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JmjC enzyme KDM2A is a regulator of rRNA transcription in response to starvation

Yuji Tanaka[1], Kengo Okamoto[1], Kwesi Teye[1], Toshiyuki Umata[2], Noriyuki Yamagiwa[3], Yutaka Suto[3], Yi Zhang[4] and Makoto Tsuneoka[1,*]

[1]Department of Molecular Pharmacy, Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Japan, [2]Radioisotope Research Center, Research Facility for Occupational and Environmental Health, University of Occupational and Environmental Health, Kitakyushu, Japan, [3]Department of Organic Chemistry, Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Japan and [4]Department of Biochemistry and Biophysics, Linberger Comprehensive Cancer Center, and Howard Hughes Medical Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

The rate-limiting step in ribosome biogenesis is the transcription of ribosomal RNA, which is controlled by environmental conditions. The JmjC enzyme KDM2A/JHDM1A/FbxL11 demethylates mono- and dimethylated Lys 36 of histone H3, but its function is unclear. Here, we show that KDM2A represses the transcription of ribosomal RNA. KDM2A was localized in nucleoli and bound to the ribosomal RNA gene promoter. Overexpression of KDM2A repressed the transcription of ribosomal RNA in a demethylase activity-dependent manner. When ribosomal RNA transcription was reduced under starvation, a cell-permeable succinate that inhibited the demethylase activity of KDM2A prevented the reduction of ribosomal RNA transcription. Starvation reduced the levels of mono- and dimethylated Lys 36 of histone H3 marks on the rDNA promoter, and treatment with the cell-permeable succinate suppressed the reduction of the marks during starvation. The knockdown of KDM2A increased mono- and dimethylated Lys 36 of histone H3 marks, and suppressed the reduction of ribosomal RNA transcription under starvation. These results show a novel mechanism by which KDM2A activity is stimulated by starvation to reduce ribosomal RNA transcription.

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Introduction

Regulation of cell growth ultimately depends on the control of new ribosome synthesis (Grummt, 2003). Although the supply of ribosomal components involves the activities of three forms of nuclear RNA polymerase (pol I, pol II, and pol III) in eukaryotic cells, pol I has a central role in the regulation of ribosome biogenesis (Laferte *et al*, 2006; Chedin *et al*, 2007; Grewal *et al*, 2007). Pol I transcribes the eukaryotic ribosomal RNA genes (rDNA) in nucleoli. rDNA code 18S, 5.8S, and 28S ribosomal RNA, which are three of the four structured RNA molecules constituting the ribosome. These three RNA result from the processing of one precursor transcript, pre-ribosomal RNA (pre-rRNA).

Many discoveries about the relationship between chromatin structures and transcription have been made during the past decade, and several chemical modifications of chromatin components, including DNA methylation and histone acetylation, have been identified (Berger, 2007). One key component of chromatin structures in biological regulation is the methylation of lysine residues in histone proteins, which has been vigorously studied over the past several years. Highly specific enzymes catalysing the synthesis of methyl marks, as well as proteins recognizing distinct methylated lysine residues, have been identified. Recently, an increasing number of histone demethylases, JmjC domain-containing enzymes, has been discovered and has highlighted the dynamic nature of the regulation of histone methylation (Kustatscher and Ladurner, 2007; Stavropoulos and Hoelz, 2007). In addition, the activities of JmjC enzymes require small molecules, including molecular oxygen, Fe(II), and α-ketoglutarate (α-KG), as co-substrates (Klose *et al*, 2006a). Therefore, these substrates and their cognate products, such as the succinate for α-KG, can affect the activities of JmjC enzymes and the transcription regulated by them. However, the roles of JmjC enzymes on rDNA chromatin have not been well studied.

Previously, we identified the JmjC protein Mina53 (Tsuneoka *et al*, 2002), which is involved in mammalian cell proliferation. The expression of the *mina53* gene is directly controlled by the oncogene *myc* (Tsuneoka *et al*, 2002) and elevated in some types of cancer (Teye *et al*, 2004, 2007; Tsuneoka *et al*, 2004; Fukahori *et al*, 2007; Ishizaki *et al*, 2007; Zhang *et al*, 2008; Komiya *et al*, 2009). Mina53 exists in nucleoli (Tsuneoka *et al*, 2002) and binds to nucleolar proteins (Eilbracht *et al*, 2005). However, the substrate of this putative enzyme has not yet been identified, and the role of JmjC proteins including Mina53 in ribosome biogenesis is still unclear.

To determine whether the dynamics of histone methylation mediated by demethylases affect ribosome biogenesis, we first attempted to identify a histone demethylase containing a JmjC domain that regulates rDNA transcription. For this, we searched the database of the ~700 human nucleolar proteins that have been identified using high sensitivity mass spectrometry (Andersen *et al*, 2005), and found a candidate JmjC enzyme, KDM2A. It had been reported that KDM2A had histone demethylase activity *in vivo* on the dimethylated Lys 36 of histone H3 (H3K36me2) and *in vitro* on the monomethylated Lys 36 of histone H3 (H3K36me1) in addition to H3K36me2 (H3K36me1/2) (Tsukada *et al*, 2006). The

*Corresponding author. Department of Molecular Pharmacy, Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki 370-0033, Japan. Tel.: +81 27 352 1180; Fax: +81 27 352 1118; E-mail: tsuneoka@takasaki-u.ac.jp

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amino acid sequence of human KDM2A is 97.4 and 87.0% identical to those of *Mus musculus* and *Gallus gallus*, respectively. These suggest that KDM2A has an essential role in higher animals, but it is not clear how KDM2A functions and on which genes. Here, we show that KDM2A represses the transcription of ribosomal RNA by binding to the rDNA promoter and demethylating H3K36me1/2, and that its activity is controlled by succinate.

Results

KDM2A gene encodes two proteins

An antibody was produced against the recombinant polypeptide from Leu 763 to Gly 855 of the human KDM2A protein (Figure 1A). When a human cell lysate was analysed by western blotting using this KDM2A-specific antibody (Figure 1A, anti-pan-KDM2A antibody), two bands were recognized (Figure 1B). The protein with the lower mobility migrated to the same spot as the polypeptide exogenously expressed by the human *KDM2A* cDNA (GenBank Accession No. NM_012308) (Figure 1C, arrowhead), indicating that this protein was KDM2A. The protein with the higher mobility may be a degradation product of KDM2A or an mRNA product with a shorter ORF coded in the *KDM2A* gene.

Using high-resolution maps of histone lysine methylations and pol II across the human *KDM2A* genome (Barski *et al*, 2007) and previously deposited sequences of EST clones, we identified a new mRNA whose transcription started from part of intron 12 of the *KDM2A* gene. Finally, we found that in addition to KDM2A protein, a smaller protein was expressed by the *KDM2A* gene (Figure 1A; a detailed description is included in the Supplementary text and Figure S1 of the Supplementary data). The protein with higher mobility had the same mobility as the polypeptide exogenously expressed from the cDNA encoding the smaller protein (Figure 1C). The cognate siRNA duplex specific for KDM2A reduced the band for KDM2A but not the band with the higher mobility (Figure 1B). These results indicate that the protein with the higher mobility was produced by mRNA with a shorter ORF coded in the *KDM2A* gene. We named the polypeptide SF-KDM2A (short-form KDM2A), and deposited the sequence in the GenBank (Accession No. AB490246). SF-KDM2A does not have a JmjC domain. Although KDM2A possessed demethylase activity for dimethylated Lys36 histone H3 (H3K36me2) as reported before, SF-KDM2A did not (Supplementary Figure S2). These results suggest that SF-KDM2A has a different function from KDM2A. To investigate the specific role of histone lysine methylation on the rDNA chromatin, we focused this study on KDM2A.

KDM2A is localized in nucleoli and binds to ribosomal RNA gene promoter

To investigate the subcellular localization of KDM2A, an antibody specific to KDM2A was produced against a recombinant polypeptide whose amino acid sequence was found in KDM2A but not in SF-KDM2A (Figure 1A). Western blot analysis showed that the antibody recognized the band that was reduced by the siRNA for KDM2A (Figure 1B). These results indicate that this antibody specifically recognized KDM2A. Immunostaining of human cells with the antibody produced signals localized in the nucleoli (Figure 2A), and the siRNA for KDM2A clearly reduced the nucleolar signals.

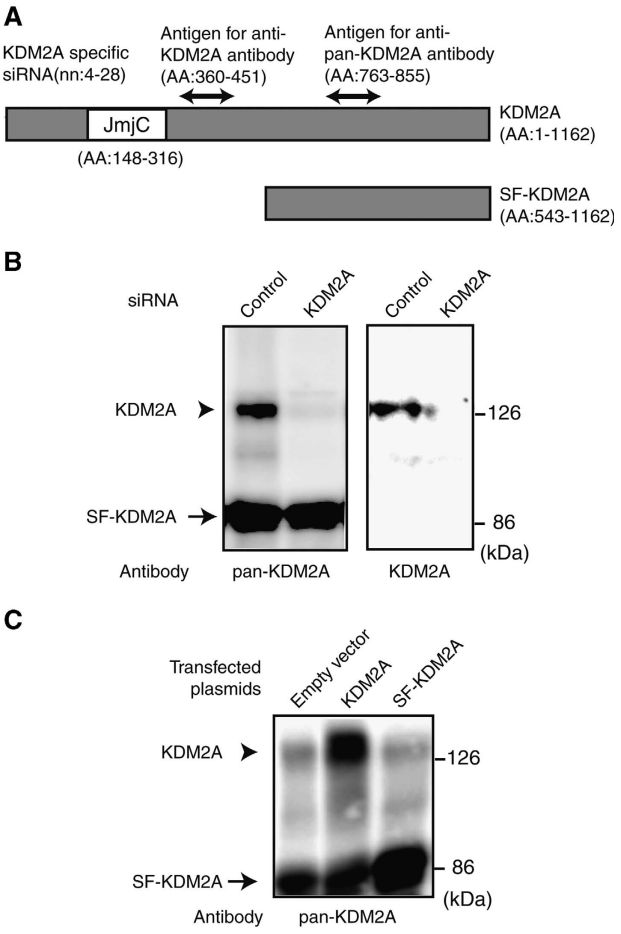

Figure 1 Proteins encoded by the *KDM2A* gene. (**A**) Diagrams of human KDM2A proteins. KDM2A (upper bar) has 1162 amino acids and contains the JmjC domain (AA148–316, shown by the white box) (GenBank Accession No. NM_012308). The numbers with AA in parentheses show amino acid numbers of KDM2A. SF-KDM2A (lower bar) has 620 amino acids and corresponds to the polypeptide from Met 543 to the end of KDM2A (AA 543–1162). The first Met for SF-KDM2A occurs in exon 14 of the *KDM2A* gene. The anti-pan-KDM2A antibody was produced against the polypeptide from Leu 763 to Gly 855 of KDM2A, and recognized both KDM2A and SF-KDM2A. An anti-KDM2A antibody was produced against the polypeptide from Ser 360 to Val 451 of KDM2A, and recognized only KDM2A. KDM2A-specific siRNA is a stealth RNA cognated to a partial nucleotide sequence for only KDM2A mRNA. The numbers with nn in parentheses show nucleotide numbers from the A of the first Met of KDM2A mRNA. (**B**) Western blot analysis to detect KDM2A proteins. Breast adenocarcinoma cell line MCF-7 cells were transfected with KDM2A-specific siRNA (KDM2A siRNA) or control siRNA. After 48 h culture, cells were lysed, and the extracts were subjected to western blotting using anti-pan-KDM2A antibody or anti-KDM2A antibody. The positions of KDM2A and SF-KDM2A are indicated by an arrowhead and arrow, respectively. The positions of protein markers with defined molecular weights are indicated on the right side of the figure. (**C**) The expression vector for KDM2A, SF-KDM2A, or the empty control vector was introduced into MCF-7 cells and analysed by western blotting as in (**B**) using anti-pan-KDM2A antibody.

Most of the signals for KDM2A overlapped with those for the nucleolar protein nucleolin (Figure 2A). It was reported that exogenously expressed KDM2A localized throughout the nucleoplasm as a heterochromatin-associated protein (Frescas *et al*, 2008). However, we observed that when KDM2A was moderately expressed exogenously but not

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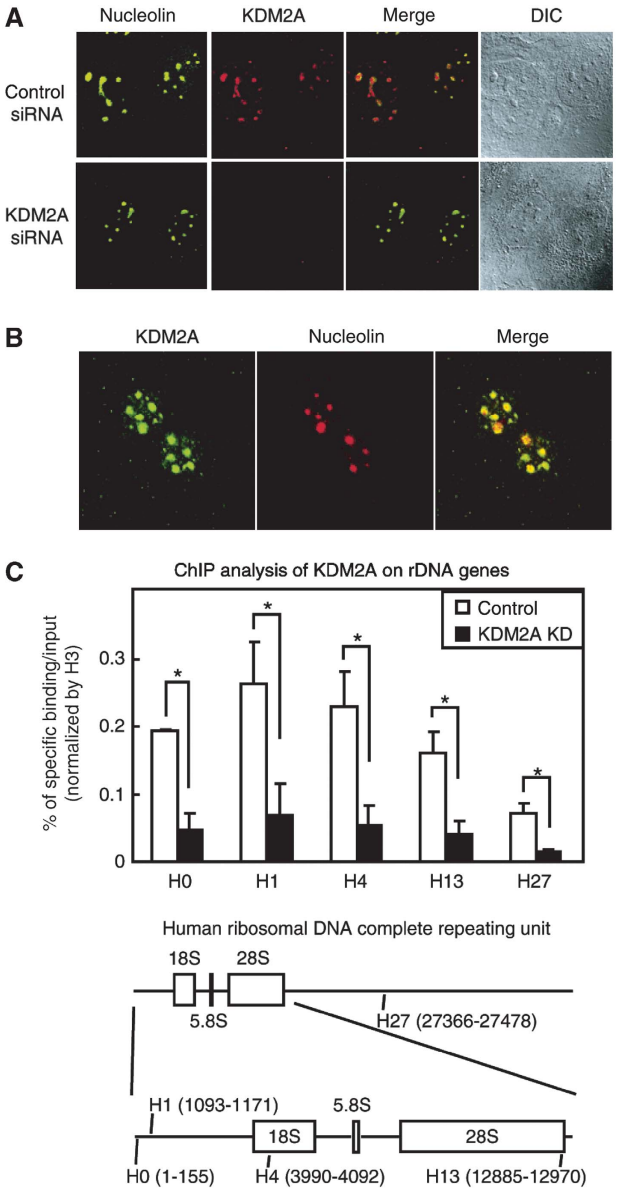

Figure 2 KDM2A with JmjC domain was localized in nucleoli and bound to rDNA. (**A**) MCF-7 cells were transfected with control or KDM2A siRNA and double-stained with anti-KDM2A (red) and mouse anti-nucleolin (green) antibodies. The specimen was observed through a fluorescence and differential interference contrast (DIC) microscope, and representative images are shown. The anti-KDM2A antibody produced signals localized in the nuclei (control siRNA). KDM2A siRNA reduced them. Most of the signals for KDM2A overlapped with those for nucleolin (merge). (**B**) HeLa cells were transfected with an expression vector encoding Flag-tagged KDM2A, and double-stained with anti-Flag (green) and rabbit anti-nucleolin antibodies (red). Representative images observed using a fluorescence microscope are shown. Most of the signals for KDM2A are co-localized with those for nucleolin when KDM2A was moderately expressed. (**C**) MCF7 cells were transfected with control or KDM2A siRNA and analysed by chromatin immunoprecipitation (ChIP) analysis using the anti-KDM2A antibody. The specific signals for the binding of KDM2A to DNA (white bars) were detected in all regions of rDNA genes including the promoter region, and the signals were reduced when cells were treated with KDM2A siRNA before ChIP analysis (black bars). The experiments were performed three times, and mean values with standard deviations are indicated. $^*P<0.05$. A diagram of human rDNA and the positions of PCR primers used in this experiment are shown at the bottom of this figure. The numbers in parentheses show nucleotide numbers in a human ribosomal DNA complete repeating unit (GenBank Accession No. U13369).

highly overexpressed, the protein was located in nucleoli (Figure 2B). These results show that KDM2A exists in nucleoli, although some part of it may exist outside of nucleoli.

Next, the binding of KDM2A to rDNA was investigated. First, the distribution of histone H3 through rDNA was examined. Chromatin immunoprecipitation (ChIP) analysis using anti-H3 antibody indicated that histone H3 was almost evenly distributed to all regions of rDNA (Supplementary Figure S3A). The anti-KDM2A antibody collected the fragment of the rDNA promoter (H0 region) (Supplementary Figure S3B). Enrichment of the fragment is dependent on KDM2A binding to the rDNA promoter, because the siRNA for KDM2A abolished recovery of the rDNA fragment by the anti-KDM2A antibody (Supplementary Figures S3B; Figure 2C). Additionally, the KDM2A binding detected here is specific, because the anti-KDM2A antibody hardly enriched the promoter and exon 5 genomic DNA fragments of the TATA-binding protein (*TBP*) gene (Supplementary Figure S3B). ChIP analysis also showed that the anti-KDM2A antibody collected DNA fragments from all regions of rDNA (Figure 2C). The siRNA for KDM2A abolished recovery of these DNA fragments, confirming the specific binding of KDM2A to the rDNA (Figure 2C).

Overexpression of KDM2A represses transcription of rDNA in demethylase activity-dependent manner

To investigate whether KDM2A regulates rDNA transcription, KDM2A was exogenously expressed, and the amount of pre-rRNA was measured by quantitative reverse transcription-mediated polymerase chain reaction (qRT–PCR). When the wild-type KDM2A was expressed, the amount of pre-rRNA was reduced (Figure 3A). KDM2A that had His 212 in the JmjC domain replaced with Ala (H212A mutant) did not show the demethylase activity (Tsukada *et al*, 2006) (Supplementary Figure S2). The H212A mutant also did not show a capacity to decrease the amount of pre-rRNA in these experimental conditions (Figure 3A). It was confirmed that comparable amounts of the wild-type KDM2A and H212A mutant were expressed on both RNA and protein levels (Figure 3A). Ongoing ribosomal RNA synthesis was also assessed by fluorouridine (FUrd) incorporation in *in situ* run-on assays (Kruhlak *et al*, 2007). Although high FUrd incorporation at nucleolar sites was observed in cells, ectopic expression of KDM2A led to a pronounced decrease in nucleolar FUrd incorporation (Figure 3B and C). This effect was not observed when *Escherichia coli* β-galactosidase targeted to the nucleus (Tsuneoka and Mekada, 1992) was expressed. Furthermore, the H212A mutant and SF-KDM2A did not show reduced FUrd incorporation (Figures 3B and C). These results indicate that the JmjC domain of KDM2A has a crucial role in the reduction. Together, these results show that KDM2A represses the transcription of rDNA in a demethylase activity-dependent manner.

JmjC enzyme is involved in reduction of rDNA transcription under starvation

Cells of the human breast adenocarcinoma cell line MCF-7 retain a good ability to change the levels of rDNA transcription in response to environmental conditions. When MCF-7 cells were cultured in starvation conditions, it was observed that starvation reduced the amount of pre-rRNA (Figure 4A). The effects of starvation on ongoing ribosomal RNA synthesis

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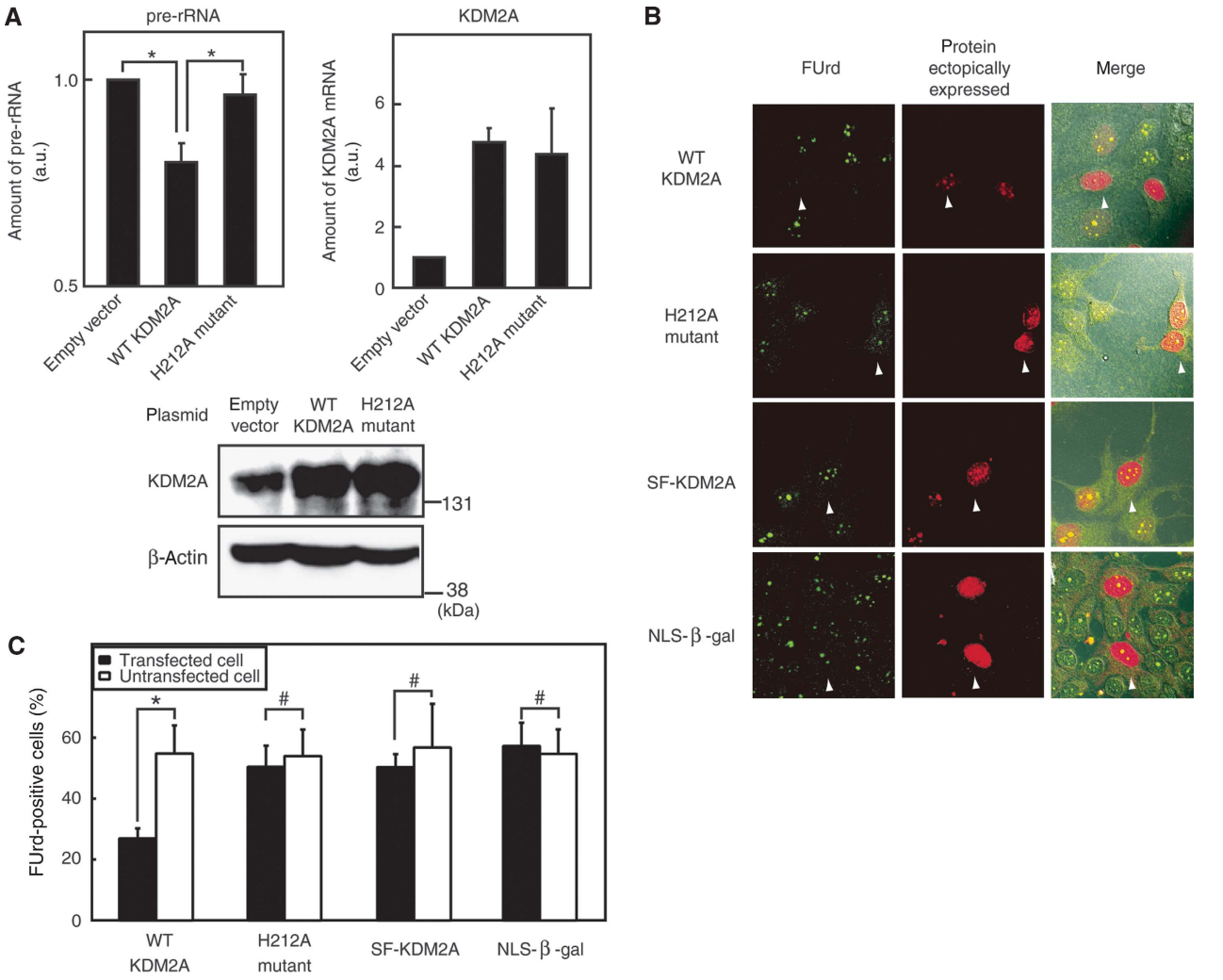

Figure 3 KDM2A reduced rDNA transcription. (**A**) MCF-7 cells were transfected with the KDM2A- or H212A mutant-expressing vector or the empty vector by electroporation and cultured for 2 days. Total RNA was isolated and analysed by quantitative real-time PCR (qRT–PCR) using specific primers for pre-rRNA, KDM2A, and RNA polymerase II subunit a (Polr2a). The values were normalized using the amounts of mRNA for Polr2a. The experiments were performed three times, and mean values with standard deviations are indicated. $^{*}P<0.05$. KDM2A protein was also detected by western blotting. β-actin was detected as a loading control. The positions of the molecular weight markers are indicated on the right side of the figure. (**B**) MCF-7 cells transfected with vector encoding KDM2A, the H212A mutant, SF-KDM2A, or nuclear-localizing *E. coli* β-galactosidase. Two days later, cells were cultured with 2 mM FUrd for 15 min, fixed, and stained for FUrd and Flag-tagged KDM2A or β-galactosidase for assessment by FUrd incorporation assays. The incorporated FUrd (green) and exogenously expressed protein (red) were observed by a fluorescence microscopy. Representative images are shown. One of the cells with positive signals for exogenous proteins in one filed is indicated by an arrowhead. (**C**) Percentages of FUrd-positive cells were calculated in cells that exogenously expressed KDM2A, the H212A mutant, SF-KDM2A, or nuclear-localizing *E. coli* β-galactosidase (black bars), or in cells that did not exogenously express each protein in the same specimen (white bars). The experiments were performed three times, and mean values with standard deviations are indicated. $^{*}P<0.05$; $^{\#}P>0.1$ (no significant difference).

were also assessed by the FUrd incorporation assays. Although high FUrd incorporation at nucleolar sites was observed in cells in growth conditions, starvation clearly reduced the incorporation (Figure 4B).

To test whether JmjC-domain enzymes are involved in the reduction of rDNA transcription by starvation, MCF-7 cells were treated with a compound to inhibit the demethylase activity of the enzymes. Succinate is produced from α-KG by the JmjC-domain enzymes during its demethylation reaction (Figure 4C). As an enzymatic activity depends on the product/substrate equilibrium, succinate may inhibit the activity of the enzymes. Indeed, we observed *in vivo* inhibition of the demethylase activity of KDM2A by a cell-permeable succinate, dimethyl succinate (DMS) (Figure 4D). As shown in Figure 4A, DMS suppressed the reduction in the amount of pre-rRNA by starvation. DMS also suppressed the reduction of FUrd incorporation at nucleolar sites by starvation (Figure 4B). Together these results suggest that a JmjC-domain enzyme regulates rDNA transcription during starvation.

Levels of KDM2A substrates, H3K36me1/2 marks, are changed on the rDNA promoter during starvation

The effects of starvation and DMS on the levels of substrates of KDM2A, H3K36me2 marks (Tsukada *et al*, 2006), were investigated. As shown in Figure 5A, starvation decreased the level of H3K36me2 marks on the rDNA promoter, and treatment with DMS increased it during starvation (Figure 5B). As

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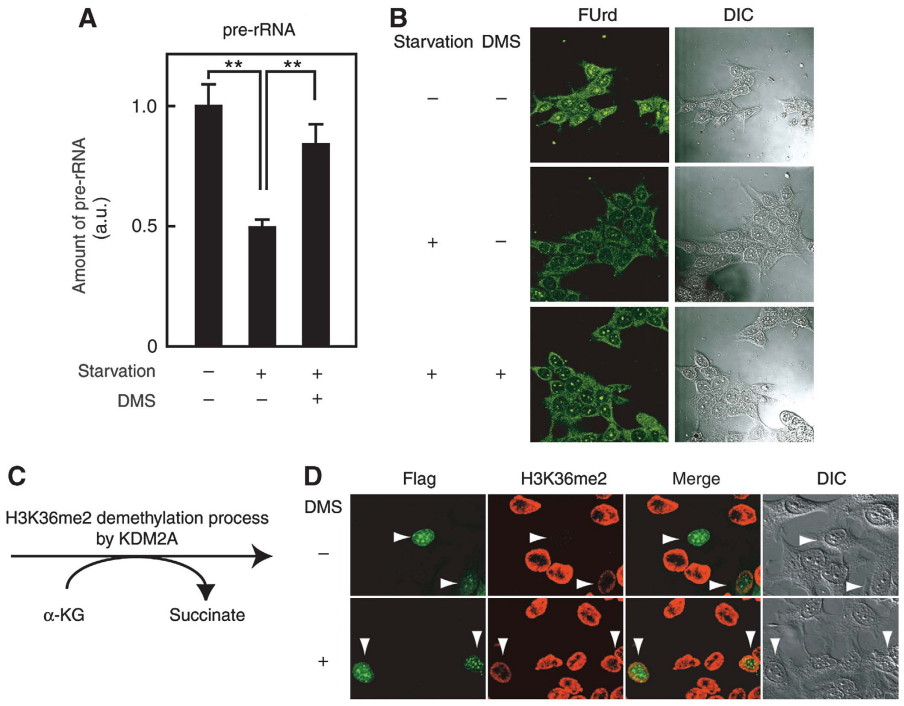

Figure 4 Levels of rDNA transcription under starvation conditions in the presence or absence of cell-permeable succinate (DMS). (**A**) MCF-7 cells were cultured with or without starvation for 9 h. Total RNA was isolated from cells, and analysed by qRT–PCR with specific primers for pre-rRNA and Polr2a mRNA, as described in Figure 3A. A cell-permeable succinate, DMS, was added at a final concentration of 50 mM during starvation in the indicated experiments. The experiments were performed three times, and mean values with standard deviations are indicated. $^{**}P<0.01$. (**B**) MCF-7 cells were cultured with or without starvation for 9 h. In the indicated experiments, DMS was added during starvation. Cells were incubated with 2 mM FUrd for 15 min, fixed, and stained for FUrd. Representative images observed through a fluorescence (FUrd) and differential interference contrast (DIC) microscopy are shown. (**C**) KDM2A produces succinate during demethylation. When JmjC-domain enzymes execute demethylation reactions, they catalyse α-ketoglutarate (α-KG) as a co-substrate to succinate (Tsukada *et al*, 2006; Klose *et al*, 2006a). Thus, excess amounts of succinate may inhibit the activity of JmjC-domain enzymes. (**D**) MCF-7 cells were transfected with a Flag-KDM2A expression vector in serum-free DMEM, cultured for 1 day, and further cultured in serum-free DMEM for another day in the presence or absence of a cell-permeable succinate, dimethyl succinate (DMS) (50 mM). Cells were stained with anti-H3K36me2 (red)- and anti-Flag (green)-specific antibodies. Representative images observed by a fluorescence and differential interference contrast (DIC) microscopy are shown. Cells with positive signals for KDM2A are indicated by arrowheads. DMS inhibited the reduction of H3K36me2 marks by the ectopic expression of KDM2A. Cells with decreased H3K36me2 levels were 59 and 28% of cells in the absence and presence of DMS, respectively. These results provide *in vivo* evidence that succinate inhibits the demethylase activity of KDM2A.

starvation and DMS treatment hardly affected the amount of KDM2A protein (Supplementary Figure S4A) and DMS inhibited the demethylase activity of KDM2A (Figure 4D), these results are consistent with the possibility that KDM2A reduces rDNA transcription in an enzyme activity-dependent manner during starvation. DMS did not increase the level of H3K36me3 marks in starvation. Interestingly, although H3K36me1 has not been identified as a substrate of KDM2A *in vivo*, starvation decreased the level of H3K36me1 marks on the rDNA promoter (Figure 5A), and treatment with DMS increased it (Figure 5B). These results suggest the possibility that H3K36me1 on the rDNA promoter may also be recognized by KDM2A as a substrate.

An H3K4me3 mark was recently reported to be an active mark for rDNA transcription and demethylated by a JmjC-domain demethylase KDM2B/JHDM1B/FbxL10 (Frescas *et al*, 2007). However, neither starvation nor DMS affected the level of H3K4me3 marks in the rDNA promoter region (Figure 5A and B), suggesting that a JmjC-domain enzyme demethylating H3K4me3 on the rDNA promoter did not work during starvation.

Next, we investigated the effects of starvation and DMS on H3K36 methylation in the other genomic regions. In the *TBP* gene, the levels of H3K36me1, H3K36me2, and H3K36me3 (H3K36me1/2/3) marks were higher in a transcribed region than in the promoter region (Supplementary Figure S4B). The levels of H3K36me1/2/3 in the two regions of the *TBP* gene did not change in response to starvation and DMS (Supplementary Figure S4B). In rDNA, the levels of H3K36me1/2/3 in the transcribed regions (H1, H4, and H13) were not higher than those in the promoter region (H0) (Supplementary Figure S5). Interestingly, starvation and DMS did not significantly change the levels of H3K36me1/2/3 marks in the transcribed and untranscribed regions (H1, H4, H13, and H27) of rDNA (Supplementary Figure S5). These results together with the results shown in Figure 5 suggest that the starvation signal is specifically transduced to an H3K36me1/2 demethylase located in the rDNA promoter region.

KDM2A regulates levels of H3K36me1/2 and rDNA transcription in response to starvation

To directly clarify the involvement of KDM2A in the regulation of H3K36me1/2 marks in the rDNA promoter region and rDNA transcription under starvation conditions, the expression of KDM2A was reduced using siRNA for KDM2A. The

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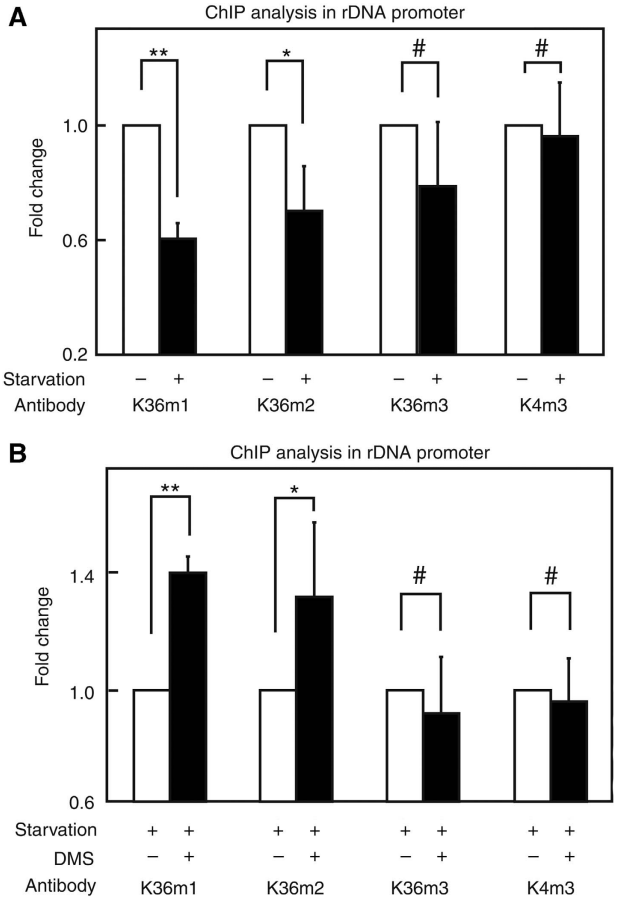

Figure 5 Levels of methyl histone marks under starvation conditions in the presence or absence of cell-permeable succinate (DMS). (**A**) MCF-7 cells were cultured with or without starvation for 9 h, and the histone methylation status on rDNA promoters was investigated by ChIP analyses using specific antibodies to H3K36me1, H3K36me2, H3K36me3, H3K4me3, and histone H3. The results were expressed as fold changes to the values without starvation. (**B**) MCF-7 cells were cultured with starvation in the presence or absence of 50 mM DMS for 9 h, and the histone methylation status on rDNA promoters was detected as in (**A**). The results are expressed as fold changes to the values without DMS. For (**A**) and (**B**), the experiments were performed at least three times, and mean values with standard deviations are indicated. $^{*}P<0.05$; $^{**}P<0.01$; $^{\#}P>0.1$ (no significant difference).

KDM2A knockdown reduced the amount of KDM2A protein in cells and on the rDNA promoter, and increased the levels of not only H3K36me2 but also H3K36me1 marks there (Figure 6A). Importantly, the KDM2A knockdown inhibited the decrease of H3K36me1/2 levels during starvation, indicating the involvement of KDM2A in the reduction of the marks under starvation.

The knockdown of KDM2A also resulted in a significant increase in the rDNA transcription both with and without starvation (Figure 6B), but its effect was stronger with than that without starvation (Figure 6C). KDM2A knockdown by another siRNA also increased the rDNA transcription with starvation (Supplementary Figure S6), confirming that the effect of the siRNAs on rDNA transcription under starvation was caused by the reduction of KDM2A expression. The KDM2A knockdown completely abolished the increase in the amount of pre-rRNA because of the treatment with DMS (Figure 6D), indicating that inhibition of KDM2A is crucial for the recovery of pre-rRNA synthesis by DMS. These results show that KDM2A decreases the levels of both H3K36me1 and H3K36me2 marks on the rDNA promoter and reduces rDNA transcription under starvation.

KDM2A knockdown increases amount of mature ribosomal RNA and rate of protein synthesis under starvation

To investigate whether KDM2A regulates ribosome biogenesis, the amounts of mature ribosomal RNA (28S rRNA) and the rate of protein synthesis were measured. KDM2A knockdown without starvation rarely or only faintly increased the amount of pre-rRNA, 28S rRNA, and the rate of protein synthesis per cell (Figure 7A–C). After 24 h starvation, cells with the KDM2A knockdown increased the transcription of pre-rRNA, amounts of 28S rRNA, and the rate of protein synthesis (Figure 7A–C). These results indicate that the elevation of pre-rRNA synthesis by KDM2A knockdown increases ribosome biogenesis and the rate of net protein synthesis.

Discussion

We found that KDM2A was localized in nucleoli and bound to the rDNA promoter. Our results indicate that KDM2A reduced H3K36me1/2 on the rDNA promoter during starvation and downregulates the level of rDNA transcription (Supplementary Figure S7). A KDM2A knockdown resulted in elevations of pre-rRNA synthesis, levels of mature ribosomal RNA, and the rate of protein synthesis. These results suggest that KDM2A functions as a regulator of ribosome biogenesis.

KDM2A controls demethylation of H3K36me1/2 on rDNA promoter

KDM2A has a demethylase activity *in vitro* on H3K36me1/2 (Tsukada *et al*, 2006). Starvation reduced the levels of H3K36me1/2 marks on the rDNA promoter, and treatment with DMS, which inhibited KDM2A demethylase activity, suppressed the reduction during starvation (Figure 5A and B). KDM2A knockdown increased the levels of H3K36me1/2 marks on the rDNA promoter (Figure 6A). These results indicate that KDM2A reduced the levels of H3K36me1/2 marks on the rDNA promoter in response to starvation. Although H3K36me1 has not been identified as a substrate of KDM2A *in vivo*, the result shown here is the first suggestion that the amount of H3K36me1 is also reduced by KDM2A *in vivo*.

There are several JmjC enzymes that demethylate H3K36me2/3, KDM4A/JHMD3A/JMJD2A (Whetstine *et al*, 2006; Klose *et al*, 2006b), KDM4B/JMJD2B (Allis *et al*, 2007), and KDM4C/JMJD2C (Allis *et al*, 2007), which may be involved in demethylation of H3K36me2 on the rDNA promoter. However, the level of H3K36me3 marks was not increased by DMS during starvation (Figure 5B), suggesting that these JmjC demethylases did not erase H3K36me2 on the rDNA promoter under starvation. KDM2B was originally reported to demethylate H3K36me1/2 *in vitro* (Tsukada *et al*, 2006), and recently its demethylase activity for H3K36me2 was identified on genes *in vivo* (He *et al*, 2008; Polytarchou *et al*, 2008; Tzatsos *et al*, 2009). KDM2B was suggested to be localized in nucleoli and bind to rDNA

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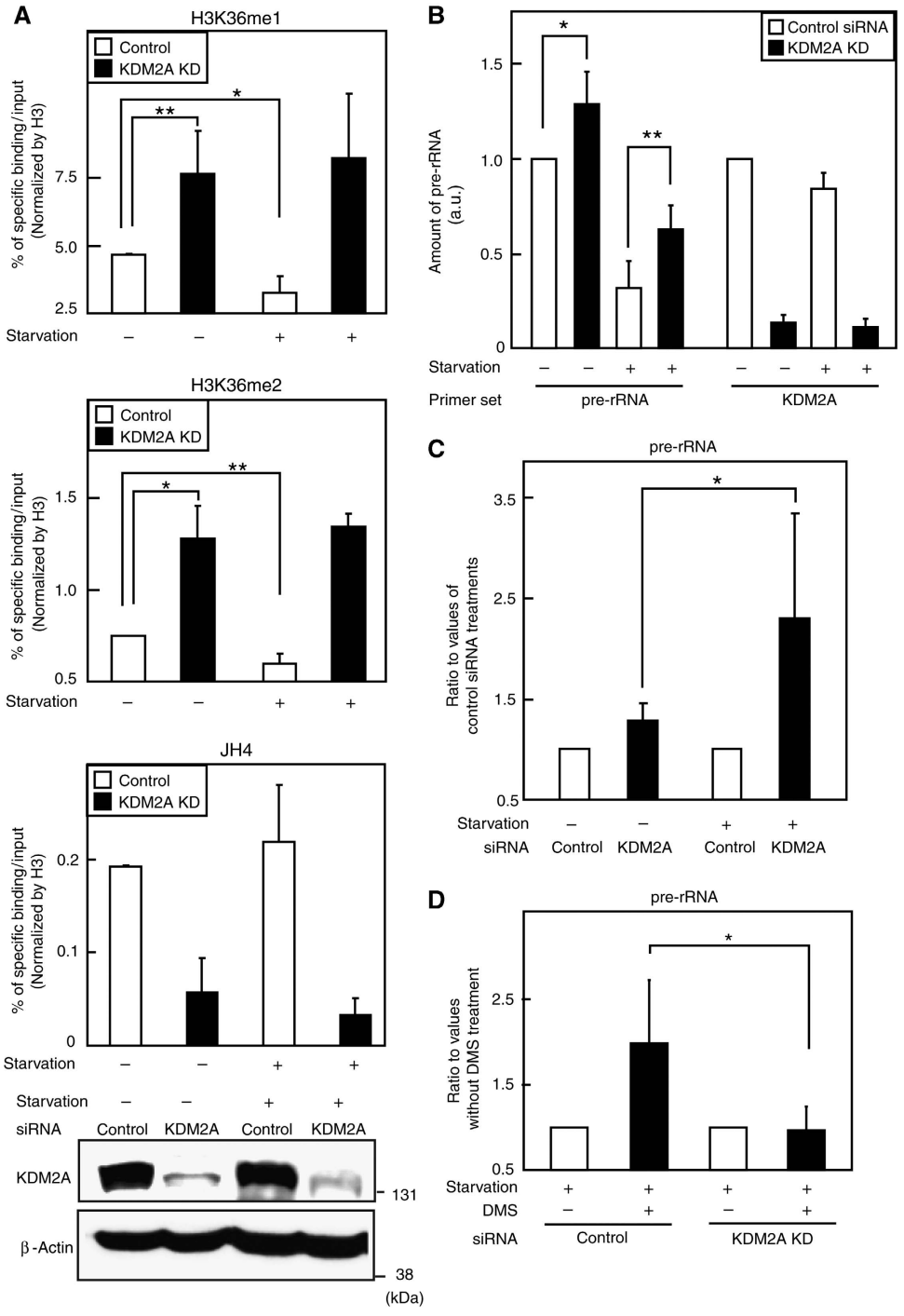

Figure 6 KDM2A was involved in the reduction of rDNA transcription by starvation, and its activity was controlled by succinate. (**A**) MCF-7 cells were transfected with control or KDM2A siRNA. Forty-eight hours after transfection, cells were further cultured 2 h with or without starvation. ChIP analyses using H3K36me1, H3K36me2, histone H3, and KDM2A antibodies were performed on the rDNA promoter as shown in Figure 2C. The experiments were performed three times, and mean values with standard deviations are indicated. $*P<0.05$; $**P<0.01$. The knockdown of KDM2A was confirmed by western blotting (lower panel). The positions of the molecular weight markers are indicated on the right side of the figure. (**B**) MCF-7 cells were transfected with control or KDM2A siRNA. Forty-eight hours after transfection, cells were further cultured 9 h with or without starvation. The amounts of pre-rRNA and KDM2A mRNA were measured by qRT–PCR as described in Figure 3A. The results are expressed as amounts relative to the values of cells treated with control siRNA and without starvation. For (**B**), (**C**), and (**D**), the experiments were performed three times, and mean values with standard deviations are indicated. $*P<0.05$; $**P<0.01$. (**C**) Increased levels of pre-rRNA transcript by KDM2A knockdown in (**B**) were expressed against the values with control siRNA. The increase of rDNA transcription with starvation was higher than that without starvation. $*P<0.05$. (**D**) Succinate functions through KDM2A to regulate rDNA transcription. Forty-eight hours after MCF-7 cells were transfected with control or KDM2A siRNA, cells were cultured in the presence or absence of DMS for 9 h with starvation. The increases of pre-rRNA transcript amounts with DMS were expressed against the values without DMS in each case (with treatment of control or KDM2A siRNA). With KDM2A knockdown, DMS did not increase the amount of pre-rRNA. $*P<0.05$.

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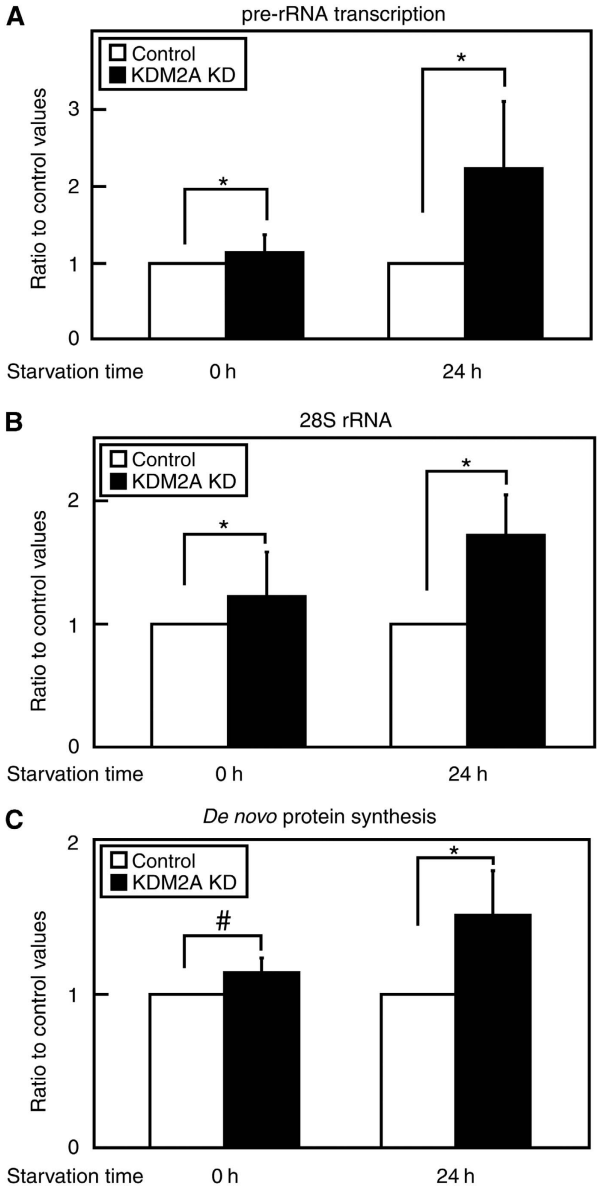

Figure 7 Effects of KDM2A knockdown on pre-rRNA synthesis, mature RNA, and the rate of protein synthesis. MCF-7 cells were transfected with siRNA for KDM2A. With or without further culturing in starvation conditions for 24 h, RNA was isolated and pre-rRNA (**A**) and mature 28S ribosomal RNA (28S rRNA) (**B**) were detected by qRT–PCR. Cells were also cultured with [^{3}H]leucine for 2 h and the radioactivity incorporated into the protein was measured (**C**). The results were normalized for cell count. The relative values with siRNA for KDM2A against these with control siRNA were expressed. The experiments were performed three times, and mean values with standard deviations are indicated. $*P<0.05$; $\#P>0.1$ (no significant difference).

(Frescas *et al*, 2007). However, KDM2B was reported to demethylate H3K4me3 on the rDNA promoter (Frescas *et al*, 2007). The knockdown of KDM2A largely increased the levels of H3K36me1/2 marks on the rDNA promoter, and completely abolished the reduction of the marks under starvation (Figure 6A). These results indicate that KDM2A is a major factor for H3K36me1/2 demethylation on the rDNA promoter region under starvation.

Although KDM2A also bound to other regions of rDNA in addition to the promoter (Figure 2C), H3K36me1/2 levels in these regions were not affected by starvation and DMS treatment. These results suggest that there is a mechanism to transduce the starvation signal specifically to KDM2A located in the rDNA promoter in rDNA. These results also suggest that KDM2A may function during conditions other than starvation to control the status of rDNA chromatin. When KDM2A functions in all regions of rDNA chromatin is unresolved.

Endogenous KDM2A was preferentially localized in nucleoli, and was detected in rDNA but not in the *TBP* gene (Figure 2; Supplementary Figure S3). The molecular mechanisms by which KDM2A accumulates in nucleoli are also important issues to be resolved in the future.

KDM2A and H3K36me1/2 marks may have role in short-term regulation on rDNA chromatin

Eukaryotes maintain several hundred copies of rDNA in each cell, but only a proportion of the genes are competent to be transcribed (Grummt and Ladurner, 2008). Actively transcribed rDNA chromatin exhibits euchromatic features, including methylation of histone H3 lysine 4 (H3K4). On the other hand, silent rDNA chromatin bears heterochromatin marks such as hypermethylated DNA at CpG residues in the promoters (Grummt and Ladurner, 2008). These epigenetic marks of rDNA chromatin are heritably maintained through DNA replication, suggesting that the epigenetic status is stable (Li *et al*, 2005). In response to environmental conditions, the transcription rate of the active rDNA is changed, which is termed short-term regulation (Grummt, 2003). In addition, in response to certain types of environmental conditions or during tumourigenesis, active genes can become silent ones and vice versa, and the proportion of active genes to silencing ones is varied, which is termed epigenetic transition (Santoro, 2005; Huang *et al*, 2006; Grummt, 2007; Grummt and Ladurner, 2008).

The level of H3K4me3 marks, generally accepted as epigenetic marks of active genes, did not change in the starvation conditions used here (Figure 5A). Digestion of rDNA by the methylation-sensitive restriction enzyme *Hpa*II showed that the copy number of the silent rDNA did not change during the starvation conditions (Supplementary Figure S8). These results suggest that the epigenetic transition from active to silent rDNA did not occur in our experimental conditions. In these conditions, H3K36me1/2 marks were reduced by KDM2A, suggesting that KDM2A and H3K36me1/2 have a role in the short-term regulation of rDNA chromatin without epigenetic transition.

In our starvation conditions, serum and glucose were depleted. Serum contains growth factors and nutrients that stimulate several transduction pathways. The mammalian target of rapamycin (mTOR) and ERK/MAPK pathways regulate pol I transcription in response to growth factors and nutrients (Grummt, 2003; James and Zomerdijk, 2004). It was suggested that these pathways regulate pol I transcription by modulating the activity of the pol I-specific transcription initiation factor TIF-IA (Zhao *et al*, 2003; Mayer *et al*, 2004). ERK/MAPK also phosphorylates the HMG boxes of UBF, a pol I factor essential for transcription enhancement, and it was shown to directly regulate elongation by inducing the remodelling of rDNA chromatin (Stefanovsky *et al*, 2006).

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In glucose starvation, the NAD^+/NADH ratio is elevated, and the NAD^+-dependent histone deacetylase SIRT1 is activated. SIRT1 controls several proteins that repress transcription of rDNA (Muth *et al*, 2001; Murayama *et al*, 2008; Zhou *et al*, 2009). These signalling molecules and pol I-specific regulatory molecules may be related to the control of KDM2A activity, and this is an important issue to be resolved in the future.

An experiment in which the rDNA immunoprecipitated by the anti-KDM2A antibody was digested by *Hpa*II indicated that KDM2A occupied both unmethylated and methylated rDNA copies (Supplementary Figure S9). These results suggest that KDM2A may also have a role in establishing and/or maintaining the silencing rDNA chromatin in addition to short-term regulation. Epigenetic transition is mediated by nucleolar remodelling complex (NoRC) (Strohner *et al*, 2001) and the recently identified energy-dependent nucleolar silencing complex (eNoSC) (Murayama *et al*, 2008). NoRC contains Snf2 h, which recruits DNA methyltransferase and histone deacetylase activities to the promoter (Santoro *et al*, 2002; Grummt and Ladurner, 2008). eNoSC is a complex consisting of a histone deacetylase, a histone methyltransferase, and a methylated histone-binding protein (Murayama *et al*, 2008). KDM2A may be related to the functions of these components.

Biological significance of methylations of histone H3 lysine 36

In the *TBP* gene, the levels of H3K36me1/2/3 were higher in a transcribed region than in the promoter region (Supplementary Figure S4B). On the other hand, in rDNA, a greater abundance of the levels of H3K36me1/2/3 marks was not detected in the transcribed regions compared with the promoter region (Supplementary Figure S5). These results suggest that H3K36me1/2/3 marks in the rDNA chromatin are distinctly different from those in a gene transcribed by pol II.

Set2, which is responsible for methylation of H3K36, was identified from *Saccharomyces cerevisiae*. Set2 and methylation at H3K36 were reported to have a function in the repression of gene transcription (Strahl *et al*, 2002). Furthermore, it was found that Set2 is recruited by pol II, which localizes H3K36 methylation to the body of genes (Krogan *et al*, 2003; Li *et al*, 2003; Schaft *et al*, 2003; Xiao *et al*, 2003); it was also reported that Rpd3S is recruited to nucleosomes with methylated H3K36 to suppress inappropriate and spurious intragenic transcription (Carrozza *et al*, 2005; Keogh *et al*, 2005). In mammals, the distribution of H3K36me3 marks is also elevated after the transcription start sites in the coding region (Barski *et al*, 2007; Li *et al*, 2007). Recently, it was reported that H3K36me3 marks were related to suppression of gene transcription in mice (Nimura *et al*, 2009). Wolf-Hirschhorn syndrome candidate 1 (WHSC1) is one of five putative Set2 homologues. Experiments using cells with a deletion of this gene showed that WHSC1 governed H3K36me3 along the euchromatin by associating with some transcription factors and repressing inappropriate transcription. In rDNA, the level of H3K36me3 in the transcribed regions (H1, H4, and H13) was not higher than that in the promoter region (H0), although it tended to be higher than it was in the untranscribed region (H27) (Supplementary Figure S5). These results suggest that the level of H3K36me3 has some relation to transcription, but the H3K36me3 mark in the rDNA promoter has a different significance from that in genes transcribed by pol II.

H3K36me2 was suggested to be different from H3K36me3 in both distribution and function. In *Drosophila*, H3K36me2 is highest adjacent to promoters and requires dMes-4, whereas H3K36me3 accumulates towards the 3′ end of genes and relies on dHypb. Reduction of H3K36me3 leads to elevated levels of acetylation, specifically at lysine 16 of histone H4 (H4K16ac). In contrast, reduction of H3K36me1/2 decreases H4K16ac (Bell *et al*, 2007). These results suggest that H3K36me1/2 have opposite effects on H4K16 acetylation against H3K36me3. Furthermore, in *Drosophila*, dKDM2 has a role in a *trans*-histone pathway involving the removal of H3K36me2 marks and formation of histone H2A ubiquitilation during polycomb group silencing (Lagarou *et al*, 2008), suggesting that H3K36me2 is an active mark. In mammals, KDM2B targets the INK4b-ARF-INK4a locus and demethylates H3K36me2 to downregulate the expression of INK4b and INK4a (He *et al*, 2008; Tzatsos *et al*, 2009). These results are consistent with our results here that H3K36me2 marks on the rDNA promoter are associated with transcriptional activation (Figure 7D).

However, KDM2B was also reported to promote the expression of NAD(P)H quinone oxidoreductase-1 and peroxiredoxin-4 by directly binding to the promoters of their genes and demethylating H3K36me2 (Polytarchou *et al*, 2008). Furthermore, H3K36me2 is sufficient to target the histone deacetylase complex, Rpd3S, *in vivo* (Li *et al*, 2009). These results suggest that H3K36me2 is also a gene repression mark, and that H3K36me2 marks may have distinct physiological significance in different genes.

KDM2A reduced H3K36me1 marks in addition to H3K36me2 marks on the rDNA promoter (Figure 6A). These results suggest that unmethylated lysine 36 histone H3 (H3K36me0) is produced by KDM2A. There is a precedent for an unmethylated lysine residue of histone H3 showing physiological significance. KDM1/LSD1 is a lysine-specific histone demethylase that demethylates histone H3 on lysine 4, produces unmethylated H3K4 (H3K4me0), and represses transcription. The plant homeodomain finger of BHC80 binds H3K4me0, and this interaction is specifically abrogated by methylation of H3K4. These findings indicate that unmodified H3K4 is part of the 'histone code' (Lan *et al*, 2007). Taking H3K4me0 as an analogy, it is possible that H3K36me0 functions as a histone code on the rDNA promoter. Clarification of each function of mono-, di-, tri-, and unmethylated H3K36 on the rDNA promoter will further the understanding of the control of gene expression.

Exogenously expressed KDM2A can decrease the H3K36me2 level in the entire region of the nuclei when H3K36me2 marks are detected under a microscope. However, it can also preferentially repress pre-rRNA transcription (Figure 3). There are two possibilities to explain this phenomenon. In the first, the change of H3K36me2 marks affects transcription of only a limited number of genes. In general, the changes of histone codes in promoter regions appear to affect transcription more effectively than those in transcribed regions. As described in Supplementary Figure S4B, the level of H3K36me2 marks in the promoter region was lower than those in a transcribed region in the *TBP* gene. These results suggest that H3K36me2 marks may not have much significance in transcription in the *TBP* gene. On the

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contrary, the levels of H3K36me2 marks in the promoter region (H0) were similar to those in the transcribed regions (H1, H4, and H13) in rDNA. In addition, the amount of H3K36me2 in the rDNA promoter was higher than that in the TBP promoter (Supplementary Figure S4B and S5). These results suggest that a demethylase for H3K36me2 may affect transcription of rDNA more strongly than transcription of TBP. Therefore, it is possible that a reduction of the H3K36me2 level in most genes in the nucleoplasm affects their transcription weakly, compared with that in rDNA.

In the second possibility, H3K36me1 or H3K36me0, but not H3K36me2, may be a histone code for transcription control. KDM2A knockdown increased the levels of H3K36me1/2 marks in the rDNA promoter. On the contrary, overexpression of KDM2A reduced overall levels of H3K36me2 but not H3K36me1 in nuclei (Tsukada *et al*, 2006), suggesting that KDM2A has a specific mechanism to reduce the H3K36me1 level in the rDNA but not in the other genes. The reduced H3K36me1 level or the increased H3K36me0 level may be related to reduction of transcription. The above two possibilities are not mutually exclusive. Determining the molecular mechanisms relating to the possibilities may lead to a better understanding of KDM2A specificity on rDNA.

KDM2A may be involved in tumourigenesis under pseudohypoxic conditions

The activity of KDM2A was inhibited by succinate. Succinate is the organic acid in the TCA cycle, and its concentration may be changed in response to external conditions. This raises the possibility that succinate may be an intracellular signalling molecule regulating KDM2A activity. Although it is not clear whether this organic acid is involved in the signal transduction in a normal cell, the control of enzyme activity by succinate had been recently proposed to occur in some specific tumours. HIFα is hydroxylated by HIFα prolyl hydroxylase (PHD) under normal oxygen conditions (normoxia), and the hydroxylated HIFα is degraded quickly (Kim and Kaelin, 2003). Hypoxia inhibits the activity of PHD, and accumulation of HIFα induces hypoxia-response genes such as vascular endothelial growth factor. A deficiency of the regulatory system induces an aberrant stabilization of HIFα under normoxic conditions, a condition termed pseudohypoxia, which predisposes to tumourigenesis. Mutations in genes coding subunits of succinate dehydrogenase (SDH) in the TCA cycle are associated with the development of paraganglioma and pheochromocytoma (Favier *et al*, 2005; Gottlieb and Tomlinson, 2005; Hao *et al*, 2009). In those tumours, succinate is accumulated (Pollard *et al*, 2005). PHD is an α-KG-dependent enzyme that catalyses α-KG to succinate during its hydroxylation reaction; in turn, the accumulated succinate inhibits PHD activity even under normoxia, which results in pseudohypoxia (Isaacs *et al*, 2005; Pollard *et al*, 2005; Selak *et al*, 2005). Pseudohypoxia is also currently reported in glioblastoma multiforme (a malignant human brain tumour) with mutations of the isocitrate dehydrogenase (*IDH1)* gene. IDH1 catalyses isocitrate to α-KG, and a dysfunction of IDH1 results in a decreased level of α-KG, lowering PHD activity and inducing pseudohypoxia (Zhao *et al*, 2009). Although the control of HIF activity under pseudohypoxic conditions fits well with the tumourigenesis that occurs under defects in certain TCA cycle enzymes and IDH1, additional mechanisms that involve other α-KG-dependent enzymes regulated by pseudohypoxic conditions are suspected to be involved in tumourigenesis (Sudarshan *et al*, 2007; Pollard and Ratcliffe, 2009; Tsuneoka *et al*, 2009). The presence of these mechanisms may be attributed to the difference between tumour predispositions conferred by mutations in distinct genes such as *IDH1* and *SDH*.

As early as 1970, changes in nucleoli were recognized as a reliable marker of cellular transformation (Gani, 1976). Several tumour suppressors reduce rDNA transcription, and some proto-oncogenes increase it. Mutations in genes that encode proteins directly involved in ribosome biogenesis have been associated with cancer (Ruggero and Pandolfi, 2003). Therefore, malignant progression may be regulated partly through altering ribosome biogenesis. KDM2A functions as a negative regulator for ribosome biogenesis by decreasing rDNA transcription (Supplementary Figure S7). Transfection of MCF-7 cells with a KDM2A-expressing vector carrying a puromycin-resistant gene greatly reduced the number of colonies produced in the presence of puromycin compared with transfection with the empty vector (Table S1). These results suggest that KDM2A has a negative effect on cell proliferation. KDM2A is an α-KG-dependent enzyme (Tsukada *et al*, 2006). The elevated level of succinate inhibits KDM2A activity (Figure 4D) and increases rDNA transcription (Figure 4A and B). KDM2A knockdown completely abolished the increase in the amount of pre-rRNA because of succinate (Figure 6D), confirming that succinate elevates rDNA transcription through inhibition of KDM2A. Succinate also increased the amounts of mature ribosome RNA in cells, and the KDM2A knockdown abolished the increase (Supplementary Figure S10). Therefore, the novel mechanism presented here, by which KDM2A reduces rDNA transcription, may be involved in tumourigenesis under the pseudohypoxic conditions induced by defects in enzymes such as SDH.

Materials and methods

Cells and cell culture

Human breast adenocarcinoma cell line MCF-7 cells and human cervical carcinoma HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Cat# D5796; Sigma-Aldrich Co.) supplemented with 10% foetal calf serum. Cells were maintained at 37°C in an atmosphere containing 5% CO and 100% humidity. The mammalian expression plasmids were introduced into human cells using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. In Figure 3A, 5 μg of plasmids was introduced by electroporation using a Gene Pulser II electroporator (Bio-Rad Laboratories) at 270 V, 100 μF, using Gene Pulser Electroporation Buffer (Cat#165-2677; Bio-Rad) according to the manufacturer's instructions.

For starvation, cells were cultured in serum-free DMEM that did not contain glucose (Cat# D5030; Sigma-Aldrich). In some experiments, cells were cultured with a cell-permeable succinate, 50 mMDMS (SO104; Tokyo Kasei Kogyo Co., Tokyo, Japan), as indicated in the figure legends.

Antibodies

Mouse monoclonal anti-nucleolin antibody (C23 (MS-3): sc-8031; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-nucleolin antibody (C23 (H-250): sc-13057; Santa Cruz), mouse monoclonal anti-β-actin (AC-15) antibody (Sigma, St Louis, MO, USA), mouse monoclonal anti-FLAG (M2) antibody (Sigma), goat anti-rabbit IgG-HRP (sc-2054; Santa Cruz), anti-monomethylated histone H3 lys36 antibody (ab9048; Abcam Cambridge, UK), anti-trimethylated histone H3 lys36 antibody (ab9050; Abcam),

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anti-trimethylated histone H3 lys4 antibody (ab8580; Abcam), anti-histone H3 antibody (ab1791; Abcam), Alexa 488-conjugated goat anti-mouse IgG (H + L) (A11029; Invitrogen, Carlsbad, CA, USA), and Alexa 568-conjugated goat anti-rabbit IgG (H + L) (A11011; Invitrogen,) were purchased.

Anti-dimethylated histone H3 lys36 antibody (#9758; Cell Signaling Technologies, Beverly, MA, USA) and control antibody (normal rabbit IgG, #2729S; Cell Signaling) were also purchased. We compared this antibody to another anti-H3K36me2 antibody (cat# 07-274; Millipore), which was shown to work in ChIP assays by the manufacturer. As shown in Supplementary Figure S11, the Cell Signaling antibody produced stronger signals than the Millipore antibody in ChIP experiments. Therefore, we used the Cell Signaling anti-H3K36me2 antibody in this study.

Antibodies to KDM2A are described in the Supplementary data.

siRNA for human KDM2A

Cells were transfected with stealth siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The siRNA oligonucleotide sequence for KDM2A was 5′-GAACCCGAAG AAGAAAGGAUUCGUU-3′. The position of the sequence is shown in Figure 1A. Cells were also transfected with control stealth RNA (Stealth RNAi Negative Control Medium GC Duplex, Invitrogen).

Western blotting and immunofluorescence staining

Cells were trypsinized and extracted in 3% SDS solution containing 100 mM Tris, pH 6.8, 0.1 M DTT, and 20% glycerol. Cell extracts were separated on SDS–PAGE and transferred to a microporous polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After treatment with antibodies, bands were detected using an enhanced chemiluminescence technique (Amersham Biosciences, Piscataway, NJ, USA).

For indirect immunofluorescence staining, cells grown on glass coverslips were fixed in methanol for 20 min at −20°C and incubated in 1% bovine serum albumin (BSA) in PBS. Rabbit antibody and/or mouse monoclonal antibody were added and incubated for 60 min at 37°C. After washing in 0.1% BSA in PBS three times, Alexa 488-conjugated anti-mouse IgG and Alexa 568-conjugated anti-rabbit IgG were added, incubated for 60 min at 37°C, and washed with 0.1% BSA three times. Finally, cells were embedded in Immunon (Thermo Shandon, Pittsburgh, PA, USA) and observed through confocal fluorescence microscopy.

RNA preparation and qRT–PCR

Total RNA was isolated from cells using RNeasy mini kit (Qiagen Inc.) according to the manufacturer's instructions. Synthesis of single-strand cDNA was performed on total RNA (1 μg) by a Superscript First-strand Synthesis system (Invitrogen) using random primers according to the manufacturer's instructions. In all, 1 μl (total 20 μl) of the resultant single-strand cDNA was used as the template for qRT–CR, using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) with Mx3000P (Stratagene, La Jolla, CA, USA) according to the manufacturers' instructions. The values were normalized using the amounts for a control mRNA, RNA polymerase II subunit a (Polr2a) mRNA (Dydensborg *et al*, 2006). The sets of PCR primers for amplification of the pre-rRNA (a sequence in the 5′ untranslated region 1–155) used were 5′-GCTGACACGCTGTCCTCTG-3′ and 5′-TCGGACGCGCGAGAGAAC-3′; for KAM2A, the primers used were 5′-TCCCCACACACATTTTGA CATC-3′ and 5′-GGGGTGGCTTGAGAGATCCT-3′; for Polr2a, the primers used were 5′-ATCTCTCCTGCCATGACACC-3′ and 5′-AGACC AGGCAGGGGAGTAAC-3′.

Statistics

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

Detection of ongoing rDNA transcription

Ongoing ribosomal RNA synthesis was measured by detecting incorporated fluorouridine (FUrd, Sigma-Aldrich) in *in situ* run-on assays (Kruhlak *et al*, 2007). Cells were incubated with 2 mM FUrd in medium for 15 min and fixed with methanol for 20 min at −20°C. Incorporated FUrd was detected by anti-BrdU antibody (Sigma-Aldrich) and Alexa 488-conjugated goat anti-mouse IgG (H + L) and observed through fluorescence microscopy. The number of cells with FUrd signals remaining in the nucleoli was counted. The results were expressed at % of FUrd-positive cells.

Chromatin immunoprecipitations

ChIP asssays were performed using a MAGnify ChIP system (Invitrogen), as described (Nelson *et al*, 2006), referring to the manufacturer's instructions. Collected DNA fragments were measured by real-time PCR. The positions of the sets of PCR primers for the amplification of rDNA are indicated in Figure 2C, The primers used for H0 were 5′-GCTGACACGCTGTCCTCTG-3′ and 5′-TCGGA CGCGCGAGAGAAC-3′; for H1, 5′-GGCGGTTTGAGTGAGACGAGA-3′ and 5′-ACGTGCGCTCACCGAGAGCAG-3′; for H4, 5′-CGACGACCCAT TCGAACGTCT-3′ and 5′-CTCTCCGGAATCGAACCCTGA-3′; for H13, 5′-ACCTGGCGCTAAACCATTCGT-3′ and 5′-GGACAAACCCTTGTGTC GAGG-3′; and for H27, 5′-CCTTCCACGAGAGTGAGAAGCG-3′ and 5′-CTCGACCTCCCGAAATCGTACA-3′.

To detect the specific binding, the values simultaneously obtained by using the control antibody (normal rabbit IgG) were subtracted from those using specific antibodies. The values for specific binding were divided by total input, and expressed at a % of specific binding/input. As shown in Supplementary Figure S3, histone H3 was almost evenly distributed over all regions of rDNA. Then the values for the specific binding were normalized by the values for H3, and expressed as a % of specific binding/input normalized by H3.

Measurement of the rate of protein synthesis

The rate of protein synthesis was assayed basically as described earlier (Tsuneoka *et al*, 1993). In brief, cells were cultured in Ham's F12 medium with 0.5 μCi/ml [^{3}H]leucine for 1 h, and the radioactivity incorporated into protein was measured. The rate of protein synthesis was expressed per cell.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: YT performed most of the experiments; KO performed the experiments shown in Figure 2C; TK produced antibodies against KDM2A; TU performed the experiments shown in Figure 7C; NY and YS provide technical advice; YT, KO, and MT analysed and discussed the data; YZ provided the expression vectors for KDM2A and discussed the manuscript; MT supervised the project and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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