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A potential common role of the Jumonji C domain-containing 1A histone demethylase and chromatin remodeler ATRX in promoting colon cancer

XIAOMENG LI[1,2], SANGPHIL OH[2,3], HOOGEUN SONG[2], SOOK SHIN[2,3], BIN ZHANG[1], WILLARD M. FREEMAN[3,4] and RALF JANKNECHT[2,3]

[1]Department of Endoscopy and Gastrointestinal Medicine, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, P.R. China; [2]Department of Cell Biology, University of Oklahoma Health Sciences Center; [3]Stephenson Cancer Center; [4]Department of Physiology, University of Oklahoma Health Sciences Center, Oklahoma, OK 73104, USA

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Abstract. Jumonji C domain-containing 1A (JMJD1A) is a histone demethylase and epigenetic regulator that has been implicated in cancer development. In the current study, its mRNA and protein expression was analyzed in human colorectal tumors. It was demonstrated that JMJD1A levels were increased and correlated with a more aggressive phenotype. Downregulation of JMJD1A in human HCT116 colorectal cancer cells caused negligible growth defects, but robustly decreased clonogenic activity. Transcriptome analysis revealed that JMJD1A downregulation led to multiple changes in HCT116 cells, including inhibition of MYC- and MYCN-regulated pathways and stimulation of the TP53 tumor suppressor response. One gene identified to be stimulated by JMJD1A was α-thalassemia/mental retardation syndrome X-linked (ATRX), which encodes for a chromatin remodeler. The JMJD1A protein, but not a catalytically inactive mutant, activated the ATRX gene promoter and JMJD1A also affected levels of dimethylation on lysine 9 of histone H3. Similar to JMJD1A, ATRX was significantly overexpressed in human colorectal tumors and correlated with increased disease recurrence and lethality. Furthermore, ATRX downregulation in HCT116 cells reduced their growth and clonogenic activity. Accordingly, upregulation of ATRX may represent one mechanism by which JMJD1A promotes colorectal cancer. In addition, the data presented in this study suggest that the current notion of ATRX as a tumor suppressor is incomplete and that ATRX might context dependently also function as a tumor promoter.

Correspondence to: Dr Ralf Janknecht, Department of Cell Biology, University of Oklahoma Health Sciences Center, 975 NE 10th Street, BRC-1464, Oklahoma, OK 73104, USA
E-mail: ralf-janknecht@ouhsc.edu

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Introduction

Jumonji C domain-containing 1A (JMJD1A), also called lysine demethylase 3A (KDM3A), is an enzyme that converts dimethylated lysine 9 on histone H3 progressively into its mono- and unmethylated form (1). Depending on the degree of this demethylation process and where throughout a gene body it occurs, this affects gene expression in different ways (2). Accordingly, in different contexts, JMJD1A was shown to activate or repress gene transcription (1,3-7). Further, JMJD1A may act independently of its enzymatic activity through binding to nucleosome remodeling complexes, thereby modulating long-range chromatin interactions (8).

Knockout of JMJD1A in mice has revealed multiple physiological functions of this histone demethylase. Complete JMJD1A knockout caused male-to-female sex reversal, most likely due to deficient transcription of the sex-determining region Y gene that is required to trigger the differentiation of the bipotential gonads into testes (9). Moreover, in a hypomorphic JMJD1A knockout mouse model, males were infertile and displayed defective spermatogenesis, indicating that JMJD1A plays an important role in adult testes, too (10). Unrelated to its reproductive role, JMJD1A is also critical for normal homeostasis because JMJD1A knockout mice became obese and developed metabolic syndrome (11,12). In addition, JMJD1A is required for the adaptation to cold stress by promoting thermogenesis (8,13).

Interestingly, the stem cell factor OCT4 appears to upregulate JMJD1A gene transcription and depletion of JMJD1A can lead to the differentiation of embryonic stem cells (14), suggesting that JMJD1A exerts more, yet-to-be-discovered tasks in development, wound repair or tissue regeneration that are all dependent on stem cell function. In addition, JMJD1A promoted stemness in breast and ovarian cancer cells that could enhance chemoresistance (15,16). In HCT116 colon cancer cells, JMJD1A was needed for efficient tumor growth in a xenograft model (17,18), possibly because of its ability to stimulate stem cells (19). Despite these findings, the role of JMJD1A in colorectal cancer that is the second leading cause of

cancer death in the Western hemisphere (20) has not been fully elucidated. In the present study, we set out to analyze JMJD1A expression in colorectal tumors, gain mechanistic insights by determining genes that are regulated by JMJD1A, and explore the role of one of these genes, α-thalassemia/mental retardation syndrome X-linked (ATRX), in colon cancer.

Materials and methods

Analysis of databases. Microarray experiments were analyzed with Oncomine (21) and respective data downloaded from www.oncomine.org. The Human Protein Atlas (22) served as a source for survival data (www.proteinatlas.org) with the corresponding RNA sequencing data originating from 'The Cancer Genome Atlas' (TCGA). To assess coexpression of ATRX and JMJD1A in colorectal adenocarcinomas, provisional TCGA RNA sequencing data were analyzed with cbioportal (www.bioportal.org).

Cloning of shRNA. The retroviral vector pSIREN-RetroQ (Clontech, Palo Alto, CA, USA) was linearized with the restriction enzymes *Bam*HI and *Eco*RI and ligated with double-stranded oligonucleotides encoding shRNAs (23). Correct cloning was verified by DNA sequencing. The control shRNA targets the sequence 5'-CAACAAGATGAAGAG CACCAA-3', which displays at least four mismatches to any known human gene. The human JMJD1A shRNAs target the sequences 5'-GCAGGTGTCAATAGTGATA-3' (#1 shRNA) and 5'-GTAGACCTAGTTAATTGTA-3' (#2 shRNA), while the human ATRX shRNAs target the sequences 5'-GGTGTT ATGATCATAGGCTAT-3' (#1 shRNA) and 5'-GGATTCAAC CTCTTGAGGATA-3' (#3 shRNA).

Cell culture and analyses. Human colorectal carcinoma cells HCT116 (CCL-247), SW480 (CCL-228), DLD-1 (CCL-221) and HT-29 (HTB-38) as well as human embryonic kidney 293T cells (CRL-3216; all American Type Culture Collection, Manassas, VA, USA) were grown in a humidified, 5% CO_2-containing atmosphere in Dulbecco's modified Eagle's medium (10-013-CV; Mediatech; Corning Inc., Corning, NY, USA) that was supplemented with 10% fetal bovine serum (S11150; Atlanta Biologicals, Flowery Branch, GA, USA) as previously described (24,25). Transfection of 293T cells was done by the calcium phosphate coprecipitation method (26,27), the precipitate washed off with phosphate-buffered saline (28), retrovirus collected from the supernatant over the next 48 h (29) and in some cases concentrated by precipitation with poly (ethylene glycol)-8000 (30). HCT116 cells were infected with retrovirus three times (31) and then selected with 1.5 μg/ml puromycin for 3-4 days (32). To measure growth, 2,000 or 2,500 cells were seeded in 96-wells and growth determined essentially as described (33,34). For clonogenic assays, 1,000 or 3,750 cells were seeded into 6-wells and colony formation assayed as described (30).

Analysis of protein expression. To generate whole cell protein extracts, cells were lysed in Laemmli sample buffer and boiled for ~10 min (35). For biochemical fractionation of cells, the NE-PER nuclear and cytoplasmic extraction kit (78833; Pierce Biotechnology, Rockford, IL, USA) was employed as described (36). Proteins were then electrophoretically separated on SDS polyacrylamide gels (37), transferred to polyvinylidene difluoride membrane (38) and incubated with primary antibodies as described (39,40). Signals on blots were revealed utilizing appropriate secondary antibodies (41) followed by detection with chemiluminescence (42). Staining of a human colon cancer tissue microarray (AccuMax A303 I, slide #40; Isu Abxis, Seongnam, South Korea) was performed employing 20 min of antigen retrieval with method 2 and a 1:100 dilution of anti-JMJD1A antibody as described (43). The stained slide was digitized and the digital image extracted with Aperio ImageScope software (Leica, Wetzlar, Germany). Diaminobenzidine staining was obtained by color deconvolution from this image, intensity of light transmission was measured with Fiji version of ImageJ software (http://fiji.sc) and the strength of JMJD1A staining was defined as 100x (log maximum intensity-log intensity). The following rabbit polyclonal antibodies were utilized: Anti-Actin (A2066; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); anti-ATRX (NBP1-83077); anti-JMJD1A (NB100-77282; both Novus Biologicals, Littleton, CO, USA); and anti-H3K27me_1 (07-448; Upstate Biotechnology, Lake Placid, NY, USA). Also used was a goat polyclonal anti-Lamin B antibody (sc-6216; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA sequencing. Total RNA from HCT116 cells was isolated employing TRIzol (44) following standard procedures (45). RNA was further purified with the RNeasy Mini kit (74104; Qiagen, Hilden, Germany) and sequenced in the Targeted DNA Methylation and Mitochondrial Heteroplasmy Core at the Oklahoma Nathan Shock Center of Excellence in the Biology of Aging (Oklahoma City, OK, USA). Reads were aligned in Strand NGS (Agilent Technologies, Inc., Santa Clara, CA, USA) against the hg38 human genome build (December 2013) with Ensemble gene annotations (v87, January 2017). Three bases were trimmed from 3' and 5' ends of reads and read quality <Q20 was discarded prior to alignment. Alignment was to the full genome build to detect novel genes and splice variants and required 90 percent identity, minimum read length of 25, and reads with multiple matches were eliminated. A screening database for Illumina adapter sequences was used to remove adapter sequences. Reads counts were normalized by DESeq and a z-test was used to determine differential expression between samples. Ingenuity Pathway Analysis (Qiagen) was performed on genes whose mRNA levels were at least 1.5-fold different upon JMJD1A downregulation compared to the control shRNA treated cells.

Luciferase assays. The human ATRX promoter spanning from -600 to +100 (the ATRX transcription start site was based on the human transcript variant 1: NCBI reference sequence NM_000489.4) was cloned into pGL2-Basic (Promega Corp., Madison, WI, USA). Human 293T and HCT116 cells, which were grown in 12-wells, were transiently transfected with 100 ng of this luciferase reporter construct, 900 ng pBluescript KS^+, and 60 ng of Flag-JMJD1A expression plasmid or empty vector pEV3S utilizing 2 μg polyethylenimine (43). Approximately 42 h after transfection, cells were lysed as described (46) and luciferase activities determined in a luminometer (47).

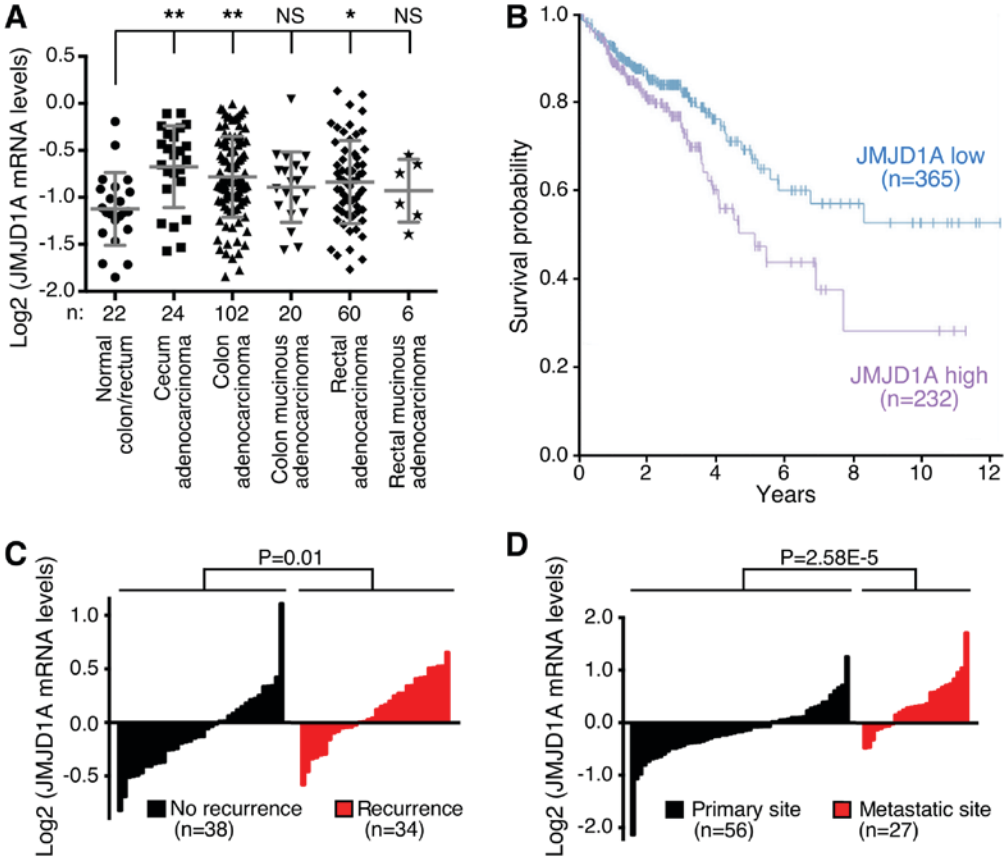

Figure 1. Overexpression of JMJD1A in colorectal cancer. (A) Levels of JMJD1A mRNA in normal and cancerous colorectal tissues in a TCGA microarray data set (reporter A_23_P258033). Means with standard deviations are indicated. One-way ANOVA with post hoc Dunnett's multiple comparisons test; *P<0.05; **P<0.01; NS, not significant compared with healthy colon/rectum tissue. (B) Kaplan-Meier survival plot based on TCGA RNA sequencing data. P=0.0132 (log-rank test). (C) Waterfall plot showing higher expression of JMJD1A mRNA (reporter 212689_s_at) in patients with recurrent disease five years after treatment compared to patients without recurrence; Student's t-test. (D) Similar, higher expression of JMJD1A at metastatic sites compared to the primary colorectal tumors. JMJD1A, Jumonji C domain-containing 1A; TCGA, The Cancer Genome Atlas; ANOVA, analysis of variance.

Chromatin immunoprecipitation. Human embryonic kidney 293T cells were grown in 10 cm dishes and transfected by the calcium phosphate coprecipitation method with 3 μg ATRX luciferase reporter plasmid, 21 μg pBluescript KS$^+$, and 6 μg Flag-JMJD1A expression plasmid or empty vector pEV3S. Cells were treated with formaldehyde and processed for chromatin preparation and immunoprecipitation as described (48,49). The following antibodies were used: Normal mouse IgG (sc-2025; Santa Cruz Biotechnology); anti-H3K9me$_2$ mouse monoclonal antibody (ab1220); and anti- H3K36me$_2$ rabbit polyclonal antibody (ab9049; both Abcam, Cambridge, MA, USA). Resultant DNA was then amplified by PCR, which was performed with the GoTaq DNA polymerase kit (M3008; Promega Corp.) and the following temperature program: 97˚C for 2 min; 8 cycles of 97˚C for 25 sec, 65˚C (-1˚C per cycle) for 25 sec, 72˚C for 35 sec; 26 cycles of 97˚C for 25 sec, 57˚C for 25 sec, 72˚C for 35 sec (+1 sec per cycle); 72˚C for 4 min followed by cooling down to 4˚C. Primers used were ATRX-for2 (5'-GTAGGT TTGTCTACCTCAGAGAGTG-3'; spanning the ATRX promoter from -332 to -308) and ATRX-rev2 (5'-ACAGCT CAAAGGCCGCTACCACTGC-3'; spanning the ATRX promoter from +117 to +141). The PCR products were electrophoresed in agarose gels and revealed by ethidium bromide staining (50).

Statistics. Statistical significance was assessed with one- or two-way analysis of variance (ANOVA) with post hoc Dunnett's or Tukey's multiple comparisons test, an unpaired Student's t-test, or a log-rank test. P<0.05 was deemed to show a statistically significant difference.

Results

JMJD1A expression in colorectal cancer. To examine the expression of JMJD1A mRNA in colorectal tumors, we first interrogated publicly available databases. In a study from TCGA (51), we found significant overexpression of JMJD1A in cecum, colon and rectal adenocarcinomas and a trend towards overexpression in mucinous tumors of the colon and rectum (Fig. 1A). Importantly, high JMJD1A mRNA levels were significantly correlated with reduced survival (Fig. 1B). Further, we discovered that JMJD1A was more highly expressed in patients with recurrent disease [Fig. 1C; microarray data retrieved from reference (52)] and at metastatic sites compared to the primary colorectal tumors [Fig. 1D; microarray data retrieved from reference (53)]. Altogether, these data indicate that JMJD1A mRNA is overexpressed in many colorectal tumors and associated with a more aggressive phenotype.

We then wanted to assess JMJD1A protein expression in colorectal cancer. Expression of JMJD1A protein was

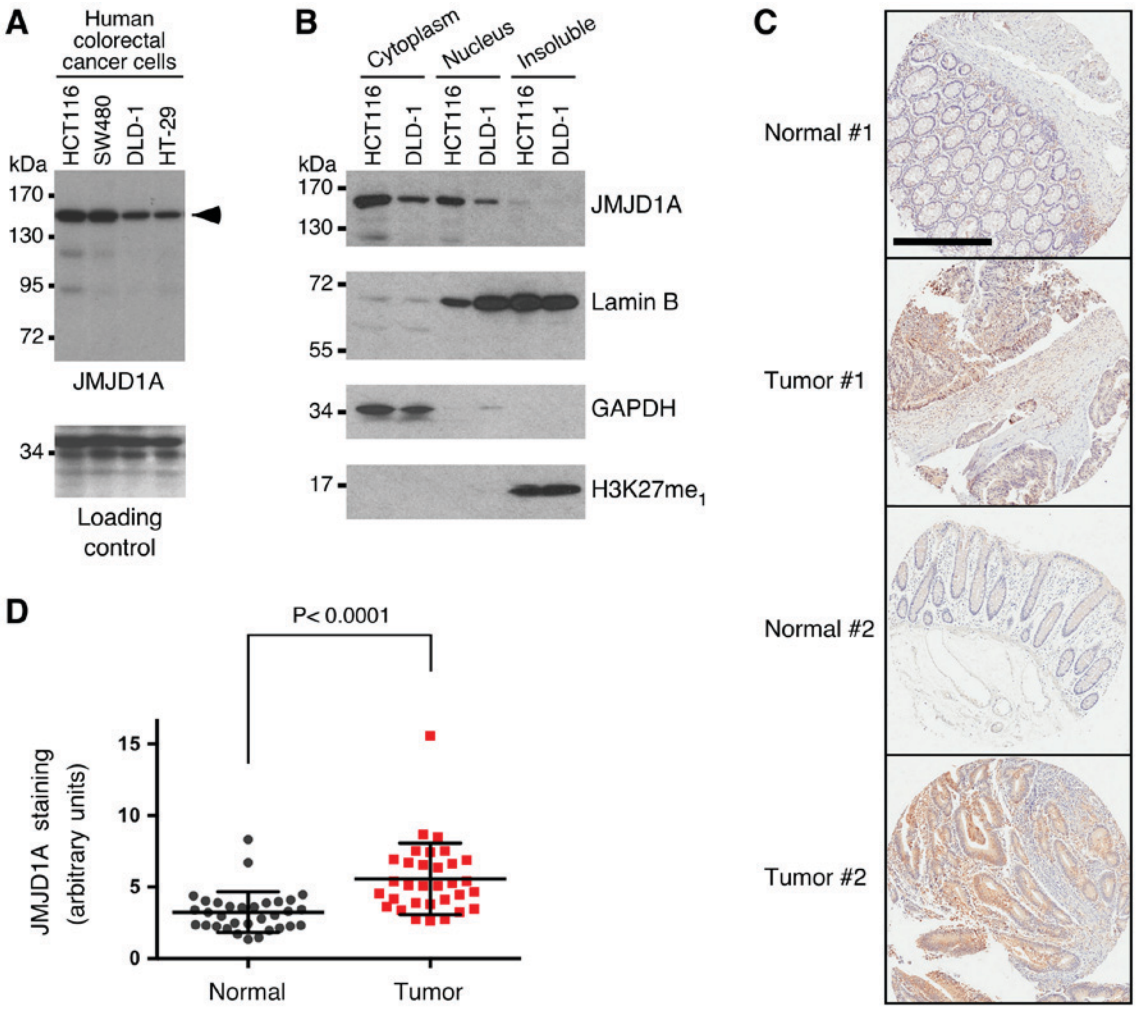

Figure 2. JMJD1A protein expression. (A) Western blot showing the expression of JMJD1A in HCT116, SW480, DLD-1 and HT-29 human colorectal cancer cells. (B) Biochemical fractionation of HCT116 and DLD-1 colorectal cancer cells. GAPDH, Lamin B and histone H3 monomethylated on lysine 27 ($H3K27me_1$) served as markers for cellular compartments. (C) JMJD1A immunostaining with hematoxylin/eosin counterstaining. Examples of two matching normal and tumor tissues of the colon. Magnification, x5; scale bar, 0.5 mm. (D) Quantitative analysis of JMJD1A immunostaining across 32 matching normal and cancerous colon tissues. Statistical significance was assessed with Student's t-test. JMJD1A, Jumonji C domain-containing 1A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

confirmed in all of the four tested human colorectal cancer cell lines by western blotting (Fig. 2A). Further, we biochemically fractionated HCT116 and DLD-1 colorectal cancer cells and found that JMJD1A was present in both the cytoplasm and nucleus, while very little JMJD1A was detectable in the insoluble fraction that primarily consists of the nuclear matrix and heterochromatin (Fig. 2B). Then, we stained a human tissue microarray consisting of 32 matching normal and cancerous colorectal tissues. Consistent with our biochemical fractionation experiments, JMJD1A staining was present in both the cytoplasm and nucleus. Importantly, a significant overexpression of JMJD1A was observed in the tumors (Fig. 2C and D), further implicating that JMJD1A overexpression may contribute to the development of colorectal cancer.

Impact of JMJD1A on HCT116 cells. To assess a potential physiological role of JMJD1A, we downregulated JMJD1A with two different shRNAs in HCT116 colorectal cancer cells. Both shRNAs induced a large reduction of JMJD1A protein levels (Fig. 3A). While JMJD1A shRNA#1 did not cause any change in HCT116 cell growth, shRNA#2 displayed a significant, yet very small reduction in cell growth (Fig. 3B). This indicates that JMJD1A downregulation has only negligible effects on HCT116 cell growth. In contrast, clonogenic activity of HCT116 colorectal cancer cells was robustly reduced upon JMJD1A downregulation with either of the two shRNAs (Fig. 3C). The latter data indicate that JMJD1A can influence the physiology of HCT116 cells in a manner that is predicted to be tumor promoting.

Transcriptome analysis. Next, we assessed how JMJD1A affects the transcriptome of HCT116 cells. To this end, we again downregulated JMJD1A with two different shRNAs (Fig. 4A) and performed RNA sequencing. Compared to control shRNA, we found that 281 genes were >1.5-fold upregulated with both JMJD1A shRNAs and 192 genes were >1.5-fold downregulated (Fig. 4B). Ingenuity Pathway Analysis revealed that multiple metabolic (Fig. 4C) and upstream regulatory pathways (Fig. 4D) were affected by JMJD1A downregulation. This indicates that JMJD1A pleiotropically affects the gene expression program of HCT116 colorectal cancer cells and may thereby be a determinant of their oncogenic potential.

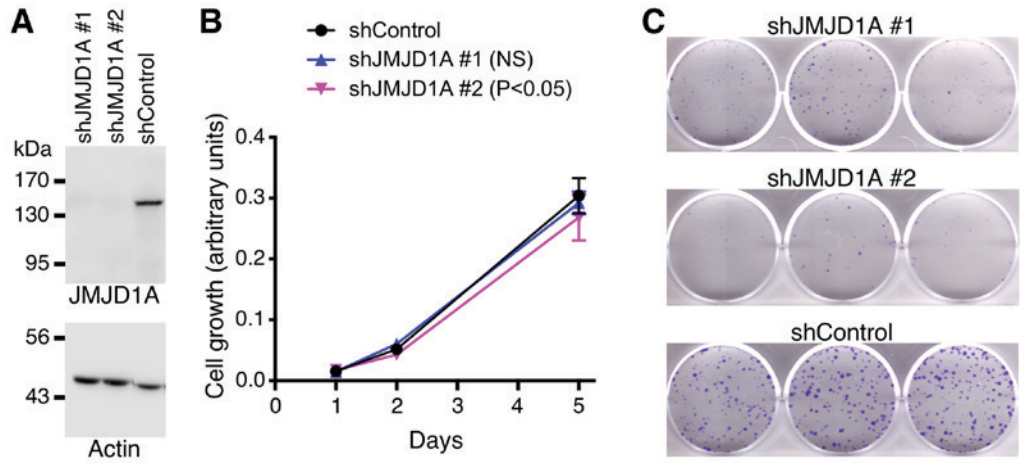

Figure 3. Role of JMJD1A in HCT116 cells. (A) Downregulation of JMJD1A with two different shRNAs. Shown are indicated western blots. (B) Measurement of cell growth. Means with standard deviations are shown (n=3). Statistical significance at day five from two-way ANOVA with post hoc Dunnett's multiple comparisons test. (C) Clonogenic assays. Representative pictures from at least three independent experiments. NS, not significant; JMJD1A, Jumonji C domain-containing 1A; ANOVA, analysis of variance.

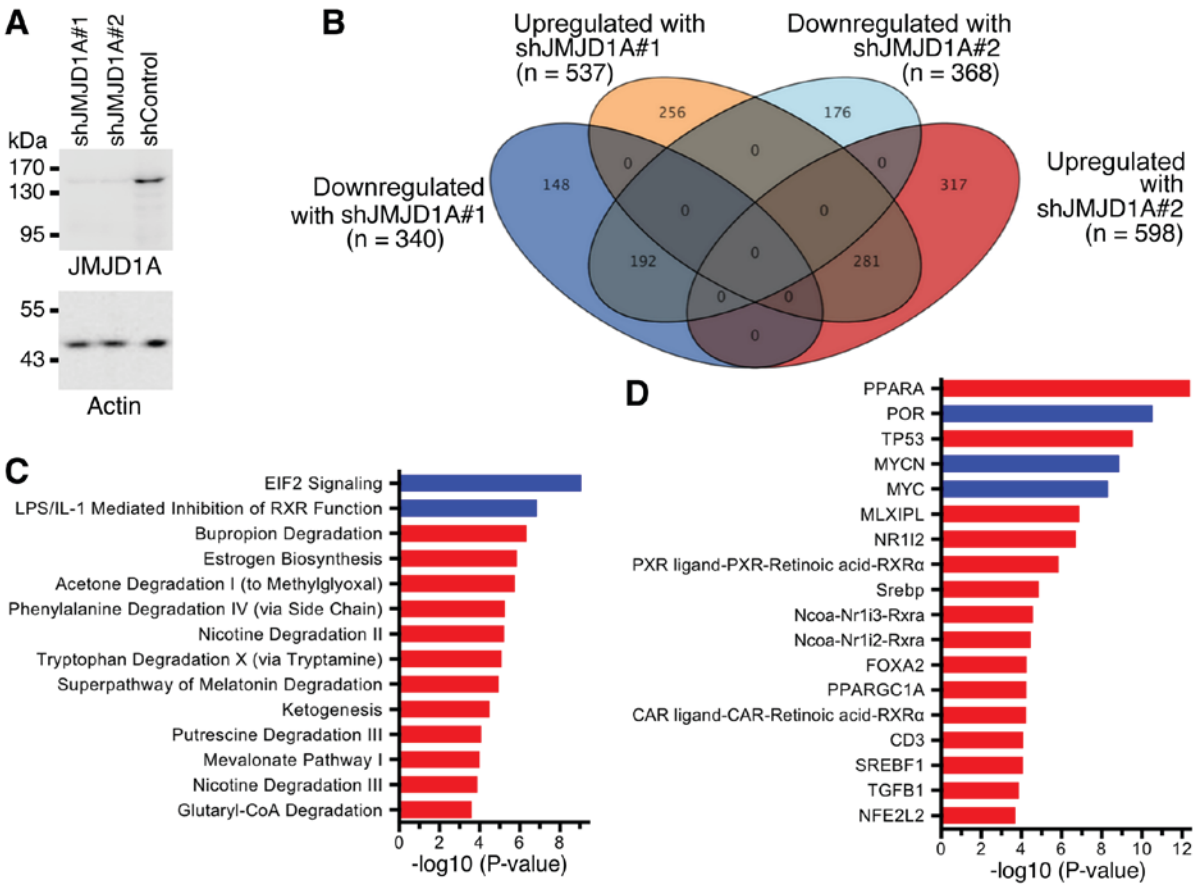

Figure 4. Transcriptome analysis. (A) Downregulation of JMJD1A in HCT116 cells upon expression of two different JMJD1A shRNAs. Western blots for JMJD1A and actin are shown. (B) Venn diagram showing the number of genes >1.5-fold up- or downregulated upon expression of two different JMJD1A shRNAs compared to control shRNA. (C) Ingenuity Pathway Analysis for canonical pathways. Red color indicates activation and blue color inhibition in the presence of JMJD1A shRNA. Results shown are limited to an absolute z-score >1.5 and -logP >3.5. (D) Analogous for upstream regulator pathways. JMJD1A, Jumonji C domain-containing 1A.

Identification of ATRX as a potential JMJD1A target gene. Since JMJD1A as a histone demethylase modulates chromatin structure, we were especially interested in other proteins involved in chromatin regulation whose genes were found to be affected by JMJD1A in our transcriptome analysis. One such gene was ATRX, which encodes for a chromatin remodeler (54). Our RNA sequencing data showed that JMJD1A downregulation by shRNA#1 or shRNA#2 led to a 2.6-fold or 3.5-fold decrease in ATRX mRNA levels, respectively (Fig. 5A). Accordingly, ATRX protein levels were also reduced in the presence of JMJD1A shRNAs (Fig. 5B). Please note that ATRX has a calculated molecular weight of

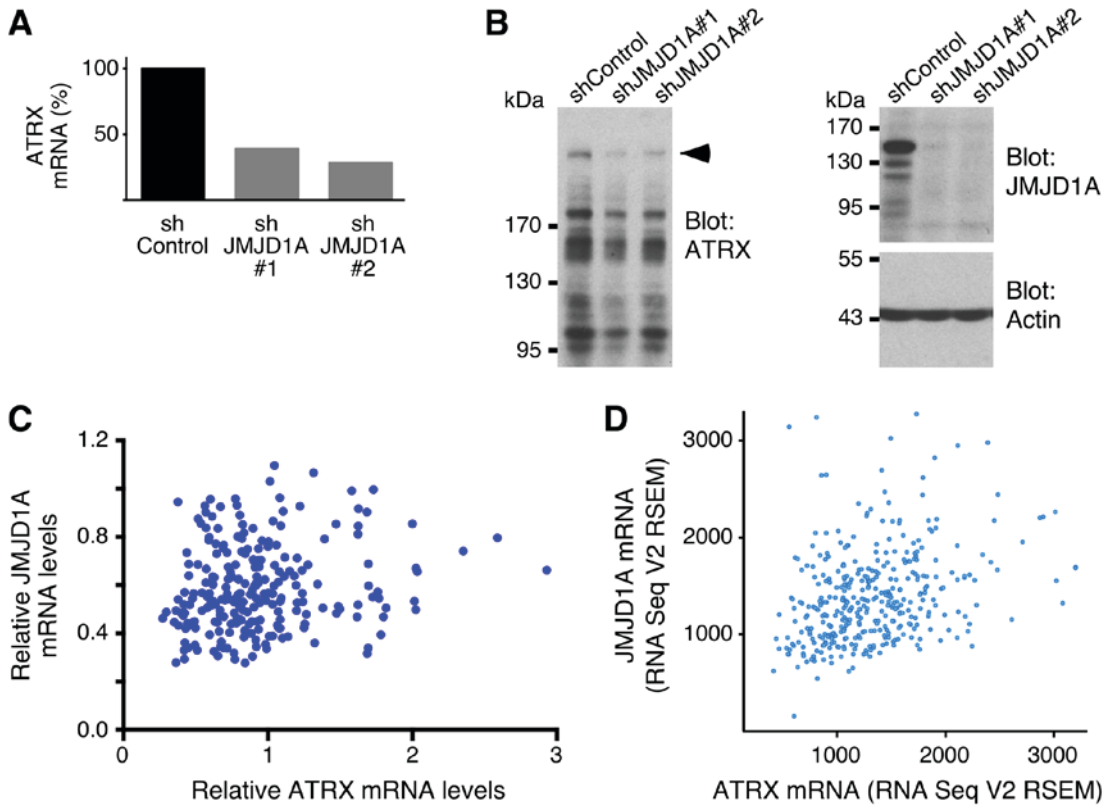

Figure 5. Regulation of ATRX by JMJD1A. (A) Relative ATRX mRNA levels in our RNA sequencing analysis. (B) Downregulation of JMJD1A in HCT116 cells leads to reduced ATRX protein levels as determined by western blot analysis. Arrowhead marks full-length ATRX. (C) Correlation of JMJD1A and ATRX mRNA levels across normal and cancerous colorectal tissue (n=237). Data from TCGA (microarray reporters A_23_P258033 and A_24_P128044). Pearson correlation coefficient=0.17; P=0.0087. (D) Likewise, provisional TCGA RNA sequencing data from colorectal adenocarcinomas. Pearson correlation coefficient=0.33; P<0.0001. JMJD1A, Jumonji C domain-containing 1A; ATRX, α-thalassemia/mental retardation syndrome X-linked; TCGA, The Cancer Genome Atlas.

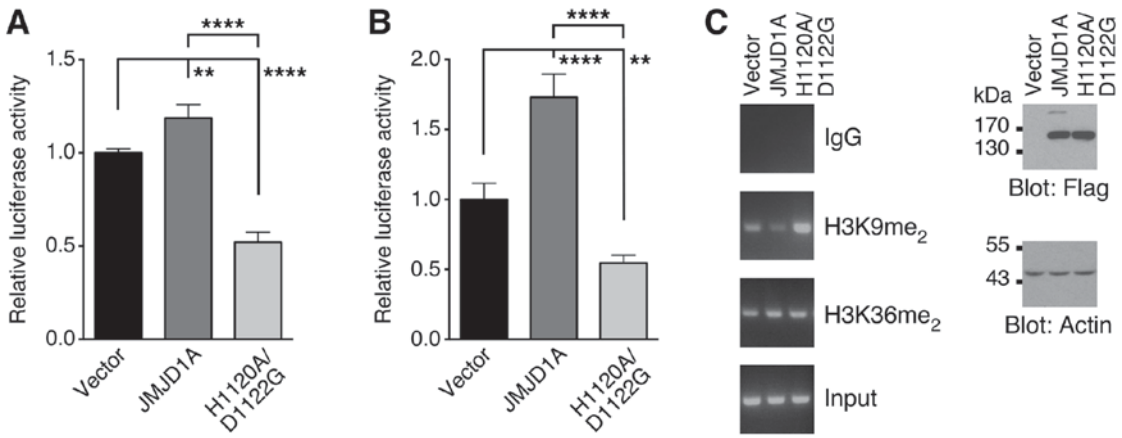

Figure 6. Role of JMJD1A at the ATRX gene promoter. (A) Human 293T or (B) HCT116 cells were transfected with an ATRX luciferase reporter construct and JMJD1A (wild-type or H1120A/D1122G catalytic mutant). Resultant relative luciferase activity is depicted. Means with standard deviations are shown (n=4). One-way ANOVA with post hoc Tukey's multiple comparisons test; **P<0.01; ****P<0.0001. (C) Chromatin immunoprecipitation assay with 293T cells transfected with indicated Flag-tagged JMJD1A expression constructs and the ATRX luciferase reporter gene. The left four panels show ethidium bromide-stained agarose gels of amplified DNA promoter fragments after immunoprecipitation with indicated antibodies or input levels of DNA. The right two panels show western blots demonstrating that comparable amounts of wild-type JMJD1A and its H1120A/D1122G mutant were expressed. JMJD1A, Jumonji C domain-containing 1A; ATRX, α-thalassemia/mental retardation syndrome X-linked; ANOVA, analysis of variance.

282.6 kDa and that the ATRX gene is composed of 35 exons, giving rise to multiple splice variants. This complex structure, and possibly protein degradation, accounted for the fact that multiple bands were detected with the anti-ATRX antibody in our western blot analyses. In total, these data suggest that JMJD1A can stimulate ATRX gene transcription. This notion is strongly supported by the fact that JMJD1A and ATRX mRNA levels were positively correlated across normal and malignant colorectal tissue specimens (Fig. 5C) or across adenocarcinomas in another data set (Fig. 5D).

To provide further evidence for a JMJD1A-ATRX axis, we cloned the human ATRX gene promoter in front of a luciferase

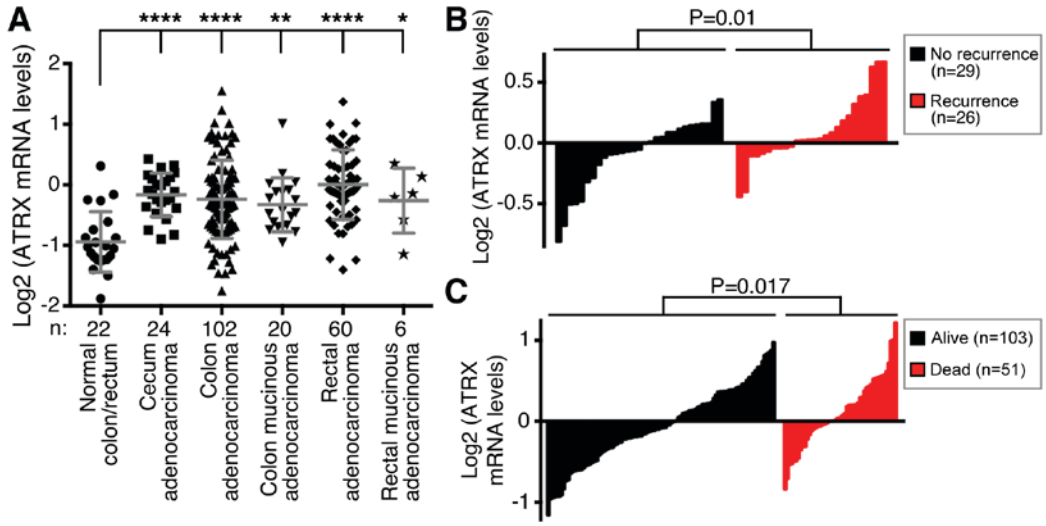

Figure 7. ATRX upregulation in colorectal cancer. (A) ATRX mRNA levels in normal and cancerous colorectal tissues in a TCGA microarray data set (reporter A_24_P128044). Means with standard deviations are indicated. One-way ANOVA with post hoc Dunnett's multiple comparisons test; *P<0.05; **P<0.01; ****P<0.0001 compared with healthy colon/rectum tissue. (B) ATRX mRNA (reporter 208861_s_at) waterfall plot showing higher ATRX expression in patients with recurrent disease five years after treatment compared to patients without recurrence; Student's t-test. (C) Similar, higher ATRX expression (reporter 208860_s_at) in patients succumbing to the disease compared to patients being alive three years after diagnosis; Student's t-test. ATRX, α-thalassemia/mental retardation syndrome X-linked; TCGA, The Cancer Genome Atlas; ANOVA, analysis of variance.

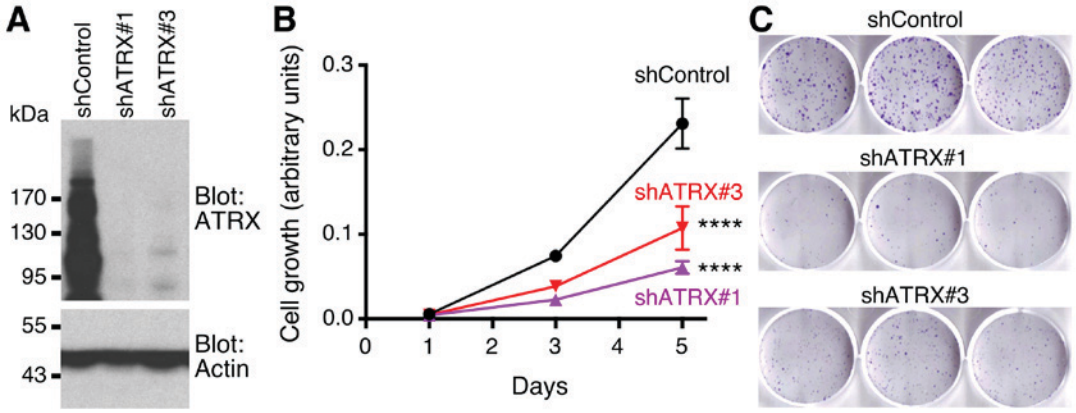

Figure 8. Impact of ATRX on HCT116 cells. (A) Western blots showing downregulation of ATRX in HCT116 cells with two different shRNAs. (B) Corresponding cell growth assays. Means with standard deviations are shown (n=3). Two-way ANOVA with post hoc Dunnett's multiple comparisons test; ****P<0.0001 compared with shControl. (C) Representative pictures of clonogenic assays independently performed in three different experiments. ATRX, α-thalassemia/mental retardation syndrome X-linked; ANOVA, analysis of variance.

reporter gene. As shown in Fig. 6A, overexpression of JMJD1A in 293T cells slightly stimulated the ATRX promoter. In addition, we also overexpressed JMJD1A-H1120A/D1122G, in which two amino acids within the JMJD1A catalytic center have been mutated rendering it inactive (1). This mutant repressed the ATRX luciferase reporter gene, presumably since it prevents endogenous JMJD1A from interacting with and thereby activating the ATRX promoter. Similar results were obtained with wild-type JMJD1A and its H1120A/D1122G mutant in HCT116 colorectal cancer cells (Fig. 6B). Moreover, we found that JMJD1A overexpression expectedly reduced dimethylation of histone H3 on lysine 9, but not dimethylation on lysine 36 (Fig. 6C). On the other hand, the H1120A/D1122G mutant predictably elevated levels of dimethylation on lysine 9 of histone H3. Collectively, these data suggest that JMJD1A interacts with the ATRX promoter and induces it by a mechanism that involves reduction of $H3K9me_2$ levels.

ATRX in colorectal cancer. The previous findings led to the question of whether ATRX is overexpressed in colorectal cancer. Indeed, similar to JMJD1A, ATRX mRNA was significantly upregulated in cecum, colon and rectal adenocarcinomas, and even mucinous adenocarcinomas of the colon and rectum displayed significant ATRX upregulation [Fig. 7A; microarray data from reference (51)]. Excitingly, high ATRX expression was positively correlated with increased disease recurrence [Fig. 7B; microarray data from reference (55)] and lethality [Fig. 7C; microarray data from reference (52)]. These data suggest that ATRX might promote colorectal cancer.

We then downregulated ATRX with two different shRNAs in HCT116 cells (Fig. 8A). This led to a significant reduction of HCT116 cell growth (Fig. 8B). In addition, both ATRX shRNAs caused a reduction in clonogenic activity of HCT116 cells (Fig. 8C). These data are further supporting the notion that ATRX is a promoter of colorectal cancer.

Discussion

In the current study, we provided evidence that JMJD1A is overexpressed at the mRNA and protein level in colorectal tumors and is associated with worse clinical outcomes, the latter being consistent with a previous report (18). Further, we demonstrated that JMJD1A has no or a minimal, effect on the *in vitro* growth of HCT116 cells. This result is consistent with data published by Krieg *et al* (17), but in contrast to Uemura *et al* (18) who reported that JMJD1A downregulation led to basically complete loss of *in vitro* HCT116 proliferation. However, it has to be noted that only one JMJD1A siRNA was utilized in the latter study, whose potential off-target effects might have caused the dramatic phenotype observed. Yet, both of our JMJD1A shRNAs caused a robust reduction in HCT116 clonogenic activity, which is the ability of single cells to form colonies that can be associated with the seeding of tumors and which is reliant on cancer stem cell properties. Hence, JMJD1A may be more important for promoting cancer stem cells than for stimulating the growth rate of tumors. Consistent with this concept, JMJD1A has been reported to foster cancer stemness in a variety of different tumors (15,16,19,56).

JMJD1A gene transcription is upregulated upon oxygen depletion by the transcription factor HIF-1, the master regulator of hypoxia (57-59). Given that most tumors are in a hypoxic environment, JMJD1A is therefore destined to become overexpressed in cancer cells. Further, JMJD1A can form complexes with the HIF-1 protein, which may particularly be important for the regulation of glycolytic enzymes and adaptation of cancer cells to a hypoxic environment (60,61). In how far JMJD1A's role in colorectal cancer is related to hypoxia and whether hypoxia is the driving force behind its overexpression remains to be studied.

Previous studies have found a predominantly nuclear localization of JMJD1A (1,3,62). In contrast, our cell fractionation experiments with two different colorectal cancer cell lines indicated that JMJD1A protein levels are comparable in the cytoplasm and cell nucleus. This result suggests that JMJD1A may perform non-nuclear functions that are independent of its histone demethylase activity. Interestingly, hypoxia was reported to reduce cytoplasmic residence of JMJD1A (62), implicating that the intracellular localization of JMJD1A and hence its epigenetic nuclear function are likely regulated through environmental cues.

Bioinformatic analyses revealed that JMJD1A downregulation affects multiple pathways in HCT116 colorectal cancer cells, suggesting that JMJD1A may perform pleiotropic functions. Notably, JMJD1A downregulation led to stimulation of TP53- and TGF-β1-regulated and inhibition of MYC- and MYCN-driven pathways (Fig. 4D). Both TP53 and TGF-β1 are tumor suppressors and mutations in respective pathways are commonly observed upon the progression of colorectal adenomas to early carcinomas (63). On the other hand, MYC and MYCN are prominent oncoproteins and transcription factors, whose overexpression is an underlying cause of cancer development in many different tissues (64,65). Further, the peroxisome proliferator-activated receptor α (PPARA) upstream regulator pathway was the most significantly stimulated pathway upon JMJD1A shRNA expression (Fig. 4D). While PPARA is known to tissue-specifically act as a tumor promoter or suppressor, current evidence points to PPARA as an inhibitor of colon cancer development, possibly by curtailing inflammation (66). Lastly, the estrogen biosynthesis pathway was enhanced upon JMJD1A downregulation (Fig. 4C). Estrogen in colonic tissue is thought to activate estrogen receptor-β, which seems to prevent colorectal cancer formation and may account for the fact that women have a lower risk for colon cancer than men (67). All of the above described transcriptional changes upon JMJD1A downregulation likely reduce the oncogenic potential of HCT116 cells. This conversely outlines potential mechanisms by which JMJD1A overexpression promotes tumorigenesis.

In addition, we discovered that ATRX levels were positively regulated by JMJD1A, which likely entailed activation of the ATRX gene promoter with concurrent removal of dimethylation on histone H3 lysine 9. ATRX has been shown to be a chromatin remodeler, which includes its role in the deposition of histone variant H3.3 at repetitive regions such as telomeres and pericentric heterochromatin, binding to and likely resolving G-quadruplexes, potentially evicting histone variant macroH2A1 and also promoting homologous recombination after DNA double-strand breaks (54,68). Interestingly, while ATRX was mostly localized to intergenic regions in mouse embryonic stem cells, the majority of ATRX was bound to promoters and gene bodies in neuroepithelial progenitors. Accordingly, knockout of ATRX could result in pleiotropic changes of the gene expression program through altering chromatin accessibility, implying that ATRX can epigenetically impact on many genes (69). *In vivo*, ATRX is essential for development, as respective knockout mice died midway through embryogenesis (70). Also, mutations in ATRX can cause α-thalassemia and mental retardation, a syndrome that manifests predominantly in males due to the fact that the ATRX gene is encoded on the X chromosome (71). Mutations in the ATRX gene thought to inactivate its function have also been found in various cancers, especially in pancreatic neuroendocrine tumors, pediatric glioblastoma multiforme and adult low-grade gliomas (72). This implicated that ATRX is a tumor suppressor. Consistently, ablation of ATRX accelerated tumor growth in a glioblastoma model (73).

In contrast, our data are indicative of a tumor-promoting role of ATRX. We demonstrate that ATRX is overexpressed in colorectal cancer, and high ATRX mRNA levels are positively correlated with a worse outcome of this disease. In line with the idea of ATRX stimulating tumorigenesis, ATRX downregulation led to significantly reduced HCT116 cell growth and clonogenic activity. Similar to our results with HCT116 cancer cells, knockout of ATRX in mouse embryonic stem cells disadvantaged their growth (70). Please note that JMJD1A knockdown had only a negligible effect on HCT116 cell growth (Fig. 3B) despite the fact that this concurrently led to a reduction in ATRX expression (Fig. 5A and B). However, the degree of ATRX downregulation was much lower upon JMJD1A knockdown compared to when we utilized ATRX shRNA (compare Figs. 5B to 8A), and this relatively weaker degree of ATRX downregulation may have been insufficient to robustly impair cell growth. Another possibility is that JMJD1A knockdown compensated by an unknown mechanism for the reduction of ATRX levels. Finally, it remains to

be studied if ATRX has the capability to stimulate cancer cells also in tissues other than the colon and rectum.

Altogether, our data suggest that JMJD1A and ATRX may act in common to promote colon cancer. In addition, to our knowledge, our data for the first time provide considerable evidence that ATRX is a tumor promoter, which challenges the dogma of ATRX solely being a tumor suppressor.

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Availability of data and materials

RNA sequencing data have been deposited in the NCBI BioProject database under accession no. PRJNA453378 and can be freely downloaded from the NCBI Sequence Read Archive (accession nos. SRX3992514, SRX3992513 and SRX3992472).

Authors' contributions

XL, SO, HS and RJ designed and performed experiments. XL, SO, HS, SS, BZ, WMF and RJ analyzed and interpreted data. RJ supervised the whole study and wrote the manuscript with input from all other authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Yamane K, Toumazou C, Tsukada Y, Erdjument-Bromage H, Tempst P, Wong J and Zhang Y: JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell 125: 483-495, 2006.
2. Kooistra SM and Helin K: Molecular mechanisms and potential functions of histone demethylases. Nat Rev Mol Cell Biol 13: 297-311, 2012.
3. Knebel J, De Haro L and Janknecht R: Repression of transcription by TSGA/Jmjd1a, a novel interaction partner of the ETS protein ER71. J Cell Biochem 99: 319-329, 2006.
4. Lockman K, Taylor JM and Mack CP: The histone demethylase, Jmjd1a, interacts with the myocardin factors to regulate SMC differentiation marker gene expression. Circ Res 101: e115-e123, 2007.
5. Fan L, Peng G, Sahgal N, Fazli L, Gleave M, Zhang Y, Hussain A and Qi J: Regulation of c-Myc expression by the histone demethylase JMJD1A is essential for prostate cancer cell growth and survival. Oncogene 35: 2441-2452, 2016.
6. Tee AE, Ling D, Nelson C, Atmadibrata B, Dinger ME, Xu N, Mizukami T, Liu PY, Liu B, Cheung B, *et al*: The histone demethylase JMJD1A induces cell migration and invasion by up-regulating the expression of the long noncoding RNA MALAT1. Oncotarget 5: 1793-1804, 2014.
7. Sechler M, Parrish JK, Birks DK and Jedlicka P: The histone demethylase KDM3A, and its downstream target MCAM, promote Ewing sarcoma cell migration and metastasis. Oncogene 36: 4150-4160, 2017.
8. Abe Y, Rozqie R, Matsumura Y, Kawamura T, Nakaki R, Tsurutani Y, Tanimura-Inagaki K, Shiono A, Magoori K, Nakamura K, *et al*: JMJD1A is a signal-sensing scaffold that regulates acute chromatin dynamics via SWI/SNF association for thermogenesis. Nat Commun 6: 7052, 2015.
9. Kuroki S, Matoba S, Akiyoshi M, Matsumura Y, Miyachi H, Mise N, Abe K, Ogura A, Wilhelm D, Koopman P, *et al*: Epigenetic regulation of mouse sex determination by the histone demethylase Jmjd1a. Science 341: 1106-1109, 2013.
10. Okada Y, Scott G, Ray MK, Mishina Y and Zhang Y: Histone demethylase JHDM2A is critical for Tnp1 and Prm1 transcription and spermatogenesis. Nature 450: 119-123, 2007.
11. Tateishi K, Okada Y, Kallin EM and Zhang Y: Role of Jhdm2a in regulating metabolic gene expression and obesity resistance. Nature 458: 757-761, 2009.
12. Inagaki T, Tachibana M, Magoori K, Kudo H, Tanaka T, Okamura M, Naito M, Kodama T, Shinkai Y and Sakai J: Obesity and metabolic syndrome in histone demethylase JHDM2a-deficient mice. Genes Cells 14: 991-1001, 2009.
13. Abe Y, Fujiwara Y, Takahashi H, Matsumura Y, Sawada T, Jiang S, Nakaki R, Uchida A, Nagao N, Naito M, *et al*: Histone demethylase JMJD1A coordinates acute and chronic adaptation to cold stress via thermogenic phospho-switch. Nat Commun 9: 1566, 2018.
14. Loh YH, Zhang W, Chen X, George J and Ng HH: Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev 21: 2545-2557, 2007.
15. Ramadoss S, Guo G and Wang CY: Lysine demethylase KDM3A regulates breast cancer cell invasion and apoptosis by targeting histone and the non-histone protein p53. Oncogene 36: 47-59, 2017.
16. Ramadoss S, Sen S, Ramachandran I, Roy S, Chaudhuri G and Farias-Eisner R: Lysine-specific demethylase KDM3A regulates ovarian cancer stemness and chemoresistance. Oncogene 36: 1537-1545, 2017.
17. Krieg AJ, Rankin EB, Chan D, Razorenova O, Fernandez S and Giaccia AJ: Regulation of the histone demethylase JMJD1A by hypoxia-inducible factor 1 alpha enhances hypoxic gene expression and tumor growth. Mol Cell Biol 30: 344-353, 2010.
18. Uemura M, Yamamoto H, Takemasa I, Mimori K, Hemmi H, Mizushima T, Ikeda M, Sekimoto M, Matsuura N, Doki Y and Mori M: Jumonji domain containing 1A is a novel prognostic marker for colorectal cancer: In vivo identification from hypoxic tumor cells. Clin Cancer Res 16: 4636-4646, 2010.
19. Li J, Yu B, Deng P, Cheng Y, Yu Y, Kevork K, Ramadoss S, Ding X, Li X and Wang CY: KDM3 epigenetically controls tumorigenic potentials of human colorectal cancer stem cells through Wnt/β-catenin signalling. Nat Commun 8: 15146, 2017.
20. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2017. CA Cancer J Clin 67: 7-30, 2017.
21. Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB, Barrette TR, Anstet MJ, Kincead-Beal C, Kulkarni P, *et al*: Oncomine 3.0: Genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. Neoplasia 9: 166-180, 2007.
22. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson Å, Kampf C, Sjöstedt E, Asplund A, *et al*: Proteomics. Tissue-based map of the human proteome. Science 347: 1260419, 2015.
23. Kim TD, Oh S, Lightfoot SA, Shin S, Wren JD and Janknecht R: Upregulation of PSMD10 caused by the JMJD2A histone demethylase. Int J Clin Exp Med 9: 10123-10134, 2016.

24. Kim TD, Oh S, Shin S and Janknecht R: Regulation of tumor suppressor p53 and HCT116 cell physiology by histone demethylase JMJD2D/KDM4D. PLoS One 7: e34618, 2012.
25. Mooney SM, Grande JP, Salisbury JL and Janknecht R: Sumoylation of p68 and p72 RNA helicases affects protein stability and transactivation potential. Biochemistry 49: 1-10, 2010.
26. Dowdy SC, Mariani A and Janknecht R: HER2/Neu- and TAK1-mediated up-regulation of the transforming growth factor beta inhibitor Smad7 via the ETS protein ER81. J Biol Chem 278: 44377-44384, 2003.
27. Bosc DG, Goueli BS and Janknecht R: HER2/Neu-mediated activation of the ETS transcription factor ER81 and its target gene MMP-1. Oncogene 20: 6215-6224, 2001.
28. Shin S, Kim TD, Jin F, van Deursen JM, Dehm SM, Tindall DJ, Grande JP, Munz JM, Vasmatzis G and Janknecht R: Induction of prostatic intraepithelial neoplasia and modulation of androgen receptor by ETS variant 1/ETS-related protein 81. Cancer Res 69: 8102-8110, 2009.
29. Berry WL, Shin S, Lightfoot SA and Janknecht R: Oncogenic features of the JMJD2A histone demethylase in breast cancer. Int J Oncol 41: 1701-1706, 2012.
30. Berry WL, Kim TD and Janknecht R: Stimulation of β-catenin and colon cancer cell growth by the KDM4B histone demethylase. Int J Oncol 44: 1341-1348, 2014.
31. Shin S, Oh S, An S and Janknecht R: ETS variant 1 regulates matrix metalloproteinase-7 transcription in LNCaP prostate cancer cells. Oncol Rep 29: 306-314, 2013.
32. Kim TD, Shin S and Janknecht R: ETS transcription factor ERG cooperates with histone demethylase KDM4A. Oncol Rep 35: 3679-3688, 2016.
33. Kim TD, Shin S, Berry WL, Oh S and Janknecht R: The JMJD2A demethylase regulates apoptosis and proliferation in colon cancer cells. J Cell Biochem 113: 1368-1376, 2012.
34. Oh S, Shin S, Lightfoot SA and Janknecht R: 14-3-3 proteins modulate the ETS transcription factor ETV1 in prostate cancer. Cancer Res 73: 5110-5119, 2013.
35. Shin S, Bosc DG, Ingle JN, Spelsberg TC and Janknecht R: Rcl is a novel ETV1/ER81 target gene upregulated in breast tumors. J Cell Biochem 105: 866-874, 2008.
36. Kim TD, Fuchs JR, Schwartz E, Abdelhamid D, Etter J, Berry WL, Li C, Ihnat MA, Li PK and Janknecht R: Pro-growth role of the JMJD2C histone demethylase in HCT-116 colon cancer cells and identification of curcuminoids as JMJD2 inhibitors. Am J Transl Res 6: 236-247, 2014.
37. Goel A and Janknecht R: Concerted activation of ETS protein ER81 by p160 coactivators, the acetyltransferase p300 and the receptor tyrosine kinase HER2/Neu. J Biol Chem 279: 14909-14916, 2004.
38. Papoutsopoulou S and Janknecht R: Phosphorylation of ETS transcription factor ER81 in a complex with its coactivators CREB-binding protein and p300. Mol Cell Biol 20: 7300-7310, 2000.
39. Mooney SM, Goel A, D'Assoro AB, Salisbury JL and Janknecht R: Pleiotropic effects of p300-mediated acetylation on p68 and p72 RNA helicase. J Biol Chem 285: 30443-30452, 2010.
40. Wu J and Janknecht R: Regulation of the ETS transcription factor ER81 by the 90-kDa ribosomal S6 kinase 1 and protein kinase A. J Biol Chem 277: 42669-42679, 2002.
41. Li X, Moon G, Shin S, Zhang B and Janknecht R: Cooperation between ETS variant 2 and Jumonji domain-containing 2 histone demethylases. Mol Med Rep 17: 5518-5527, 2018.
42. Janknecht R: Regulation of the ER81 transcription factor and its coactivators by mitogen- and stress-activated protein kinase 1 (MSK1). Oncogene 22: 746-755, 2003.
43. Kim TD, Jin F, Shin S, Oh S, Lightfoot SA, Grande JP, Johnson AJ, van Deursen JM, Wren JD and Janknecht R: Histone demethylase JMJD2A drives prostate tumorigenesis through transcription factor ETV1. J Clin Invest 126: 706-720, 2016.
44. Oh S and Janknecht R: Histone demethylase JMJD5 is essential for embryonic development. Biochem Biophys Res Commun 420: 61-65, 2012.
45. Goel A and Janknecht R: Acetylation-mediated transcriptional activation of the ETS protein ER81 by p300, P/CAF, and HER2/Neu. Mol Cell Biol 23: 6243-6254, 2003.
46. Kim TD, Shin S and Janknecht R: Repression of Smad3 activity by histone demethylase SMCX/JARID1C. Biochem Biophys Res Commun 366: 563-567, 2008.
47. Janknecht R and Hunter T: Activation of the Sap-1a transcription factor by the c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase. J Biol Chem 272: 4219-4224, 1997.
48. Goueli BS and Janknecht R: Regulation of telomerase reverse transcriptase gene activity by upstream stimulatory factor. Oncogene 22: 8042-8047, 2003.
49. Goueli BS and Janknecht R: Upregulation of the catalytic telomerase subunit by the transcription factor ER81 and oncogenic HER2/Neu, Ras, or Raf. Mol Cell Biol 24: 25-35, 2004.
50. Kim J, Shin S, Subramaniam M, Bruinsma E, Kim TD, Hawse JR, Spelsberg TC and Janknecht R: Histone demethylase JARID1B/KDM5B is a corepressor of TIEG1/KLF10. Biochem Biophys Res Commun 401: 412-416, 2010.
51. Cancer Genome Atlas Natwork: Comprehensive molecular characterization of human colon and rectal cancer. Nature 487: 330-337, 2012.
52. Smith JJ, Deane NG, Wu F, Merchant NB, Zhang B, Jiang A, Lu P, Johnson JC, Schmidt C, Bailey CE, *et al*: Experimentally derived metastasis gene expression profile predicts recurrence and death in patients with colon cancer. Gastroenterology 138: 958-968, 2010.
53. Tsuji S, Midorikawa Y, Takahashi T, Yagi K, Takayama T, Yoshida K, Sugiyama Y and Aburatani H: Potential responders to FOLFOX therapy for colorectal cancer by Random Forests analysis. Br J Cancer 106: 126-132, 2012.
54. Ratnakumar K and Bernstein E: ATRX: The case of a peculiar chromatin remodeler. Epigenetics 8: 3-9, 2013.
55. Lin YH, Friederichs J, Black MA, Mages J, Rosenberg R, Guilford PJ, Phillips V, Thompson-Fawcett M, Kasabov N, Toro T, *et al*: Multiple gene expression classifiers from different array platforms predict poor prognosis of colorectal cancer. Clin Cancer Res 13: 498-507, 2007.
56. Nakatsuka T, Tateishi K, Kudo Y, Yamamoto K, Nakagawa H, Fujiwara H, Takahashi R, Miyabayashi K, Asaoka Y, Tanaka Y, *et al*: Impact of histone demethylase KDM3A-dependent AP-1 transactivity on hepatotumorigenesis induced by PI3K activation. Oncogene 36: 6262-6271, 2017.
57. Wellmann S, Bettkober M, Zelmer A, Seeger K, Faigle M, Eltzschig HK and Bührer C: Hypoxia upregulates the histone demethylase JMJD1A via HIF-1. Biochem Biophys Res Commun 372: 892-897, 2008.
58. Pollard PJ, Loenarz C, Mole DR, McDonough MA, Gleadle JM, Schofield CJ and Ratcliffe PJ: Regulation of Jumonji-domain-containing histone demethylases by hypoxia-inducible factor (HIF)-1alpha. Biochem J 416: 387-394, 2008.
59. Beyer S, Kristensen MM, Jensen KS, Johansen JV and Staller P: The histone demethylases JMJD1A and JMJD2B are transcriptional targets of hypoxia-inducible factor HIF. J Biol Chem 283: 36542-36552, 2008.
60. Mimura I, Nangaku M, Kanki Y, Tsutsumi S, Inoue T, Kohro T, Yamamoto S, Fujita T, Shimamura T, Suehiro J, *et al*: Dynamic change of chromatin conformation in response to hypoxia enhances the expression of GLUT3 (SLC2A3) by cooperative interaction of hypoxia-inducible factor 1 and KDM3A. Mol Cell Biol 32: 3018-3032, 2012.
61. Wan W, Peng K, Li M, Qin L, Tong Z, Yan J, Shen B and Yu C: Histone demethylase JMJD1A promotes urinary bladder cancer progression by enhancing glycolysis through coactivation of hypoxia inducible factor 1α. Oncogene 36: 3868-3877, 2017.
62. Sar A, Ponjevic D, Nguyen M, Box AH and Demetrick DJ: Identification and characterization of demethylase JMJD1A as a gene upregulated in the human cellular response to hypoxia. Cell Tissue Res 337: 223-234, 2009.
63. Jones S, Chen WD, Parmigiani G, Diehl F, Beerenwinkel N, Antal T, Traulsen A, Nowak MA, Siegel C, Velculescu VE, *et al*: Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci USA 105: 4283-4288, 2008.
64. Dang CV: MYC on the path to cancer. Cell 149: 22-35, 2012.
65. Rickman DS, Schulte JH and Eilers M: The expanding world of N-MYC-driven tumors. Cancer Discov 8: 150-163, 2018.
66. Gao J, Yuan S, Jin J, Shi J and Hou Y: PPARα regulates tumor progression, foe or friend? Eur J Pharmacol 765: 560-564, 2015.
67. Williams C, DiLeo A, Niv Y and Gustafsson JÅ: Estrogen receptor beta as target for colorectal cancer prevention. Cancer Lett 372: 48-56, 2016.
68. Juhász S, Elbakry A, Mathes A and Löbrich M: ATRX promotes DNA repair synthesis and sister chromatid exchange during homologous recombination. Mol Cell 71: 11-24.e7, 2018.

69. Danussi C, Bose P, Parthasarathy PT, Silberman PC, Van Arnam JS, Vitucci M, Tang OY, Heguy A, Wang Y, Chan TA, *et al*: Atrx inactivation drives disease-defining phenotypes in glioma cells of origin through global epigenomic remodeling. Nat Commun 9: 1057, 2018.
70. Garrick D, Sharpe JA, Arkell R, Dobbie L, Smith AJ, Wood WG, Higgs DR and Gibbons RJ: Loss of Atrx affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues. PLoS Genet 2: e58, 2006.
71. Gibbons RJ, Picketts DJ, Villard L and Higgs DR: Mutations in a putative global transcriptional regulator cause X-linked mental retardation with alpha-thalassemia (ATR-X syndrome). Cell 80: 837-845, 1995.
72. Dyer MA, Qadeer ZA, Valle-Garcia D and Bernstein E: ATRX and DAXX: Mechanisms and mutations. Cold Spring Harb Perspect Med 7: pii: a026567, 2017.
73. Koschmann C, Calinescu AA, Nunez FJ, Mackay A, Fazal-Salom J, Thomas D, Mendez F, Kamran N, Dzaman M, Mulpuri L, *et al*: ATRX loss promotes tumor growth and impairs nonhomologous end joining DNA repair in glioma. Sci Transl Med 8: 328ra28, 2016.