Oncogenic features of the JMJD2A histone demethylase in breast cancer

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Abstract. Estrogen receptor α (ERα) plays a pivotal role in the genesis of the majority of breast tumors. Consequently, endocrine therapy is now routinely utilized in the clinic for the treatment of ERα-positive breast cancer patients. However, how ERα activity becomes dysregulated in breast cancer cells remains to be elucidated. The aim of this study was to show that the histone demethylase JMJD2A, also known as KDM4A, is capable of forming a complex with ERα *in vivo*. Moreover, wild-type JMJD2A, but not a catalytically impaired mutant, was able to strongly coactivate ERα-mediated transcription. Consistently, the downregulation of JMJD2A in human T47D breast cancer cells led to a decreased expression of cyclin D1, a prominent ERα target gene and cell cycle regulator. The downregulation of JMJD2A induced a reduction in the growth of T47D cells. In addition, we found that JMJD2A is overexpressed in human breast tumors both at the mRNA and protein level. Taken together, these data indicate that the overexpression of JMJD2A may contribute to breast tumor formation by stimulating ERα activity and that JMJD2A may be a breast-relevant oncoprotein. As such, small molecule drugs targeting the catalytic center of JMJD2A might be useful in breast cancer adjuvant therapy.

Introduction

Tumors are characterized by a change of the transcriptome, which occurs to a large extent due to alterations in the chromatin structure. A key element of chromatin regulation is the post-translational modification of histones, including their acetylation and methylation on lysine residues. While histone acetylation is normally correlated with increased gene transcription, histone methylation is able to induce silencing or activation of genes depending on which histone lysine residue is modified and whether this lysine becomes mono-, di- or trimethylated (1). A prominent family of histone lysine-modifying enzymes comprises the Jumonji C domain (JMJD) demethylases, whose dysregulation has been suggested in cancer development (2,3).

One subfamily of JMJD proteins consists of JMJD2A, JMJD2B, JMJD2C and JMJD2D (4). These four JMJD2 proteins share extensive homology at their N-termini harboring the catalytic domain, however, JMJD2D is different from the other three JMJD2 proteins in that it lacks their extensive C-terminus which contains PHD and TUDOR domains involved in binding to modified histone residues (5,6). In addition, whereas JMJD2A, B and C are capable of demethylating histone 1.4 on lysine 26 as well as histone 3 on lysines 9 and 36, JMJD2D is unable to demethylate the latter lysine residue (7-13). Moreover, compared to JMJD2A and JMJD2C, JMJD2B seems to be much less catalytically active (7,10). Thus, the four JMJD2 family members exhibit different biochemical properties and are thought to perform distinct physiological functions.

The mRNA expression of JMJD2B, but not of JMJD2A or JMJD2C, is induced by estrogen and JMJD2B is therefore potentially important in estrogen receptor α (ERα)-positive tumors. Consistently, JMJD2B is more highly expressed in ERα-positive compared to ERα-negative human breast tumors and is required for the maximal proliferation of estrogen-dependent MCF7 breast cancer cells (14-16). Furthermore, only JMJD2B and JMJD2C appear to be overexpressed in medulloblastomas and their enzymatic activities may contribute to the neoplastic transformation of brain cells (17). In the present study, we focused on JMJD2A, also known as KDM4A (lysine demethylase 4A), and assessed its potential role in breast cancer.

Materials and methods

Immunohistochemistry. Tissue microarrays consisting of 12 (AccuMax A712[14]) or 20 (AccuMax A312) matching samples of human breast tumors and normal tissues were purchased from ISU Abxis. Staining with JMJD2A antibodies (Bethyl A300-861A or Abcam ab70786) was performed as described in a previous study (18) and graded by a board-certified pathologist.

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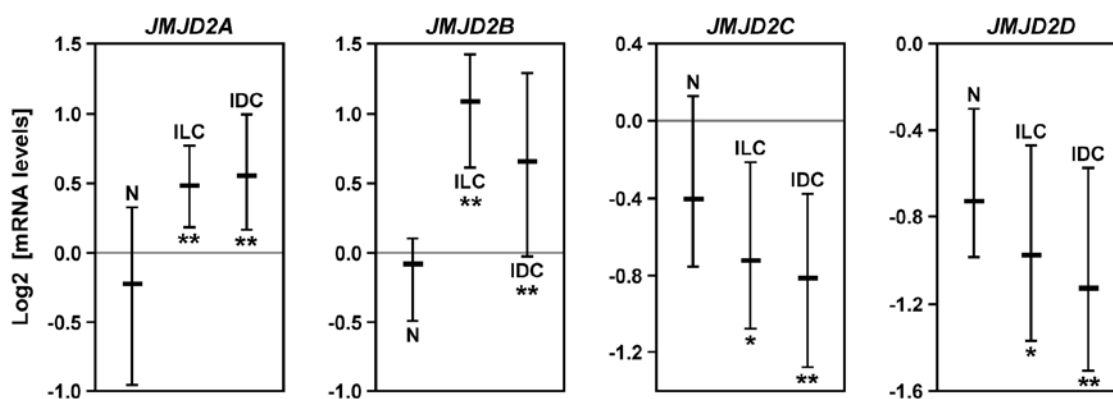E-mail: ralf-janknecht@ouhsc.edu

Figure 1. Expression of JMJD2 mRNAs in 61 normal (N), 36 invasive lobular breast carcinomas (ILC) and 392 invasive ductal breast carcinomas (IDC). Shown are log2-transformed mRNA levels with the median and a 25-75% percentile range. Statistical significance (Student's t-test) of difference in mRNA expression compared to normal breast tissue is indicated: $^*p < 0.05$; $^{**}p < 0.0001$. Microarray data were retrieved from The Cancer Genome Atlas, National Cancer Institute, and analyzed through the Oncomine web portal (www.oncomine.org).

Coimmunoprecipitation assays. MCF7 cells were grown in the absence or presence of 1 nM estradiol (19). Cells were lysed in 50 mM Tris (pH 7.4), 50 mM NaF, 150 mM NaCl, 0.5% Igepal CA-630, 0.1 mM DTT, 0.5 mM PMSF, 0.25 mM Na_3VO_4, 10 μg/ml leupeptin, 2 μg/ml aprotinin and 1 μg/ml pepstatin A (20). Immunoprecipitations were performed with ERα antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; sc-543), as previously described (21). Coprecipitated JMJD2A was then detected by western blotting utilizing JMJD2A antibodies (Bethyl Laboratories, Inc., Montgomery, TX, USA; A300-861A) and enhanced chemiluminescence (22). Similarly, coimmunoprecipitation experiments with transiently transfected 293T cells were conducted (23).

Luciferase assays. CV-1 cells were grown in 12-well plates and transiently transfected utilizing the calcium phosphate coprecipitation method (24). The reporter plasmid ERE-luc (500 ng), pSG5-ERα (2.5 ng), Flag-JMJD2A (500 ng) or empty vector pEV3S (500 ng), and pBluescript KS$^+$ (1500 ng) were cotransfected (25). After transfection, cells were incubated for 36 h in phenol red-free media containing 5% charcoal-stripped serum with or without 1 nM estradiol. The cells were then lysed (26) and luciferase activities determined in a luminometer, as described in a previous study (27). Averages with standard errors of triplicate experiments were determined.

RNA interference. Small hairpin RNA (shRNA) directed against JMJD2A was cloned into pSIREN-RetroQ (Clontech). Sequences targeted within the human JMJD2A mRNA were 5'-GUUGAGGAUGGUCUUACCU-3' (shRNA #3) and 5'-GGACUUAGCUUCAUAACUA-3' (shRNA#5). Retrovirus was then produced in 293T cells, as previously described (28). The resultant retrovirus was employed to infect T47D cells (29). Infected cells were selected with 2.5 μg/ml puromycin for three days, after which cells were lysed by boiling in Laemmli buffer (30). Protein extracts were subjected to SDS polyacrylamide gel electrophoresis, followed by western blotting (31). The following antibodies were employed for the detection of proteins: JMJD2A (Bethyl A300-861A), actin (GenScript A00730), c-Jun (Santa Cruz Biotechnology sc-45) and cyclin D1 (Cell Signaling #2926).

Cell proliferation assay. Infected, puromycin-selected T47D cells (as mentioned above) were seeded into 96-well plates in DMEM supplemented with 10% fetal calf serum. One day after seeding, the cell number was counted for the first time (defined as day 0) and two days later, the cell number was counted again. The TACS MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] kit (Trevigen Inc., Gaithersburg, MD, USA) was employed to count the cell numbers, according to the manufacturer's recommendations. Averages with standard errors of triplicate experiments were determined.

Results

Overexpression of JMJD2A in breast tumors. To study the expression pattern of JMJD2 genes in human breast tumors, publicly available microarray data retrieved from The Cancer Genome Atlas were analyzed through the Oncomine web portal (www.oncomine.org). Although JMJD2A and B mRNA expression was robustly upregulated in invasive lobular and ductal carcinomas compared to the normal breast tissue, the opposite behavior was noted for JMJD2C and D (Fig. 1). These data suggest that JMJD2A and B have different functions in breast tumors compared to JMJD2C and D and are consistent with the hypothesis that as with JMJD2B, JMJD2A is a potential breast-relevant oncoprotein.

To substantiate this view, we compared the degree of JMJD2A protein overexpression between normal and cancer breast tissue. To this end, we stained a total of 32 pairs of human breast tumors and matching normal breast tissues with two different JMJD2A antibodies. The two antibodies exhibited a similar staining pattern showing JMJD2A overexpression in 19 (Abcam antibody) or 20 (Bethyl antibody) tumors out of the 32 matching samples (Fig. 2). Thus, approximately 60% of all human breast tumors overexpress the JMJD2A protein.

Interaction of JMJD2A with ERα. The majority of breast tumors are ERα-positive and therefore endocrine therapy is applicable to most breast cancer patients (32). Thus, one mechanism by which JMJD2A is likely to contribute to breast tumor formation is through the coactivation of ERα. A prerequisite for this would be an interaction of the two proteins. To explore
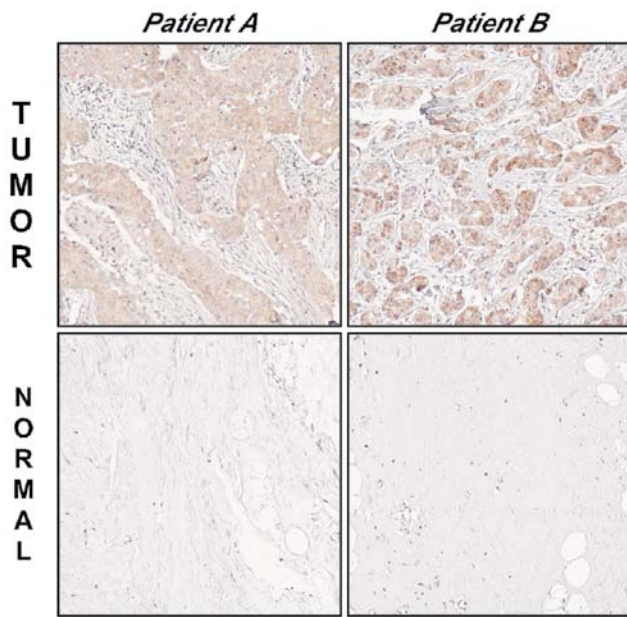
Figure 2. Overexpression of JMJD2A protein in human breast tumors. Matching normal and cancer human breast tissues were stained with JMJD2A antibody (brown color) and counterstained with hematoxylin (blue color). Two representative matched pairs of tumor and normal breast tissue stained with JMJD2A antibody from Bethyl (A300-861A) are shown.

Figure 4. Activation of estrogen-dependent transcription by JMJD2A. JMJD2A (wild-type or H188A mutant) was expressed in CV-1 cells as indicated. Additionally, an ERα-driven luciferase reporter plasmid (ERE-luc) and ERα expression vector were cotransfected and resultant luciferase activities were determined.
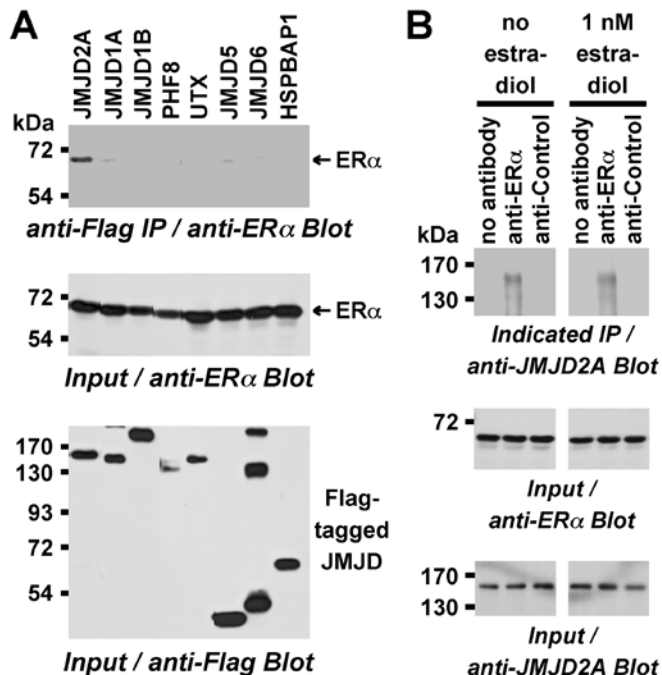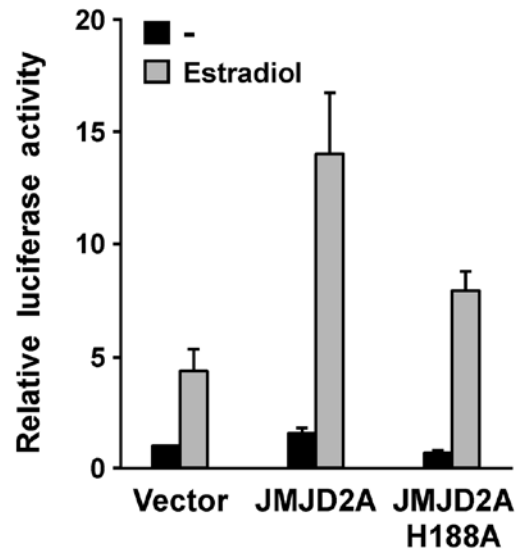

Figure 3. Interaction of JMJD2A with ERα is shown. (A) Indicated Flag-tagged JMJD proteins were coexpressed with ERα in 293T cells. After anti-Flag immunoprecipitation (IP), coprecipitated ERα was revealed by western blotting (top panel). The bottom two panels show input levels of ERα or Flag-tagged proteins. (B) Coimmunoprecipitation of endogenous JMJD2A with endogenous ERα in MCF7 breast cancer cells treated with or without 1 nM estradiol is shown.

this, Flag-tagged JMJD2A was overexpressed with ERα in human 293T cells, performed anti-Flag immunoprecipitations and assayed for any coprecipitated ERα by respective

western blotting. ERα was found to robustly coprecipitate with JMJD2A (Fig. 3A). This is not an unspecific interaction of JMJD2A with ERα, since seven other JMJD proteins tested (JMJD1A, JMJD1B, PHF8, UTX, JMJD5, JMJD6, HSPBAP1) did not significantly form complexes with ERα (Fig. 3A).

To confirm that endogenous JMJD2A interacts with endogenous ERα, human MCF7 breast cancer cells that express both proteins were examined. Furthermore, we tested whether or not such an interaction would be dependent on estradiol. To this end, ERα complexes were immunoprecipitated from MCF7 cells treated either with or without estradiol. Estradiol was added to all buffers utilized to immunoprecipitate ERα complexes from estradiol-treated cells. JMJD2A was found to coimmunoprecipitate with ERα both in the absence and presence of estradiol (Fig. 3B), suggesting that JMJD2A forms complexes with the hormone-bound and hormone-free receptor. As a validation for specificity, JMJD2A was not precipitated with no or control antibodies (Fig. 3B). Therefore, JMJD2A forms complexes with ERa *in vivo* and thus potentially functions as a transcriptional cofactor of ERα.

Stimulation of ERα-dependent gene transcription by JMJD2A. Whether or not the interaction between JMJD2A and ERα is functionally relevant was assessed. To this end, we employed a luciferase reporter construct (ERE-luc) that is inducible by hormone-stimulated ERα (33). When ERE-luc and ERα were transfected together into CV-1 cells, stimulation with estradiol led to a ~5-fold induction of luciferase activity, as expected (Fig. 4). In the absence of estradiol, JMJD2A cotransfection had only a slight impact on luciferase activity, whereas estradiol-dependent luciferase activity was strongly induced by JMJD2A.

We then determined whether this coactivation of ERα by JMJD2A is dependent on the catalytic activity of JMJD2A. To this end, we employed a mutant of JMJD2A (H188A), in which a critical histidine residue within its catalytic center was
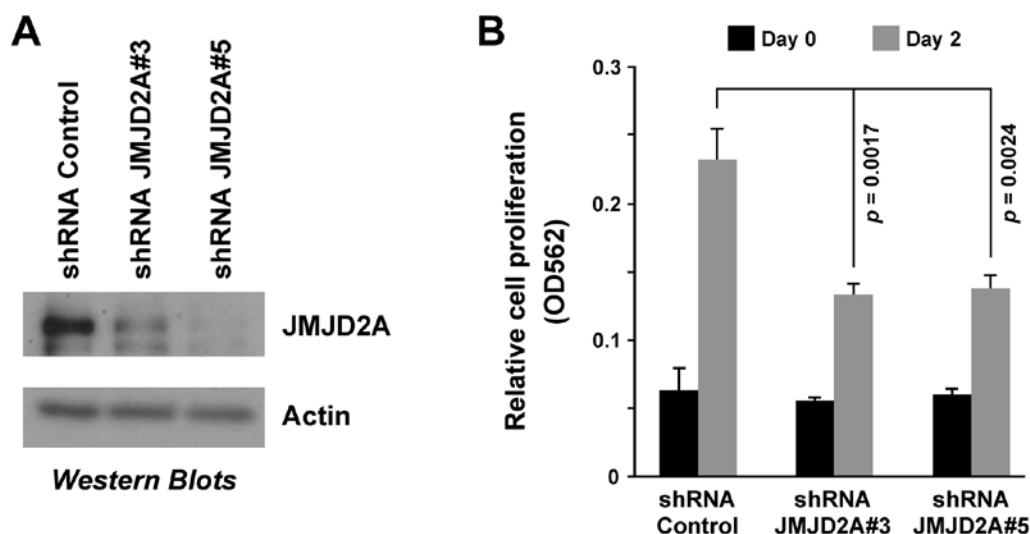
Figure 5. Impact of JMJD2A on T47D cell proliferation. (A) T47D cells were infected with retrovirus expressing a control shRNA or two different JMJD2A shRNAs (#3 and #5). Downregulation of JMJD2A was confirmed by western blotting. (B) Cell growth in T47D cells challenged with indicated shRNAs was monitored with an MTT assay. Statistical significance was determined with an unpaired, two-tailed t-test.
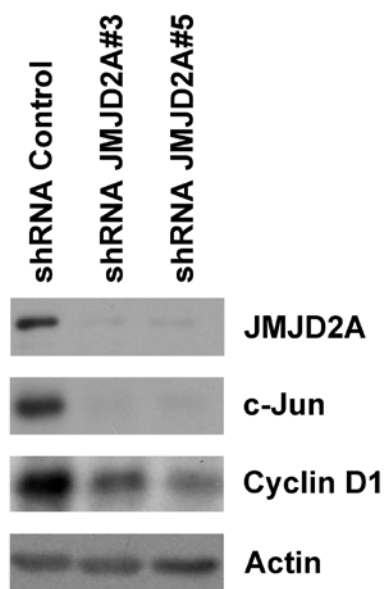

Figure 6. Downregulation of JMJD2A affects the expression of endogenous oncoproteins (c-Jun, cyclin D1) in T47D breast cancer cells.

mutated to alanine (7). Replacing wild-type JMJD2A with this H188A mutant led to a reduction of the coactivation potential of JMJD2A (Fig. 4), clearly demonstrating that the catalytic activity of JMJD2A is required for its ability to maximally stimulate estrogen-dependent gene transcription.

Impact of JMJD2A on cell growth. To assess the role of JMJD2A in breast cancer cells, JMJD2A was downregulated with two different shRNAs (#3 and #5) in human ERα-positive T47D breast cancer cells. The two shRNAs effectively reduced the expression of endogenous JMJD2A protein (Fig. 5A). Of note, T47D cell growth was severely impaired by the two JMJD2A shRNAs compared to control shRNA (Fig. 5B). Thus, JMJD2A exerts a pro-proliferative role in T47D breast cancer cells.

Effects of JMJD2A downregulation on gene transcription. A target gene of ERα is cyclin D1 (34), which is a crucial cell cycle regulator and prominent oncogene. Thus, whether or not JMJD2A downregulation would result in decreased cyclin D1 expression in T47D cells was analyzed. Cells expressing either of the two utilized JMJD2A shRNAs exhibited a reduced cyclin D1 expression (Fig. 6). In addition, we found that another oncoprotein, c-Jun (35), became downregulated by JMJD2A ablation (Fig. 6). Thus, JMJD2A may regulate T47D cell growth through influencing the expression of at least two oncoproteins, cyclin D1 and c-Jun.

Discussion

In this study, we identified the JMJD2A histone demethylase as a novel coactivator of ERα, since i) JMJD2A formed a complex with ERα *in vivo*, ii) JMJD2A overexpression enhanced estrogen-dependent transcription, and iii) the downregulation of JMJD2A reduced transcription of a seminal ERα target gene, cyclin D1. Moreover, our analyses revealed that JMJD2A mRNA as well as protein are overexpressed in human breast tumors and that JMJD2A supports breast cancer cell growth. As such, JMJD2A exhibits features of an oncoprotein, whose action may be particularly relevant in ERα-positive breast tumors.

In addition to JMJD2A, our microarray analyses showed that JMJD2B is overexpressed in invasive breast tumors, suggesting that JMJD2A and JMJD2B may perform similar functions. Consistently, it was previously reported that JMJD2B coimmunoprecipitated with ERα and that JMJD2B downregulation suppressed growth of the ERα-positive breast cancer cell lines, MCF7 and T47D (14-16). Similarly, JMJD2A and B (but not JMJD2C or D) are able to perform comparable roles in the DNA damage recognition and repair pathway (36). Notably, JMJD2B downregulation had no effect on the ability of ERα-negative MDA-MB-231 breast cancer cells to induce tumorigenesis in a nude mouse model (16), and similarly JMJD2A siRNA had a very modest impact on the proliferation of MDA-MB-231 cells

in vitro (37). Thus, JMJD2A and B are likely to significantly promote tumorigenesis only in ERα-positive breast tissue, which probably involves their ability to coactivate ERα.

However, in contrast to our data showing the downregulation of JMJD2C in invasive breast tumors at the mRNA level, another report posits that JMJD2C mRNA is upregulated in breast tumors (38). One reason for this discrepancy may be the use of different breast tumor samples. Regardless, JMJD2C mRNA expression was reportedly higher in ERα-negative compared to ERα-positive tumors (38), which is opposite to the expression pattern of JMJD2B (14,15) and is suggestive of JMJD2C not being a promoter of estrogen-dependent breast tumorigenesis.

Cyclin D1 gene transcription is induced by estradiol in breast cancer cells, which involves activation of its promoter by a complex of ERα and the AP-1 transcription factor (39,40). Notably, cyclin D1 is frequently overexpressed in breast tumors and a respective transgenic mouse model develops breast cancer (41). Consistent with JMJD2A being an ERα coactivator, the downregulation of JMJD2A led to a reduced cyclin D1 expression in our study, indicating that the overexpression of JMJD2A likely contributes to breast tumor formation by inducing this oncogene.

Another oncoprotein, whose expression was reduced upon JMJD2A downregulation, was c-Jun. This transcription factor is not known to be directly regulated by ERα, however, it has important functions in breast tumors. Its overexpression in MCF7 breast cancer cells stimulated their ability to migrate, invade and form tumors in a xenograft model, while its inactivation caused cell cycle arrest (42,43). Furthermore, inappropriately activated c-Jun has been found in invasive human breast tumors and c-Jun was also critical for cell invasion in ErbB2-induced mouse mammary tumors (44,45). Therefore, JMJD2A overexpression may lead to the overexpression of c-Jun that is predicted to cause an invasive, aggressive phenotype of breast tumors.

Notably, JMJD2A does not only coactivate ERα, but also the androgen receptor (46). As with estrogen receptors, the androgen receptor belongs to the nuclear hormone receptor superfamily and is crucial for the development of prostate tumors (47,48). As such, one may predict that JMJD2A also contributes to the development of prostate cancer. Currently, no data supporting this hypothesis have been published, and the same holds true for JMJD2B. In addition, JMJD2A likely contributes to the development of colorectal tumors, since its downregulation impairs the proliferation and survival of colon cancer cell lines (49). In contrast to breast and prostate cancer, nuclear hormone receptors are not prime drivers of tumorigenesis in colorectal cancer. Thus, it remains to be elucidated which other pivotal DNA-binding transcription factor(s) become dysregulated by JMJD2A in colon cancer.

Originally, JMJD2A was described as a transcriptional repressor (50,51). However, published data (46) and findings of this study clearly indicate that JMJD2A also functions as a transcriptional coactivator in conjunction with the androgen receptor or ERα. Thus, the ability of JMJD2A to regulate gene transcription may depend on which transcription factor recruits JMJD2A to a particular gene regulatory element. Mechanistically, JMJD2A particularly demethylates trimethylated lysines 9 and 36 on histone 3 (2,4). Although the demethylation of histone 3 on lysine 9 has been associated with the activation of gene transcription (1), the function of lysine 36 methylation on histone 3 remains to be elucidated (52). Moreover, JMJD2A is capable of demethylating histone 1.4 on lysine 26 (12), and this demethylation is predicted to also induce gene transcription (53,54). Thus, JMJD2A overexpression should lead to the activation of ERα target genes, as observed with the ERE-luc reporter construct in this study. The reduced expression of the ERα target gene cyclin D1 upon JMJD2A downregulation in T47D cells is consistent with the hypothesis that the function of JMJD2A is to open up chromatin, thereby facilitating estrogen-dependent transcription. Accordingly, we found that catalytically inactive JMJD2A was compromised in its ability to stimulate ERα-mediated transcription. This latter result strongly suggests that inhibitors of JMJD2A enzymatic activity may be beneficial for the treatment of ERα-positive breast tumors.

In conclusion, our data have identified JMJD2A as a novel *bona fide* ERα coactivator, thus deepening our understanding of estrogen-dependent transcriptional processes. In addition, the fact that JMJD2A is overexpressed in human breast tumors and required for efficient growth of breast cancer cells suggests JMJD2A as a novel potential drug target.

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