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## The histone demethylase JMJD2B is regulated by estrogen receptor alpha and hypoxia and is a key mediator of estrogen induced growth

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### Abstract

ER $\alpha$  plays an important role in breast cancer. Up-regulation of HIF-1 $\alpha$  in ER $\alpha$ -positive cancers suggests that HIF-1 $\alpha$  may cooperate with ER $\alpha$  to promote breast cancer progression and consequently affect breast cancer treatment. Here we show the histone demethylase JMJD2B is regulated by both ER $\alpha$  and HIF-1 $\alpha$ , and drives breast cancer cell proliferation in normoxia and hypoxia, and epigenetically regulates the expression of cell cycle genes such as *CCND1*, *CCNA1* and *WEE1*. We also demonstrate that JMJD2B and the hypoxia marker CA9 together stratify a subclass of breast cancer patients and predicts a worse outcome of these breast cancers. Our findings provide a biological rationale to support the therapeutic targeting of histone demethylases in breast cancer patients.

### Keywords

Hypoxia; ER $\alpha$ ; HIF-1 $\alpha$ ; Histone demethylase; JMJD2B; breast cancer

### Introduction

Estrogen plays an essential role in the establishment and progression of breast cancer. The biological actions of estrogens are mediated by two members of the nuclear receptor family, estrogen receptors  $\alpha$  (ER $\alpha$ ) and  $\beta$  (ER $\beta$ ) (1, 2). ER $\alpha$  is critical to mammary epithelial cell division and breast cancer progression while ER $\beta$  suppresses transcriptional activity of ER $\alpha$  (2, 3). Hypoxia-inducible factor 1 is a master regulator of oxygen homeostasis, functioning

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by transactivating hundreds of genes that are involved in angiogenesis, cell survival, metabolism and invasion or metastasis (4-6). In response to physiological or pathological hypoxic stress, the subunit HIF-1 $\alpha$  is stabilized and heterodimerizes with HIF-1 $\beta$ , forming a functional HIF-1 transactivator which binds the hypoxia response element (HER) to initiate gene expression. In normoxia, the HIF-1 $\alpha$  subunit is post-translationally hydroxylated by a class of enzymes called prolyl hydroxylases (PHDs), resulting in VHL-mediated proteasomal degradation, thus maintaining the activity of HIF-1 at low levels (7, 8). HIF-1 $\alpha$  is upregulated in many solid tumors as a consequence of hypoxia. Nevertheless, HIF-1 $\alpha$  may also be upregulated in normoxia by oncogenic stimulation, mitochondrial metabolic dysfunction or loss of the tumor suppressor VHL (9).

HIF-1 $\alpha$  has been associated with an aggressive phenotype of breast cancer, with large tumor size, high grade, high proliferation and lymph node metastasis (10). Increased HIF-1 $\alpha$  expression is also associated with ER $\alpha$  positivity (10) and hypoxia can trigger ligand-independent ER $\alpha$  activation (11), possibly by direct HIF-1 $\alpha$  and ER $\alpha$  interaction. Two studies suggest that hypoxia and estrogen can cooperate to regulate gene expression in T47D breast cancer cells (12) or increase transcriptional activity of ERE (13), but whether HIF-1 $\alpha$  is involved in this process is unknown. Hypoxia is an important determinant of tumor resistance to chemotherapy and radiotherapy. Tamoxifen and aromatase inhibitors are important antagonists or suppressors of estrogen activity in breast cancer and our recent clinical study has indicated that HIF-1 $\alpha$  is significantly associated with endocrine therapy resistance in ER $\alpha$  positive breast cancers (14).

Collectively, these observations suggest that an unidentified mechanism exists by which interaction of the HIF-1 $\alpha$  and ER $\alpha$  signaling pathways could coordinate to promote breast cancer progression. However, the molecular mechanism of coordinated gene regulation by HIF-1 $\alpha$  and ER $\alpha$  in breast cancer has not been elucidated. In this study, we found that HIF-1 $\alpha$  and ER $\alpha$  regulate a common signaling pathway engaged by the histone lysine demethylase JMJD2B. JMJD2B is upregulated in hypoxia in a HIF-1 $\alpha$ -dependent manner. ER $\alpha$  is critical for JMJD2B induction in hypoxia in ER-positive breast cancer cells. JMJD2B is functional in hypoxia and regulates histone demethylation on the promoters of its target genes which include multiple signaling pathways and biological processes that are involved in cancer. JMJD2B is critical to breast cancer cell survival in normoxia and hypoxia, partly via regulation of cell cycle progression, is highly expressed in ER $\alpha$  positive primary breast cancers and is an adverse prognostic factor in hypoxic breast cancers.

## Materials and Methods

### Cell Culture and Reagents

Breast cancer cell lines MCF7, T47D, MDA-MB-361, MDA-MB-468, and MDA-MB-231 were cultured in Dulbecco's modified Eagle's medium (DMEM). All cell culture medium were supplemented with 10% fetal bovine serum, penicillin (50 IU/ml) and streptomycin sulphate (50  $\mu$ g/ml). For hypoxia incubation, cells were exposed to hypoxic conditions (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub>) in a Heto-Holten CellHouse 170 incubator (RS Biotech, Irvine, Scotland). 17 $\beta$ -estradiol (E2) was purchased from Calbiochem. ICI 182780 was purchased

from Tocris Bioscience. 4-hydroxy-tamoxifen and Desferrioxamine (DFX) were purchased from Sigma.

### siRNA Transfection

siRNAs were transfected into subconfluent cells using HiPerfect transfection reagent (Qiagen) according to the manufacturer's instructions. The target sequences for HIF-1 $\alpha$  and HIF-2 $\alpha$  were synthesized by Eurogentec (Eurogentec, Southampton, UK) were as follows: HIF-1 $\alpha$ , 5-UCAAGUUGCUGGUCAUCAGdTdT-3; HIF-2 $\alpha$ , 5-ACUGCUAUAACAAGAUGCUGdTdT-3. The siRNA oligos for JMJD2B were purchased from Dharmacon with sequences as follows: JMJD2B#1, 5-GCGCAGAAUCUACCAACUU-3, JMJD2B#2, 5-CAAAUACGUGGCCUACAUA-3. The SMARTpool siRNA oligos for ER $\alpha$  were purchased from Dharmacon. The siRNA non-targeting control was purchased from Dharmacon. For the cell viability assay, long-term transfection protocol was used according to the Qiagen manufacturer's instructions.

### Plasmids and Transfection

HA-JMJD2B plasmid was described previously (15). HA-HIF-1 $\alpha$  was introduced into pCMV $\beta$  vector. To transfect plasmids into cells, FuGene6 transfection reagent (Roche) was used according to the manufacturer's protocol.

### Western Blotting and Immunoprecipitation

Methods and Antibodies were listed in Supplemental table.

### Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays was performed according to the manufacturer's protocol (EZ-CHIP, Upstate, Millipore). Briefly, MCF cells were crosslinked with 1% paraformaldehyde for 10 minutes, and quenched for 5 minutes with 125 mM of glycine. After sonification, cell lysates were spin down and 100  $\mu$ l of supernatant was diluted to 1ml for immunoprecipitation. Samples were pre-cleared with 60  $\mu$ l of protein G beads. Appropriate concentrations of antibodies were used for immunoprecipitation of the cross-linked DNA-protein complexes. After a serial washing, DNA-protein crosslink was reversed and DNA was extracted for polymerase chain reaction (PCR). PCR primers and antibodies used for ChIP were listed in Supplemental Data

### Subcellular Fractionation

Cellular cytoplasmic and nuclear fractions were prepared using the NE-PER kit (Perbio) according to the manufacturer's protocol.

### Cell Viability and Colony Formation Assays

Cell viability and colony formation were assessed as described in Supplemental Methods.

### qRT-PCR

Quantitative RT-PCR was performed as previously described (16). Detailed methods and primers were described in supplemental data.

## Immunofluorescence

Cells were washed three times with PBS and fixed in 4.0% paraformaldehyde in PBS for 1 hour at room temperature. Cells were permeabilized for 10 min using Triton X-100 (0.5%), and then incubated with 1% BSA for 20 min. Cells were incubated overnight with primary antibodies with a dilution of 1:1000 in 1% BSA. After three consecutive 5 min washes with PBS, cells were incubated with secondary antibodies for 1 hour before being washed with PBS and mounted in a mounting medium (Dako) containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Imaging of the cells was carried out using a Zeiss immunofluorescence microscope.

## GSEA

Enrichment analysis of ER $\alpha$  target genes was performed as previously described (17) using the ER $\alpha$  gene signature (18) from molecular signature database (GSEA v2.0 at <http://www.broad.mit.edu/gsea>) and gene expression profiles of HIF-1 $\alpha$  knockdown in MCF7 cells (19).

## Gene Expression Profiling

RNA was extracted from MCF7 cells using RNeasy mini kit (Qiagen). Labeling and hybridization of samples to Illumina gene expression chip were performed using standard methodology. Data analysis was performed using GenePattern (20).

## Clinical data

Patients and tissue samples were described in detail in the Supplemental data.

## Immunohistochemistry

Immunohistochemistry was performed as described previously for CA9 (Cancer Res 2001;61:6394-9. Virology 1992;187:620-6.). Methods are described in brief for JMJD2B. Antigen retrieval was performed by incubating slides in Tris-EDTA, pH 9.0, under 15 psi pressure for 2 minutes using a Decloaking Chamber (Biocare Medical, Concord, CA). Sections were incubated for 1 hour at room temperature with a primary anti-JMJD2B (Bethyl Laboratories, A301-478A) polyclonal rabbit antibody at 0.4  $\mu$ g/mL. Bound antibody was labelled with anti-rabbit/mouse Envision (DAKO, Carpinteria, CA). Antibody complexes were visualized using 2,3-diaminobenzidine chromogen, and sections were counterstained with Mayer's haematoxylin.

Intensity of JMJD2B expression was scored separately in the cytoplasm and nuclei of neoplastic cells as either positive or negative. The highest intensity score among replicate cores was used as the score for each patient.

## Statistics

The  $\chi^2$ -test was used to evaluate associations between categorical variables. Details are described in the supplemental data.

## Results

### Identification of an Interaction Between the ER $\alpha$ and HIF-1 $\alpha$ Pathways

We found a functional association of HIF-1 $\alpha$  and ER $\alpha$  using molecular concept ([www.oncomine.com](http://www.oncomine.com)) analysis (Fig. *S1A*). To further understand the interaction between the HIF-1 $\alpha$  and ER $\alpha$  signaling pathways, we used Gene Set Enrichment Analysis (GSEA) (21) to analyze the association of genes differentially regulated by HIF-1 $\alpha$  in MCF7 cells from a recent publication (19) with an ER $\alpha$ -positivity gene signature (18). Using this method, we found that ER $\alpha$ -regulated genes such as *CA12* and *CCNG2* were enriched in the HIF-1 $\alpha$  target gene signature (Fig. *S1B,C*), suggesting an interaction between HIF-1 $\alpha$  and ER $\alpha$ . Among the genes that are regulated by both ER $\alpha$  and HIF-1 $\alpha$  (Fig. *S1A, B*), we focused on *JMJD2B* gene in this study for several reasons. *JMJD2B* encodes a histone demethylase that has been recently shown to be a HIF-1 $\alpha$  target gene by us and others (22-25). However, the functions of the most histone demethylases including *JMJD2B* are not clear (especially in hypoxia) and how histone demethylase epigenetically regulates gene expression in hypoxia has not well been demonstrated. Importantly, elucidation of the function of *JMJD2B* may help to find novel therapeutic targets for breast cancers.

### ER $\alpha$ -Dependent Regulation of *JMJD2B*

The histone demethylase *JMJD2* family has potential oncogenic functions (26). However, the molecular mechanism by which *JMJD2*s promote cancer progression is unknown. In addition, how *JMJD2* oncogenes respond to proliferative signals (such as hormone stimulation) and antagonize environmental stress (such as hypoxia) has not been reported. To address the role of *JMJD2B*, we analyzed *JMJD2B* expression in primary breast cancers and found that *JMJD2B* is significantly expressed in ER-positive breast cancers (Fig. *S1D*). To test these observations in human breast cancer cell lines, we examined *JMJD2B* expression in ER positive (T47D and MCF7) and ER negative (MDA-MB-231, MDA-MB-468) breast cancer cells. Real-time PCR and western blotting showed that *JMJD2B* was highly expressed in ER-positive cells in comparison to ER-negative cells (Fig. *1A*, and data not shown), further indicating that *JMJD2B* expression is correlated with ER $\alpha$  status.

To examine whether ER $\alpha$  regulates *JMJD2B* expression in a ligand-dependent manner, we treated estrogen deprived T47D and MCF7 cells with 17 $\beta$ -estradiol (E2) for 16 hours and assessed the expression of *JMJD2B*. E2 treatment caused an increase in *JMJD2B* expression at mRNA levels and protein levels in T47D cells (Fig. *1B*) and in known ER $\alpha$  target genes *c-Myc*, *CCND1* and *pS2 (TFF1)*. Similarly, E2 treatment induced *JMJD2B* expression in MCF7 cells, though to a lesser extent at the 16 hour time point (Fig. *S2A,B*). To further examine the role of ER $\alpha$  in regulation of *JMJD2B* expression, T47D cells were treated with the ER $\alpha$  inhibitor ICI182780. This inhibitor suppressed expression of ER $\alpha$  (Fig. *S3A*) and *c-Myc*, *CCND1* and *pS2 (TFF1)* (Fig. *1C*). Importantly, ICI182780 inhibited *JMJD2B* expression at both mRNA and protein levels (Fig. *1C, S3A*). Similarly, ICI182780 treatment led to *JMJD2B* downregulation in MCF7 cells (Fig. *S2C, D*). To further confirm ER $\alpha$ -dependent expression of *JMJD2B*, small interfering RNA (siRNA) was used to knock down the expression of ER $\alpha$  in T47D and MCF7 cells. Loss of ER $\alpha$  in T47D and MCF7 cells resulted in a great reduction in *JMJD2B* expression (Fig. *1D*). To determine whether ER $\alpha$

directly binds to *JMJD2B* gene, we analyzed *JMJD2B* gene locus and found an ER $\alpha$  binding site in the first intron of *JMJD2B* gene (Fig. S3B). Our CHIP-PCR assay revealed that ER $\alpha$  bound this region *in vivo* (Fig. S3C), suggestive of a functional ER $\alpha$  binding element. In agreement with our findings, a genome-wide analysis of ER $\alpha$  binding sites also showed an ER binding in the first intron of *JMJD2B* (27). In addition, another global analysis of ER $\alpha$  binding showed that *JMJD2B* is an ER-regulated gene (28)

Collectively, these data indicate that *JMJD2B* is a bona fide target of ER $\alpha$  and its expression in ER-positive breast cancer cells is mainly dependent on ER $\alpha$ .

### ER $\alpha$ Is Important for *JMJD2B* Expression in Hypoxia

We and others have shown that *JMJD2B* can be upregulated in hypoxia in a HIF-1 $\alpha$ -dependent manner (Fig. S4A-F) (22-25), raising the question that whether ER $\alpha$  is still required for *JMJD2B* induction in hypoxia. To address this, we treated estrogen deprived T47D cells with E2 in normoxia and hypoxia. E2 treatment increased *JMJD2B* expression in both normoxia and hypoxia (Fig. 2A). Suppression of ER $\alpha$ , by either treating cells with ICI182780 or RNAi, not only reduced *JMJD2B* expression in normoxia but significantly downregulated *JMJD2B* expression in hypoxia in ER-positive breast cancer cells (Fig. 2B-D), indicating that ER $\alpha$  still functions to regulate *JMJD2B* expression in hypoxia. The ER depletion in hypoxia was more effective at inhibiting *JMJD2B* induction than the HIF siRNA. Of note, suppression of ER $\alpha$  also reduced HIF-1 $\alpha$  expression (Fig. 2B-D), suggesting that ER $\alpha$  is important for HIF-1 $\alpha$  induction or protein stabilization.

### *JMJD2B* Regulates Multiple Biological Processes Including the Cell Cycle

To analyze the functions of *JMJD2B*, we performed genome-wide expression profiling to identify *JMJD2B* target genes in MCF7 cells. Depletion of *JMJD2B* downregulated 300 genes in normoxia but only 142 genes in hypoxia, 59 of which were commonly downregulated in normoxia and hypoxia (Fig. 3A). Intriguingly, depletion of *JMJD2B* only upregulated 33 genes in normoxia, but 137 genes in hypoxia, 22 of which were commonly upregulated (Fig. 3A). These data have several implications: (1) *JMJD2B* can regulate a hypoxia-specific transcriptional response; (2) *JMJD2B* predominantly regulates transcriptional activation in normoxia since loss of *JMJD2B* causes downregulation of gene expression, though this transcriptional competence of *JMJD2B* is compromised in hypoxia; (3) transcriptionally repressive and active functions of *JMJD2B* co-exist in hypoxia since depletion of *JMJD2B* causes differential expression approximately in a 1:1 ratio; (4) *JMJD2B* maintains a basal activity in normoxia and regulates expression of a substantial number of genes, preserves this ability under hypoxia stress, as evidenced by a set of common genes such as *CCNA1* and *SOCS2* that are regulated by *JMJD2B* in both normoxia and hypoxia (Fig. 3C).

Gene ontology analysis revealed that *JMJD2B* regulates multiple signaling pathways and biological processes (Fig. 3B). For example, *JMJD2B* regulates Wnt, TGF $\beta$ , Notch, VEGF, PI3K pathways and angiogenesis, all of which play important roles in tumorigenesis or cancer progression. Biological processes engaged by *JMJD2B* target genes include signal transduction, cell proliferation and differentiation, cell adhesion and oncogenesis.

Importantly, JMJD2B regulates cell cycle genes such as *CCND1*, *CCNA1* and *WEE1* (Fig. 3C), indicating that JMJD2B is important for regulation of breast cancer cell proliferation. *CCND1* encodes cyclin D1, which complexes with cyclin-dependent kinases CDK4/CDK6 to regulate G1-phase progression. *CCNA1* encodes cyclin A1, which can activate CDK2 or CDK1 and may play a role in driving cells to enter S phase and G2/M phase transition. Wee1 is a CDK-inhibitory kinase that phosphorylates CDK1, thereby suppressing G2/M phase transition. In this study we found that JMJD2B positively regulates *CCNA1* expression and negatively regulates *WEE1* expression (Fig. 3C), suggesting that JMJD2B can promote G2/M phase transition. Interestingly, JMJD2B negatively regulates *CCND1* expression, suggesting that JMJD2B might be also involved in G1 phase regulation to coordinate cell cycle progression. *PGR*, which encodes progesterone receptor PR, is also positively regulated by JMJD2B (Fig. 3C). PR has been involved in cell cycle regulation of breast cancer cells (30) and, JMJD2B might also regulate breast cancer cell proliferation via PR.

### JMJD2B Is Important for Breast Cancer Cell Proliferation

Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired alterations to genes that directly regulate their cell cycles (31, 32). The evidence that JMJD2B gene is amplified in medulloblastoma (33) and herein that it regulates expression of cell cycle genes, indicate that JMJD2B may play a role in oncogenesis by promoting cell proliferation. We therefore analysed the role of JMJD2B in cells grown with full serum and phenol red-containing medium, i.e. in presence of estrogen and other growth factors. Loss of JMJD2B caused a significant decrease in cell proliferation, as assessed by counting cell numbers (Fig. 4A), by cell viability assay (Fig. S5A), and by colony formation assay (Fig. 4B) in the presence of estrogen. FACS analysis revealed that depletion of JMJD2B caused a significant G2/M phase arrest in normoxia and hypoxia (Fig. 4C,D), which is consistent with the western blotting results that loss of JMJD2B upregulated *WEE1*, *Cyclin B1*, and *Cyclin D1* (Fig. S5B). These data indicate that JMJD2B drives breast cancer cell proliferation in an estrogenic environment through direct or indirect coordination of the G2/M phase and G1 phases of the cell cycle.

### Histone Demethylase Function of JMJD2B in Hypoxia

JMJD2B is a JmjC-domain containing histone lysine demethylase, which belongs to the dioxygenase family, whose activities require iron,  $\alpha$ -ketoglutarate and oxygen (34). Thus, the induction of JMJD2B by hypoxia raises an important question: does JMJD2B exert an enzyme-independent function in hypoxia or use limited oxygen to catalyze the reaction? To address these questions, we assessed the enzymatic activity of JMJD2B by overexpressing HA-tagged JMJD2B in MCF7 cells. JMJD2B is a specific H3K9me3/me2 demethylase. Therefore we focused on studies of H3K9 methylation. JMJD2B erased the H3K9me3 mark in normoxia (Fig. 5A). Interestingly, this function was similar under 1% O<sub>2</sub> (Fig. 5A). H3K9me2 levels were also reduced by JMJD2B, though to a much lesser extent (Fig. 5A). H3K9me1 levels were increased presumably due to the conversion of H3K9me3/me2 to H2K9me1 (Fig. 5A). Immunostaining and subcellular fractionation results revealed that endogenous JMJD2B was mainly localized in the nucleus in normoxia and hypoxia in MCF7 cells (Fig. 5B and data not shown), consistent with its role as a histone demethylase

and transcriptional regulator. Nevertheless, neither induction of endogenous JMJD2B in hypoxia nor depletion of JMJD2B caused a significant global change in H3K9me3/me2 levels (Fig. 5C), suggesting that physiological JMJD2B acts locally to control specific gene expression or to maintain the chromatin environment in specific regions.

To directly examine the histone demethylase functions of JMJD2B we assessed H3K9me3 levels on its target gene promoters using ChIP-PCR (Fig. 5D). We chose the genes including *CCNA1*, *PGR*, *PMP22*, *CCND1* and *WEE1* that were positively or negatively regulated by JMJD2B. We found that, after depletion of JMJD2B either in normoxia or in hypoxia, H3K9me3 levels were significantly enriched on those gene promoters that are positively regulated by JMJD2B such as *CCNA1*, *PGR* and *PMP22*, indicating that JMJD2B can directly bind its target gene promoters and erase silencing marks from these promoters, thereby activating gene transcription in response to hypoxic stress. However, for the genes that are negatively regulated by JMJD2B such as *WEE1* and *CCND1*, the H3K9me3 marks were not consistent with this pattern after JMJD2B depletion. We found that the H3K9me3 mark on *CCND1* promoter in normoxia was below detection but was significantly enriched in hypoxia. Loss of JMJD2B reduced the H3K9me3 mark on the *CCND1* promoter in hypoxia, suggesting that for some genes the increase in JMJD2 expression in hypoxia can result not only in maintenance of activity but also increased activity. However the effects on the suppressed promoters in hypoxia were minimal compared to those upregulated.

### Clinical Assessment of JMJD2B Protein Induction in Breast Cancer

To further confirm the role of JMJD2B in breast cancer, we performed immunohistochemistry on tissue microarrays (TMAs) to observe the in situ expression of JMJD2B protein. Since JMJD2B is an ER $\alpha$  target gene, we assessed the JMJD2B expression according to the ER status. JMJD2B was significantly associated with ER $\alpha$  when it was predominantly localized in the nucleus ( $P < 0.001$ , Fig. 6A, and Table S1). However, we also observed that JMJD2B was localized in the cytoplasm of cancer cells in a fraction of tumors (data not shown); cytoplasmic expression of JMJD2B was not associated with ER $\alpha$ \* status ( $P = 0.69$ , Table S1), suggesting that JMJD2B function might also be regulated by an ER-dependent mechanism which can regulate the subcellular localization of JMJD2B. To study the association of JMJD2B and hypoxia, we used CA9 as a hypoxia marker. Nuclear JMJD2B and membranous/cytoplasmic CA9 colocalized in serially cut whole sections (Fig. 6B). Nuclear JMJD2B was highly expressed in perinecrotic hypoxic regions of breast cancer, where there was often strong membranous CA9 staining. Of the 54 CA9 positive cases, 32 were ER positive. Of those 32 CA9+/ER + cases, 19 were positive for JMJD2B nuclear staining.

After correcting for multiple testing, cytoplasmic JMJD2B expression was not statistically significantly associated with clinicopathological variables or CA9 expression ( $P = 0.71$ ) (Table S1). Nuclear JMJD2B expression was independent of other clinicopathological variables such as tumor grade, nodal status or HER2 status (Table S1). We analyzed whether JMJD2B alone could predict clinical outcomes. Again, cytoplasmic JMJD2B expression was not significantly associated with relapse-free or overall survival. Nuclear JMJD2B expression was not significantly associated with relapse-free or overall survival. In



multivariate analyses (including all statistically significant clinical and pathological variables by univariate analysis), neither cytoplasmic nor nuclear JMJD2B are significantly associated with relapse-free (Table S2) or overall survival (Table S3). The significance of JMJD2B expression in these multivariate survival analyses is not altered if chemotherapy, radiotherapy or hormonal therapy received is included in the models (data not shown). ER $\alpha$  positivity usually predicts a better survival as we have shown here (Table S2, S3). However, hypoxia or HIF-1 $\alpha$  is usually associated with worse survival and resistance to therapy. As JMJD2B expression may be regulated by both ER $\alpha$  and HIF-1 $\alpha$ , we combined JMJD2B expression and CA9 expression as a prognosis prediction marker. We found that those patients who are JMJD2B positive and CA9 positive showed a significantly worse outcome in overall survival than CA9 positive JMJD2B negative groups (Fig. 6C, P=0.026) and a worse tendency in relapse-free survival (Fig. 6D, P=0.12), suggesting that JMJD2B plays an important role in breast cancer progression mediated by both ER $\alpha$  and HIF-1 $\alpha$ . CA9 was chosen as a marker of HIF-1 $\alpha$  function because of its much greater stability and extensive validation in prognosis.

## Discussion

Aberrant histone methylation has been linked to cancer (34). A recent study has shown that JMJD2 family of histone demethylases have an oncogenic function and are highly expressed in human cancers (15). The involvement of the JMJD2 proteins in tumorigenesis has been supported further by recent studies that JMJD2C works as an essential coactivator of androgen receptor (AR)-induced transcription and cellular growth via a functional interaction between JMJD2C and the AR in prostate carcinomas (35). JMJD2B and JMJD2C are also amplified in medulloblastomas (36). Nevertheless, how JMJD2 is regulated by upstream oncogenic signals and the nature of its downstream effectors has not been elucidated (15, 22, 23).

In this study, we have shown that JMJD2B is highly expressed in ER-positive primary breast cancers and breast cancer cell lines whose expression can be driven by E2 treatment. The correlation of JMJD2B gene expression with ER $\alpha$  in primary breast cancers and the dependence on ER $\alpha$ /E2 for enhanced expression of JMJD2B in breast cancer cells and ChIP analysis demonstrate that JMJD2B is a bona fide target of ER $\alpha$ .

Hypoxia can cause chromatin alterations such as histone methylation (37). Although JMJD2B requires oxygen for its enzymatic activity as a dioxygenase, 1% O<sub>2</sub> does not affect its enzymatic function in our model systems. Overexpression of JMJD2B in hypoxia erases global H3K9me3 marks and loss of JMJD2B results in an increase in H3K9me3 levels on its local target gene promoters such as *CCNA1* and *PGR* in hypoxia, suggesting that JMJD2B is able to function under relatively low oxygen levels. Nevertheless, regulation of JMJD2B by HIF-1 does not affect the importance of ER $\alpha$  in hypoxia in ER-positive breast cancer cells, as loss of ER $\alpha$  significantly attenuated (or nearly abolished) JMJD2B expression in hypoxia, suggesting that HIF-1 and ER synergistically regulate JMJD2B in hypoxia.

Epigenetic suppression of gene transcription is potentially an important mechanism for cell survival under hypoxic conditions. Indeed, the number of genes suppressed by JMJD2B

increases from 33 in normoxia to 137 in hypoxia although we do not understand how exactly JMJD2B initiates this program. Nevertheless, the activities of essential genes to ensure cell proliferation and survival should be maintained under either normal conditions or hypoxia, although extreme stress could significantly suppress cell proliferation or cause cell death. In addition to regulating important signaling pathways such as Wnt, Notch, TGF-beta and angiogenesis, JMJD2B coordinately regulates cell cycle gene expression by upregulating *CCNA1* expression and suppressing *CCND1* and *WEE1* expression in both normoxia and hypoxia. Therefore, JMJD2B seems to positively promote G2/M phase transition but negatively regulates the G1 phase. Since posttranslational modifications such as proteasomal degradation play a critical role in regulating cell cycle, our findings that JMJD2B epigenetically regulates cyclin expression in breast cancer cells provides a new insight for understanding the functions of JMJD2 family members. However, this work does not provide direct evidence these genes are immediately downstream of JMJD2B, which will need individual analysis of promoters.

Given that JMJD2B is highly expressed in ER $\alpha$ -positive breast cancers and is essential for breast cancer cell proliferation/survival under estrogen stimulation conditions, by directly or indirectly promoting cell cycle progression, targeting JMJD2B may be an alternative strategy to block the ER $\alpha$  signaling pathway. Importantly, tamoxifen-resistant ER $\alpha$  is still functional in driving JMJD2B expression in normoxia and hypoxia for breast cancer cell proliferation (data not shown). The availability of a crystal structure of the JmjC domain of JMJD2A (38), which exhibits high homology to that of JMJD2B, may accelerate the identification of small molecule inhibitors for this class of enzymes. Although JMJD2B is an ER $\alpha$  target gene, it is also highly expressed in other cancers such as prostate cancer and bladder cancer (data not shown)

By using JMJD2B and CA9 in combination, we have successfully stratified a subclass of breast cancers that show a worse outcome in overall survival. This suggests that the evaluation of JMJD2B and CA9 may be of value in patient selection if such therapies are developed.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

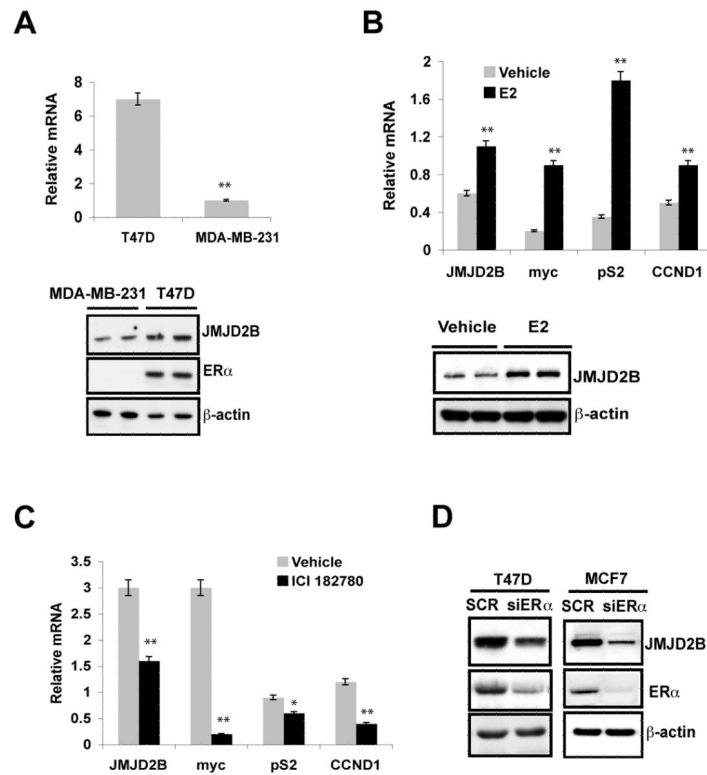
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**Figure 1. ER $\alpha$ -dependent regulation of JMJD2B**

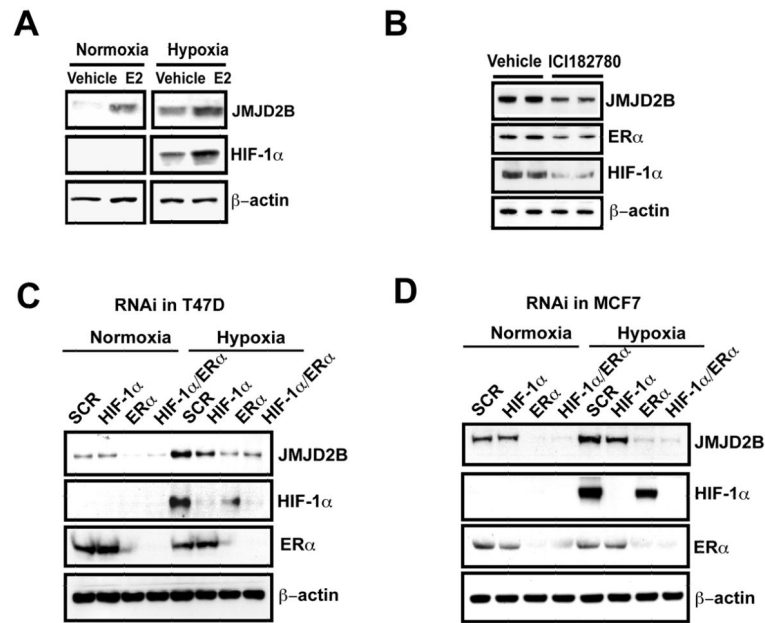
*A*, Quantitative RT-PCR (top panel) and immunoblotting (bottom panel) show JMJD2B mRNA and protein expression, respectively, in T47D and MDA-MB-231 cells.

*B*, After 3 days hormonal starvation, T47D cells were treated with 10 nM of E2 for 16 hours. Quantitative RT-PCR was performed to assess the expression of the indicated genes (top panel). Immunoblotting shows JMJD2B expression from above treatment (bottom panel)

*C*, T47D cells in normal medium containing serum and phenol red i.e. estrogen present, were treated with 1  $\mu$ M of ICI182780 for 16 hours. Quantitative RT-PCR was performed to assess the expression of the indicated genes.

*D*, T47D and MCF7 cells were transfected with smartpool siRNA oligos against ER $\alpha$ . Immunoblotting was used to assess the JMJD2B expression.

(\*P<0.05; \*\*P<0.01)



**Figure 2. ER $\alpha$  is critical for JMJD2B induction in hypoxia**

*A*, T47D cells were treated with 10 nM of E2 in normoxia and hypoxia for 16 hours.

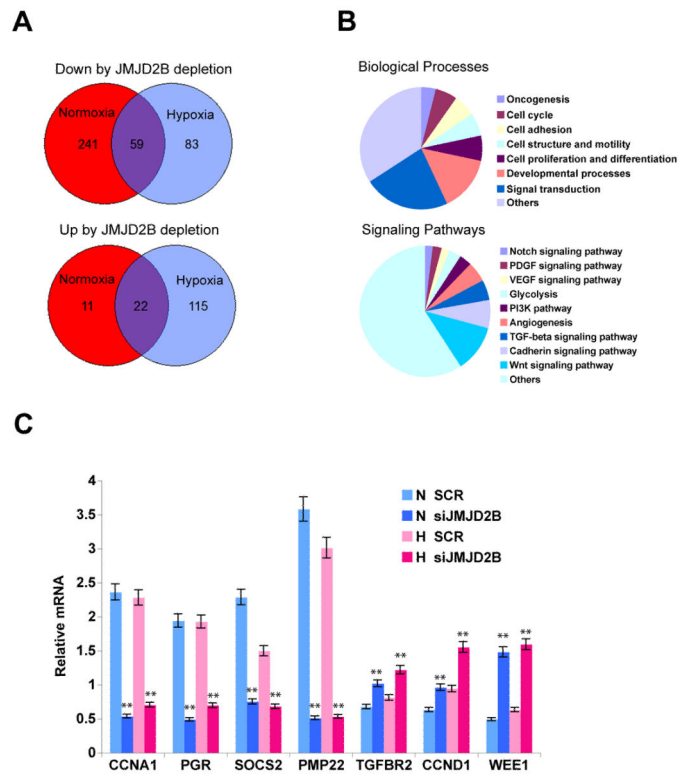
Immunoblotting was used to assess the JMJD2B expression.

*B*, T47D cells were treated with 1  $\mu$ M of ICI182780 in hypoxia for 16 hours.

Immunoblotting was used to assess the proteins with indicated antibodies.

*C*, T47D cells were transfected with siRNA oligos against ER $\alpha$  or HIF-1 $\alpha$ . After 36 hours transfection, cells were incubated in normoxia and hypoxia for 16 hours. Immunoblot was used to assess the JMJD2B expression.

*D*, MCF7 cells were treated the same as in (*C*).

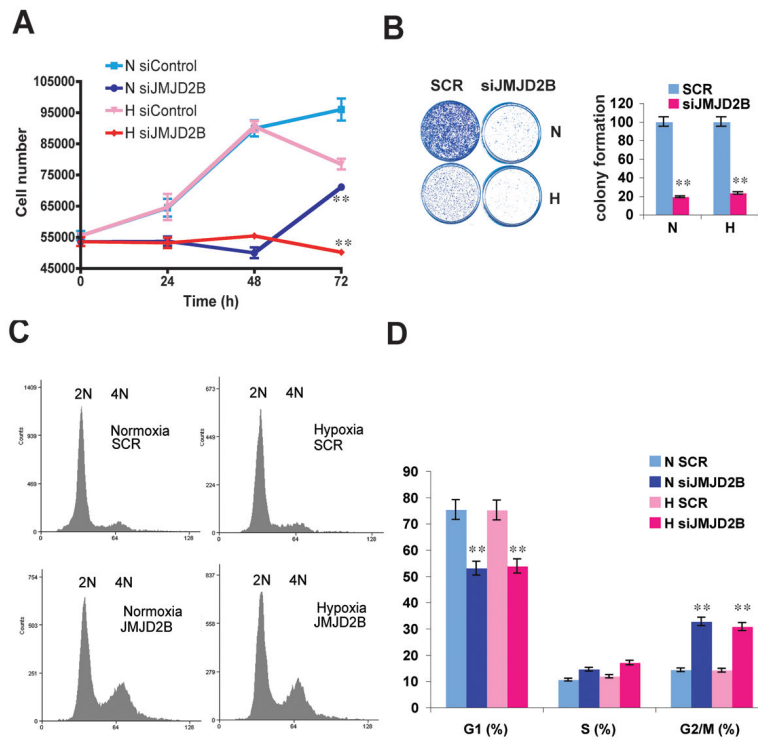


**Figure 3. JMJD2B regulates multiple biological processes and signaling pathways including cell cycle**

*A*, After 48 hour depletion of JMJD2B in MCF7 cells, global gene expression profiling reveals that JMJD2B can both positively (top) and negatively (bottom) regulates gene expression in normoxia and hypoxia.

*B*, Gene ontology analysis with Panther program shows that genes regulated by JMJD2B are involved in multiple biological processes and signaling pathways.

*C*, Quantitative RT-PCR was performed to validate gene expression regulated by JMJD2B. (\*\*P<0.01)



**Figure 4. JMJD2B is essential for breast cancer cell proliferation**

*A*, After 48 hour depletion of JMJD2B, the MCF7 cell proliferation was measured by cell number counting.

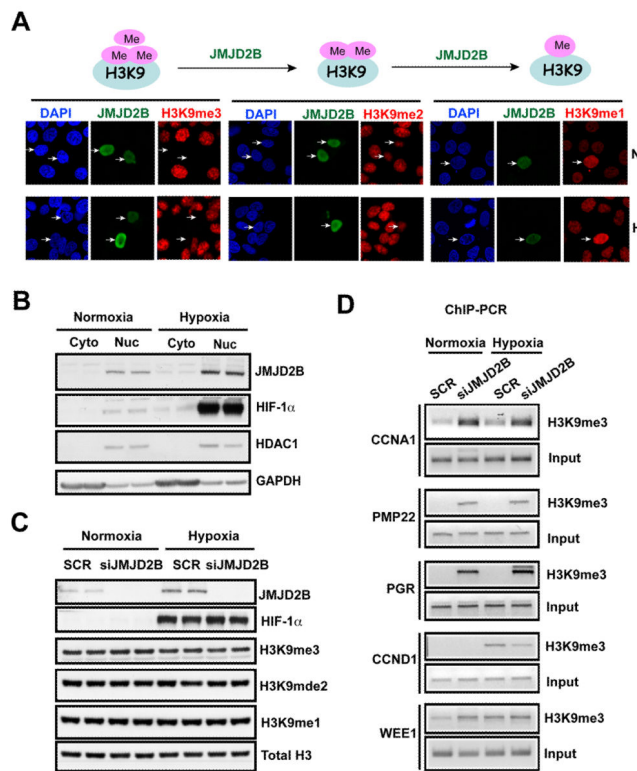
*B*, Colony formation assay was used to assess cell proliferation after depletion of JMJD2B in MCF7 cells (left) and was quantified (right).

*C*, Cell cycle profile analysis after JMJD2B depletion in normoxia and hypoxia.

*D*, Percentage analysis of DNA histogram data (*C*) were derived from mathematical analysis of (*C*) with software called Summit Version 4.3 by gating for single-cell population with FS/Pulse Width, and analyse with “counts vs area of laser”.

(\*\* $P < 0.01$ )





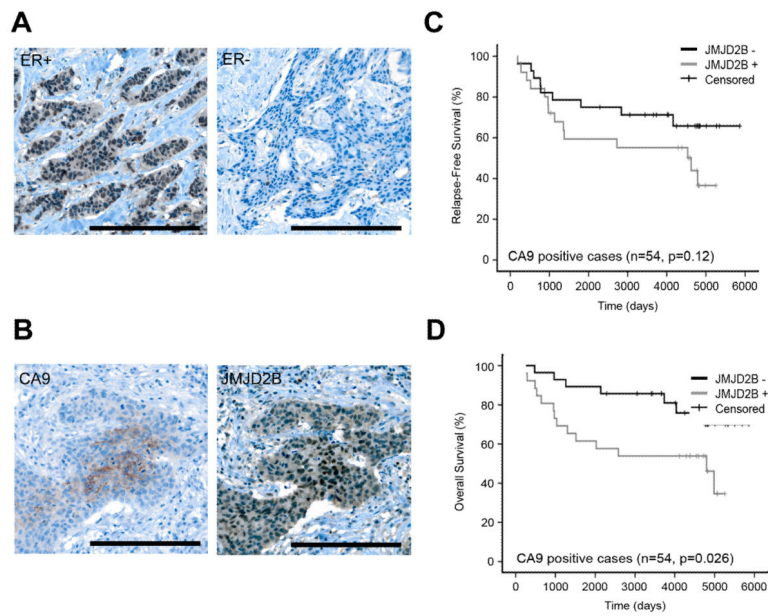
**Figure 5. JMJD2B is functional in hypoxia to exert its histone demethylase activity**

*A*, Immunofluorescence shows a global loss of H3K9me3 marks in normoxia (N) and hypoxia (H) after overexpression of HA-tagged JMJD2B in MCF7 cells.

*B*, Subcellular localization experiment demonstrates that JMJD2B is a nuclear protein in normoxia and hypoxia in MCF7 cells. (cyto =cytoplasm; Nuc = nucleus).

*C*, JMJD2B was depleted in MCF7 cells using siRNA knockdown. The indicated histone methyl marks were analyzed by immunoblot.

*D*, ChIP-PCR shows that JMJD2B regulates H3K9me3 mark on promoters of its target genes in normoxia and hypoxia in MCF7 cells.



**Figure 6. Clinical assessment of JMJD2B**

*A*, Immunohistochemistry shows JMJD2B expression in ER-positive and ER-negative breast cancer

*B*, Immunohistochemistry shows JMJD2B and CA9 expression in a serial sections

*C*, Kaplan-Meier analysis of relapse-free survival of CA9+ breast cancer with or without JMJD2B expression.

*D*, Kaplan-Meier analysis of overall survival of CA9+ breast cancer with or without JMJD2B expression.