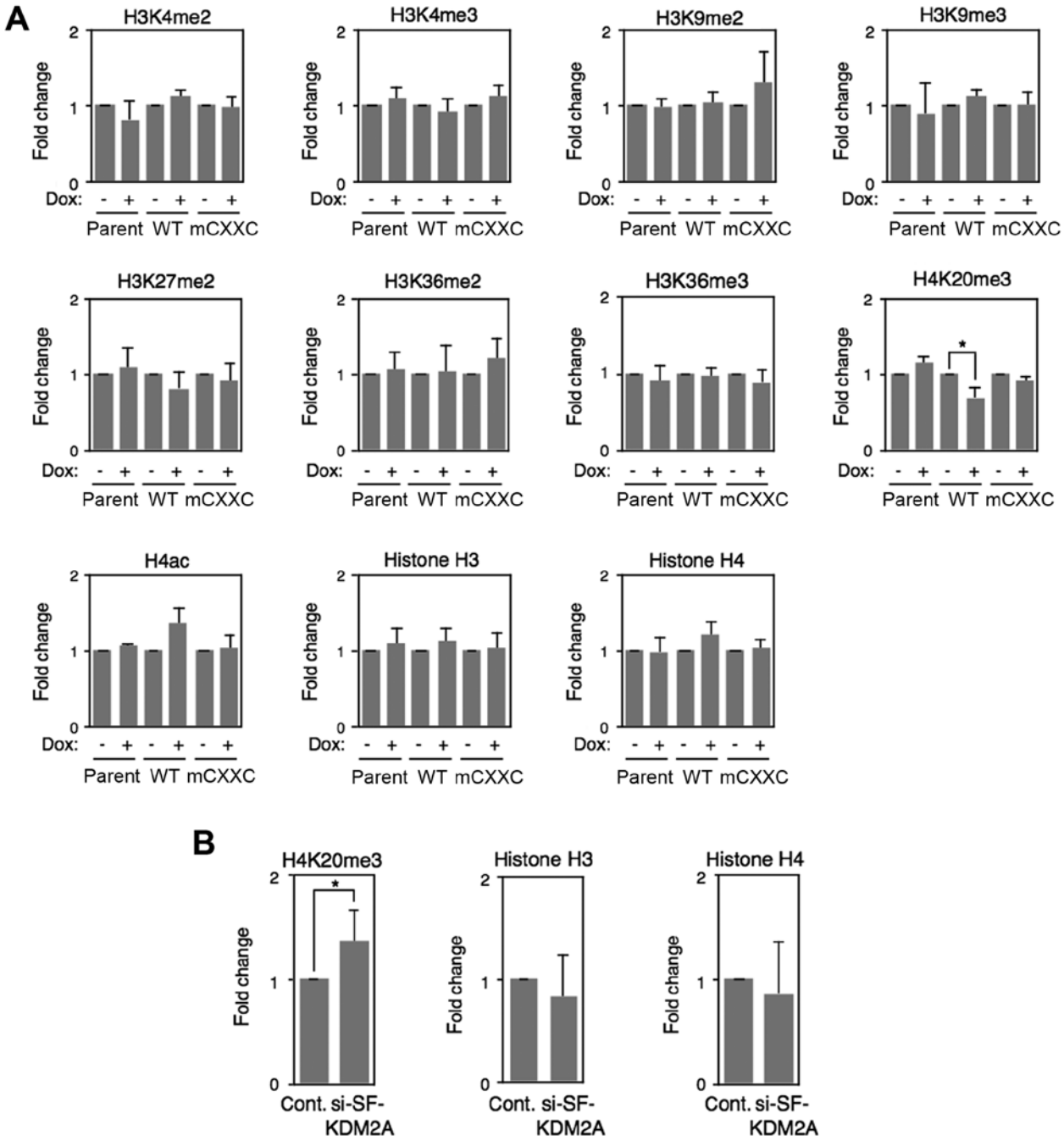

Figure 6. Changes of histone modifications in rDNA promoter. (A) MCF-7tet-SF-KDM2A cells (WT), MCF-7tet-mCXXC-SF-KDM2A cells (mCXXC), and MCF-7tet-on cells (parent) were cultured in the presence or absence of Dox (1 μg/ml) for 72 h. ChIP analyses were performed to detect the amounts of H3K4me2, H3K4me3, H43K9me2, H3K9me3, H3K27me2, H3K36me2, H3K36me3, H4K20me3, H4ac, histone H3 and histone H4 in the rDNA promoter. The results are expressed as fold-changes of the values without Dox. (B) MCF-7 cells were treated with SF-KDM2A specific siRNAs (#2), or control siRNA for 72-96 h, and analyzed by ChIP to detect the amounts of H4K20me3, histone H3 and histone H4 in the rDNA promoter. The results are expressed as fold-changes of the values for control siRNA. All experiments were performed three times and mean values with standard deviations are indicated. *P<0.05.

is required to maintain cell proliferation in breast cancer cells.

Discussion

In the present study, we investigated the functions of SF-KDM2A, which is produced by the *KDM2A* gene, but does not have the domain for histone demethylase activity (Fig. 1). SF-KDM2A localized in the nucleoli (Figs. 2 and 3) and bound to the rDNA promoter via its zf-CXXC domain in a breast cancer cell line MCF-7 (Fig. 3) as full-length KDM2A did (12,13). Overexpression of SF-KDM2A stimulated rRNA transcription, which depended on the zf-CXXC motif (Fig. 5). Knockdown of SF-KDM2A reduced rRNA transcription (Fig. 5). Furthermore, we found that overexpression of SF-KDM2A reduced the level of a repressive histone mark, H4K20me3, in the rDNA promoter, and knockdown of SF-KDM2A increased H4K20me3 marks in the rDNA promoter in MCF-7 cells (Fig. 6). It has been shown that rRNA transcription levels are associated with

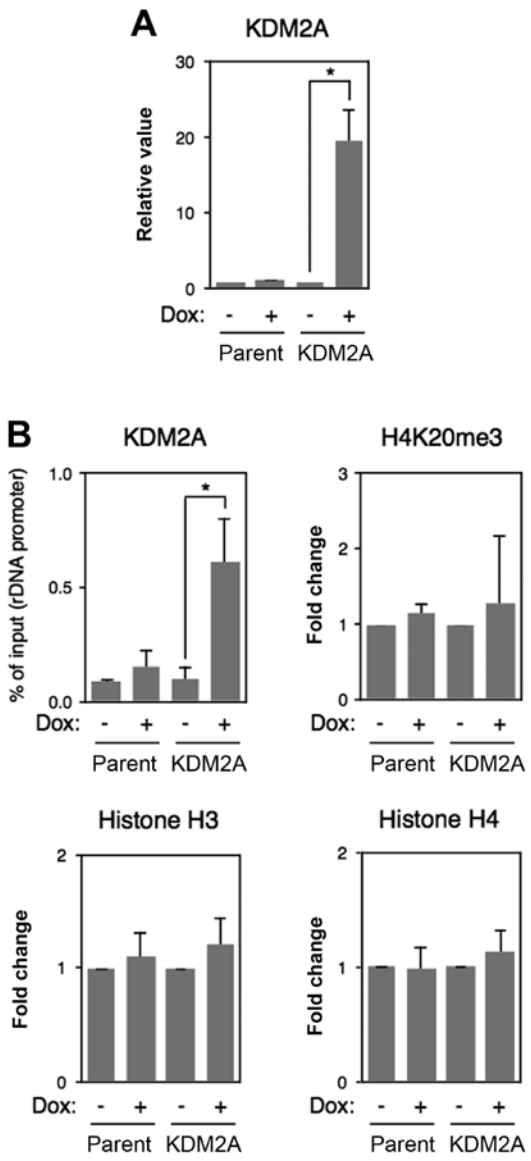

Figure 7. (A) MCF-7tet-KDM2A cells (KDM2A) and MCF-7tet-on cells (parent) were cultured in the presence or absence of Dox for 72 h. Total RNA was isolated and analyzed by qRT-PCR to detect pre-rRNA, KDM2A mRNA (12). (B) Cells were cultured as described in (A), and ChIP was analyzed in the rDNA promoter using anti-pan-KDM2A, anti-H4K20me3, anti-histone H3 and anti-histone H4 antibodies. The amount of KDM2A is shown as a percentage of input. In MCF-7tet-KDM2A cells in the presence of Dox, the anti-pan-KDM2A antibody collected rDNA promoter fragments similar to those in MCF-7tet-SF-KDM2A cells (compare to Fig. 4D). Other results are expressed as fold-changes of the values for control siRNA. All experiments were performed at least three times, and mean values with standard deviations are indicated. *P<0.05.

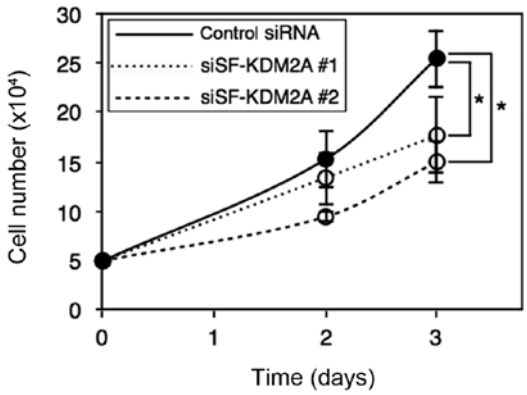

Figure 8. SF-KDM2A knockdown reduces cell proliferation. MCF-7 cells were transfected with SF-KDM2A siRNAs (#1 and #2) or control siRNA. Cells were replated, cultured for the indicated number of days and cell numbers were counted.

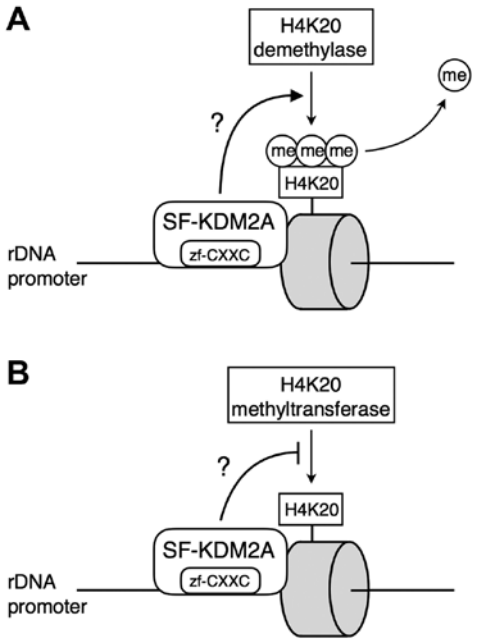

Figure 9. Model for control of H4K20 methylation in rDNA promoter by SF-KDM2A. SF-KDM2A binds to rDNA promoter via the zf-CXXC domain, and may activate or recruit histone demethylases in rDNA promoter (A), or inhibit the activity of histone H4K20 methyltransferase in rDNA promoter (B).

cell proliferation (1-3), and we found that knockdown of SF-KDM2A reduced cell proliferation (Fig. 8). These results suggest that SF-KDM2A regulates cell proliferation through controlling rRNA transcription by regulating the level of H4K20me3 marks in the rDNA promoter. Induction of SF-KDM2A expression in MCF-7tet-SF-KDM2A cells did not change cell proliferation (data not shown). These results suggest that rRNA expression levels supported by endogenous SF-KDM2A are sufficient for proper cell proliferation, and further upregulation does not increase cell proliferation in the experimental conditions tested here.

A recent report suggests that elevated expression of the *KDM2A* gene is significantly associated with short survival of breast cancer patients, that SF-KDM2A is more abundant than full-length KDM2A in a subset of breast cancers, and thus SF-KDM2A may have oncogenic potential (15). The functions of KDM2A have been shown to be associated with its demethylase activity (12-14). Therefore, SF-KDM2A should affect cell activities in ways different from KDM2A. We observed that overexpression of KDM2A in MCF-7 cells did not change the level of H4K20me3 in the rDNA promoter (Fig. 7). These results suggest that the activities of SF-KDM2A in the rDNA promoter are different from those of KDM2A. Because there are no domains expected to function as a histone demethylase in SF-KDM2A, SF-KDM2A may affect other factors in rDNA

promoter to reduce H4K20me3 levels. It was suggested that a JmjC enzyme PHF2 was associated with rDNA promoter (17), and that PHF2 reduced H4K20me3 at TLR4-responsive promoters (18), although the H4K20me3 demethylase activity of PHF2 was not reported in rDNA promoter. Suv4-20h2 was detected in rDNA promoter and induced H4K20me3 methylation (19). Since SF-KDM2A binds to rDNA promoter, it is possible that SF-KDM2A specifically affects the activities of these H4K20me3 modifying enzymes to change the status of H4K20 methylation in rDNA promoter (Fig. 9). The reduced levels of H4K20me3 may relax chromatin compaction to increase gene expression (19).

Methylation of H4K20 is important for biological processes (20). H4K20me3 is found in constitutive heterochromatin regions, and is enriched in regions of chromatin containing silenced genes (21,22). The levels of H4K20me3 marks are globally decreased in cancer cells in non-small cell lung and breast cancers (23-27). In breast carcinomas, the levels of H4K20me3 are associated with clinicopathological status (27), and a decreased H4K20me3 level is associated with shorter disease-free survival in breast cancer patients (27). Therefore, our finding that the reduction of H4K20me3 observed in SF-KDM2A expressing cells is consistent with the fact that H4K20me3 is decreased in malignant breast cancer.

The *KDM2B* gene, encoding a paralogue of KDM2A, also produces two forms of KDM2B like the KDM2A gene (28). The larger one is the full-length isoform of KDM2B, and the smaller one is a short isoform of KDM2B lacking a JmjC domain (SF-KDM2B). While the expression level of full-length KDM2B is elevated only in early embryos, the short form of KDM2B is ubiquitously expressed during embryonic development in mice (29). Recent reports suggest that SF-KDM2B cannot rescue the early differentiation phenotypes induced by KDM2B knockdown (30). These results suggest that SF-KDM2B has different functions from full-length KDM2B, although the function of SF-KDM2B is unclear. FbxL19, a member of the F-box proteins, contains zf-CXXC, a PHD domain and three leucine-rich repeats, but not a jmjC domain. This domain structure of FbxL19 is quite similar to that of SF-KDM2A. FbxL19 functions as E3 ubiquitin ligases. The Skp1-Cullin-F-box complex, SCF^{FbxL19}, mediates degradation of Rho family proteins (31,32). It is an open question whether SF-KDM2A mediates poly-ubiquitination and degradation of protein.

To the best of our knowledge, there are no published studies that described the functional roles of SF-KDM2A in human cancers besides breast cancers. On the other hand, there are some reports, which described the roles of KDM2A in human malignancies. Expression of KDM2A was elevated in lung cancer tissues and overexpression of KDM2A in lung cancer increased tumorigenesis in its demethylase activity dependent manner (33), probably through epigenetic repression of expression of dual-specificity phosphatase 3 (33) and HDAC3 (34). The expression level of KDM2A was increased in gastric cancer tissues. Forced expression of KDM2A in gastric cancer cells promoted cell growth and migration (35). RUNX3-mediated upregulation of miR-29b inhibited the proliferation and migration of gastric cancer cells by targeting KDM2A (36). An *in vitro* clonogenic assay showed that knockdown of KDM2A significantly inhibited the colony forming ability of human prostate adenocarcinoma (37). These results suggest that KDM2A have tumor-promoting functions. However, in some studies PCR primers used to detect KDM2A mRNA can amplify both KDM2A and SF-KDM2A mRNAs, and KDM2A siRNA that could reduce expression of both full-length KDM2A and SF-KDM2A was used to investigate KDM2A functions. It is also sometimes unclear whether antibody used to detect KDM2A recognized only KDM2A but also SF-KDM2A. Therefore, it is not clear whether some results reported as KDM2A functions to date were due to full-length KDM2A, SF-KDM2A or both. The careful experiments to investigate two *KDM2A* gene products separately may clarify the functions of this gene.

In summary, the *KDM2A* gene produces KDM2A and SF-KDM2A. SF-KDM2A shares plural domains with KDM2A except the JmjC domain. We found that SF-KDM2A bound to the rDNA promoter via a zf-CXXC motif as full-length KDM2A and is involved in regulation of rRNA synthesis, but the effects of SF-KDM2A on rRNA transcription are not the same as those of full-length KDM2A. SF-KDM2A reduces the transcriptional repressive histone mark H4K20me3 in the rDNA promoter and activates rRNA transcription. Knockdown of SF-KDM2A reduces cell proliferation. Our results suggest that SF-KDM2A may contribute to tumorigenesis through stimulation of rRNA transcription.

Acknowledgements

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