

Regulation of the Histone Demethylase JMJD1A by Hypoxia-Inducible Factor 1 α Enhances Hypoxic Gene Expression and Tumor Growth^{∇†}

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The hypoxia-inducible transcription factors (HIFs) directly and indirectly mediate cellular adaptation to reduced oxygen tensions. Recent studies have shown that the histone demethylase genes *JMJD1A*, *JMJD2B*, and *JARID1B* are HIF targets, suggesting that HIFs indirectly influence gene expression at the level of histone methylation under hypoxia. In this study, we identify a subset of hypoxia-inducible genes that are dependent on *JMJD1A* in both renal cell and colon carcinoma cell lines. *JMJD1A* regulates the expression of adrenomedullin (ADM) and growth and differentiation factor 15 (GDF15) under hypoxia by decreasing promoter histone methylation. In addition, we demonstrate that loss of *JMJD1A* is sufficient to reduce tumor growth *in vivo*, demonstrating that histone demethylation plays a significant role in modulating growth within the tumor microenvironment. Thus, hypoxic regulation of *JMJD1A* acts as a signal amplifier to facilitate hypoxic gene expression, ultimately enhancing tumor growth.

Cellular hypoxia occurs when the demands of growth and metabolism of a tissue surpass the vascular oxygen supply. In response to hypoxia, cells undergo specific alterations in gene expression patterns geared to promote cell survival and maintain homeostasis. This response not only is important in normal development but also is a critical part in the progression of cancers (7). Hypoxia has been implicated in activating the metabolic shift to anaerobic glycolysis, promoting the epithelial-to-mesenchymal transition (EMT), inducing the secretion of proangiogenic factors, and remodeling the extracellular matrix. Although several transcription programs are activated in response to hypoxia, the hypoxia-inducible factors (HIFs) regulate a critical repertoire of genes, making them central regulators of the cellular response to hypoxia (10, 34).

The HIFs are heterodimeric transcription factors consisting of an oxygen-sensitive alpha subunit (HIF-1 α , HIF-2 α , or HIF-3 α) and a constitutively expressed HIF-1 β subunit (also known as the arylhydrocarbon nuclear translocator [ARNT]). Under conditions where oxygen concentration is not limiting, HIF- α subunits are hydroxylated by prolyl-hydroxylases, targeting them for ubiquitin-mediated degradation by the von Hippel-Lindau tumor suppressor (VHL) (18, 19). Under hypoxic conditions, HIF- α protein is stabilized, translocates to the nucleus, dimerizes with ARNT, and binds hypoxia-responsive elements (HREs) in the regulatory regions of target genes (51). HIF-1 α and HIF-2 α will bind the same sequences in cells but do not have completely overlapping abilities to regulate genes (5, 17,

44). Under certain conditions, HIF-3 α functions as a dominant negative, antagonizing the activity of HIF-1 and HIF-2 (32).

Several hundred genes are induced in response to hypoxia, and a great deal of research has been focused on identifying direct HIF target genes (34). The massive transcriptional reorganization mediated by hypoxia and HIFs suggests that changes in histone modification would create epigenetic reinforcement of this phenotype (20). HIF-1 α function has been shown to influence and be influenced by histone deacetylases (22, 33), yet comparatively little is known regarding HIF-dependent dynamics of histone methylation (8, 21). In a screen for HIF-regulated changes in gene expression, we and others have identified several Jumonji C-domain-containing histone demethylase (JHDM) promoters as direct binding targets of HIF-1 α and HIF-2 α (3, 43, 53, 55). Histone demethylases constitute a large and diverse family of enzymes, each with a specific ability to influence transcriptional activation or repression that is dependent on the specific histone residue targeted for demethylation (46); however, the specific roles of Jumonjis in modulating transcriptional responses to hypoxia remain unknown.

Using microarray analysis and chromatin immunoprecipitation, we identified HIF targets including the adrenomedullin gene (*ADM*) and the growth and differentiation factor 15 gene (*GDF15*) and show that *JMJD1A* reduces histone H3K9 methylation at these promoters during hypoxia. Furthermore, we demonstrate that loss of *JMJD1A* is sufficient to reduce tumor growth *in vivo*, consistent with its role in regulating histone methylation during hypoxia. These studies identify a transcriptional regulatory circuit where induction of *JMJD1A* by HIF-1 α acts as an epigenetic signal amplifier to enhance cellular responses to hypoxia.

MATERIALS AND METHODS

Cell lines and culture conditions. All cell lines were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum

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(FBS). Small interfering RNA (siRNA) transfections were carried out using Dharmacon Smart Pools with Dharmafect 1 transfection reagent according to manufacturer's protocol (Dharmacon, Lafayette, CO). Short hairpin RNA (shRNA) retroviral constructs against *JMJD1A* were purchased from Open Biosystems (Huntsville, AL). Retroviral constructs were packaged and introduced into cells as described previously (57). For hypoxia treatments, cells were plated at the desired density 12 h prior to placement in a hypoxia chamber (Invivo2-400; Ruskin Technologies, Leeds, United Kingdom) maintained at 0.5 to 2% oxygen for 0 to 20 h, depending on the experiment. For *in vitro* growth assays, cells (10^5) were plated in triplicate in 6-cm plates and counted every 3 days as described previously (52).

ChIP. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (26) with the following modifications. Hypoxia-treated cells were fixed within the chamber to avoid reoxygenation. For HIF-1 α and HIF-2 α immunoprecipitations, 120 μ g of sonicated chromatin (measured as protein) was incubated with 10 μ g of anti-HIF-1 α or HIF-2 α antibodies, respectively. For histone ChIPs, sonicated chromatin was immunoprecipitated according to guidelines provided by the manufacturer (Abcam). Data were analyzed in a fashion similar to that described by Johnson et al. (21). Relative enrichment was measured by quantitative real-time PCR using a titration of pooled input samples as a standard curve. Histone H3 methylation signals were normalized to input and bulk histone H3 signal, the corresponding IgG/H3 ratio was subtracted, and the fold change in methylation relative to the normoxic control cells was calculated. For calculation of fold difference of methylation (see Fig. 5D), the H3 normalized methylation signal with *JMJD1A* knockdown was divided by the corresponding signal from the nonsilencing control cells for each time point.

For high-throughput ChIP (ChIP-chip) analysis, immunoprecipitated DNA was amplified as described previously (37) and hybridized to NimbleGen tiled promoter arrays (Roche NimbleGen, Madison, WI) spanning approximately 23,000 promoter regions in the human genome. Promoter summary reports provided by NimbleGen were linked to expression information in Microsoft Access to correlate HIF binding to *RCC4* expression (see Table S1 in the supplemental material). Signal Map was used to confirm binding and to extract the coordinates of putative binding peaks for validation experiments. Antibodies used for ChIPs were anti-HIF-1 (BD Biosciences), anti-HIF-2 (Novus), histone H3 (Abcam), histone H3K9me1 (Abcam), histone H3K9me2 (Abcam), histone H3K9me3 (Abcam), and *JMJD1A* (Abcam). Rabbit IgG (Sigma) was used for a nonspecific IgG control. Primer sequences are available upon request.

Expression microarray analysis. All microarray sample preparations and hybridizations were carried out with Stanford Human Exonic Evidence Based Oligo (HEEBO) arrays according to protocols available from Pat Brown's lab (<http://cmgm.stanford.edu/pbrown/protocols/index.html>). HEEBO arrays contain probes for 31,000 unique genes and 8,500 alternate transcripts. Data from scanned microarrays were entered into the Stanford Microarray Database (SMD) for normalization and analysis.

For comparing expression in *RCC4* cells to that in *RCC4* cells with reconstituted VHL expression (*RCC4*+VHL cells), amino-allyl-labeled cDNA was reverse transcribed from 25 μ g of total RNA and coupled to Cy dyes as described at <http://cmgm.stanford.edu/pbrown/protocols/RTaminoAllylCoupling.html>. cDNA from *RCC4* or *RCC4*+VHL cells was labeled with Cy5 and hybridized to common reference cDNA from *RCC4*+VHL cells labeled with Cy3. Labeled cDNA was hybridized and washed as described at http://cmgm.stanford.edu/pbrown/protocols/Direct_Label_Protocol1.html. Expression changes were calculated by averaging data from duplicate *RCC4* samples and calculating fold change in expression compared to *RCC4*+VHL controls.

For profiling the effect of *JMJD1A* siRNA knockdown in *RCC4*+VHL cells, RNA was amplified from 1 μ g total RNA using the Amino Allyl MessageAmp II aRNA kit (Ambion) and labeled with Cy dyes as described at http://cmgm.stanford.edu/pbrown/protocols/Amplified_Protocol1.html. Samples were from independent triplicate experiments and were labeled with Cy5 and hybridized to common reference cDNA from untreated *RCC4*+VHL cells. Data were analyzed using multicomponent Significance analysis of microarrays (SAM) (50) to identify genes significantly changed among the siRNA transfections. Genes with a false-discovery rate lower than 0.5% were extracted to calculate fold changes relative to the normoxic siControl samples. Fold change was calculated to identify genes induced greater than 1.5-fold by hypoxia (SiCon-Hypox) and genes where hypoxic expression with si*JMJD1A* is at least 1.5 times lower than that with the equivalent hypoxic siControl (SiD1A-Hypox). These two groups were imported into GeneVenn (42) to identify regions of overlap (see Table S2 in the supplemental material).

QRT-PCR. Gene expression was measured using quantitative real-time PCR (QRT-PCR) exactly as described previously (26) using the 18S rRNA or TATA-

binding protein (TBP) gene as an internal control. Primer sequences are available upon request.

In vivo experiments. All animal experiments were performed in accordance with institutional and national guidelines and approved by Stanford University's Administrative Panel on Laboratory Animal Care (APLAC). Male SCID mice, 4 to 6 weeks old, were obtained from Charles River Laboratories. HCT116 cells stably expressing shRNA to *JMJD1A* or a nonsilencing control sequence were counted and resuspended in sterile phosphate-buffered saline (PBS). Three million cells were injected into the flank of the animal and measured with calipers at regular intervals.

RESULTS

Jumonji domain histone demethylase genes are direct HIF target genes. To identify direct and functionally relevant HIF target genes involved in chromatin regulation, we performed a directed screen by combining high-throughput chromatin immunoprecipitation (ChIP-chip) with gene expression analysis. The *RCC4* clear-cell renal cell carcinoma line was used as a model system because of abundant normoxic expression of HIF-1 α and HIF-2 α due to genetic inactivation of von Hippel-Lindau (VHL) (35). We probed tiled arrays representing 23,000 human promoter regions for HIF-1 α and HIF-2 α binding. Using a global average of promoter enrichment (provided as NimbleGen promoter summaries), we biased our search to identify promoter regions with the highest HIF enrichment. Using gene expression profiling, we identified genes induced greater than 1.5-fold in *RCC4* cells compared to *RCC4* cells with reconstituted VHL expression (*RCC4*+VHL cells). These genes were then sorted for HIF-1 α or HIF-2 α binding (Fig. 1A shows a schematic representation; see Table S1 in the supplemental material for complete tabulated results). A common phenomenon with genome-wide ChIP experiments is nonfunctional binding, where a transcription factor is shown to bind but fails to regulate nearby genes (29). Even though our initial filter comparing VHL-repressed expression to HIF binding would be expected to select functional HIF targets, VHL has also been shown to regulate several cellular processes independently of HIF (4, 49). Therefore, we followed up potential HIF targets only if they were also induced by hypoxia in *RCC4* cells expressing functional VHL (*RCC4*+VHL cells).

The screen identified several known HIF target genes, including those for vascular endothelial growth factor (*VEGF*), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*PFKFB3*), and stanniocalcin 2 (*STC2*), providing validity for our ChIP-chip analysis (12, 28, 36). HIF binding at these promoters was confirmed by ChIP-PCR (Fig. 1B). The Jumonji C-terminal-domain-containing histone demethylase (JHDM) gene *JMJD1A* was also identified as a putative HIF target. JHDMs have received a great deal of attention for their ability to demethylate histones and either activate or repress transcription (46). *JMJD1A* (also known as *JHDM2A* and *KDM3A*) has recently been shown to be a coactivator for nuclear hormone receptors and myogenic factors by demethylating dimethyl lysine 9 on histone H3 (H3K9me2) of target promoters (30, 31, 38, 56).

We then searched through our binding data and identified *JMJD2B* as another strong target of HIF binding. *JMJD2B* recognizes tri- and dimethylated lysine 9 (H3K9me3/2) on histone H3, reducing both modifications to the monomethylated state (11, 24, 54). Both HIF-1 α and HIF-2 α bind near the *JMJD1A* and *JMJD2B* promoters (Fig. 1B), confirming recent reports (3, 43, 53, 55).

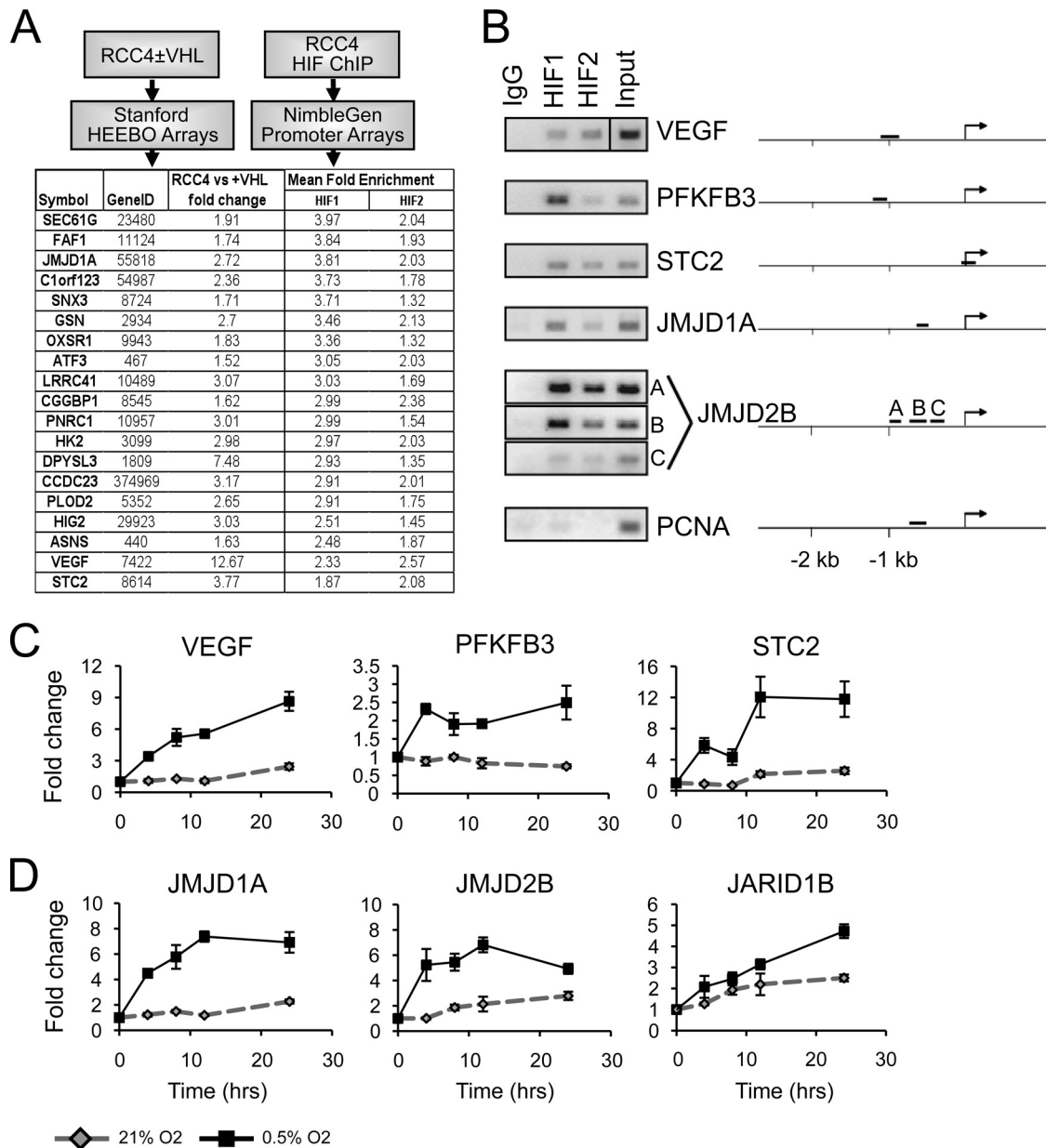


FIG. 1. Jumoni domain histone demethylases are hypoxia inducible in a HIF-dependent manner. (A) Experimental scheme of the HIF binding to expression screen. RCC4 renal cell carcinoma cells were processed for ChIP-chip as described in Materials and Methods. Promoters corresponding to genes induced greater than 1.5-fold with loss of VHL were sorted by mean HIF binding. An illustrative set of data is shown (the full data set is in Table S1 in the supplemental material). (B) Validation of HIF-1 α and HIF-2 α binding to selected promoters. *VEGF* serves as experimental validation control for both HIF-1 α and HIF-2 α . Multiple amplicons were used to screen promoters containing widely spaced HREs and are denoted A, B, or C as necessary. (C) QRT-PCR analysis of *VEGF*, *PFKFB3*, and *STC2* expression in RCC4+VHL cells exposed to either 21% or 0.5% O₂ for 0, 4, 8, 12, or 24 h. (D) QRT-PCR analysis for *JMJD1A*, *JMJD2B*, and *JARID1B* expression. Data for panel C and D are plotted as fold change normalized to TBP gene expression. Error bars represent standard deviations.

Specificity of our screen was validated by ChIP-PCR to the promoter of *PCNA*, a region with no visible HIF enrichment and no hypoxic regulation. We then retrospectively searched our expression data to identify additional Jumoni domain proteins that might be regulated by hypoxia. Expression of the histone H3 lysine 4 (H3K4me3) demethylase *JARID1B* was increased in RCC4+VHL cells during hypoxia (1.7-fold) (see Table S2 in the supplemental material). Consistent with our

finding, recent studies have also identified *JARID1B* as a hypoxia-inducible gene (16, 55).

Hypoxic transcriptional induction of the JHDMs was confirmed in RCC4+VHL cells. Expression of *JMJD1A*, *JMJD2B*, and *JARID1B* was induced and sustained for at least 24 h upon cellular exposure to hypoxia (Fig. 1D), similar to the case for *VEGFA*, *PFKFB3*, and *STC2* (Fig. 1C). Our approach combining high-throughput ChIP and gene expression analysis has

identified at least three different JHDMs as potential HIF targets, suggesting that HIF plays an important role in regulating widespread epigenetic phenomena through alterations in histone methylation.

Jumonji domain histone demethylases are hypoxia inducible in a HIF-dependent manner. To determine if hypoxic induction of histone demethylases is dependent on HIF-1 α , we next utilized wild-type and HIF-1 α -deficient mouse embryonic fibroblasts (MEFs). Previous studies have indicated that MEFs have nonfunctional HIF-2 α protein (40). Real-time PCR analysis demonstrated that the hypoxic induction of *JMJD1A*, *JMJD2B*, and *JARID1B* was significantly reduced in HIF-1 α -deficient MEFs compared to wild-type MEFs, demonstrating dependence on HIF-1 α for hypoxic induction (Fig. 2A). *JMJD1A* expression was also robustly induced in MCF-7 breast cancer and HCT116 colon cancer cells, as was that of *JMJD2B* and *VEGF* (Fig. 2B).

Transfection of siRNAs to HIF-1 α , HIF-2 α , and ARNT reduced the hypoxic induction of *JMJD1A* in RCC4+VHL cells in a statistically significant manner, indicating that in certain cell types, *JMJD1A* may be regulated by either HIF-1 α or HIF-2 α (Fig. 2C). In contrast siRNAs to HIF-1 α and ARNT, but not HIF-2 α , resulted in a statistically significant reduction of *JMJD1A* expression under hypoxia in MCF-7 cells. *VEGF* expression was disrupted by knockdown of HIF-2 α and ARNT, confirming functional knockdown of HIF-2 and previous reports that *VEGF* is primarily a target of HIF-2 α in renal cell carcinoma (17, 44). In MCF-7 cells, hypoxic expression of *VEGF* was reduced in a statistically significant manner by knockdown of HIF-1, HIF-2, and ARNT, demonstrating functional knockdown of HIF-1 α and HIF-2 α . These experiments demonstrate that the regulation of *JMJD1A* by HIF-1 α is a conserved phenomenon in both mice and humans, while HIF-2 α may regulate *JMJD1A* in certain cellular contexts. Identification of *JMJD1A* as a HIF-1 α target reveals an important mechanistic link between the cellular microenvironment, HIF activation, and epigenetic regulation of biological phenomena.

JMJD1A regulates hypoxic gene expression. Of the three JHDM genes identified in our experiments, *JMJD1A* seemed the most likely to have a direct effect on hypoxic gene induction. *JMJD1A* was one of the strongest HIF-1 α peaks identified by the ChIP-chip experiment (Fig. 1A), and its ability to function as a coactivator has been well characterized (56). Since many coactivators identified within specific transcription pathways were later found to enhance HIF-1 α activity (6), we hypothesized that *JMJD1A* might also mediate hypoxic gene expression.

In order to identify potential *JMJD1A* targets during hypoxia, RCC4+VHL cells were transfected with siRNA to *JMJD1A* or control siRNA and exposed to hypoxia (0.5% O₂) or normoxia (21% O₂), and gene expression was analyzed using Stanford HEEBO microarrays (see Materials and Methods). Knockdown of *JMJD1A* mRNA was approximately 3-fold under normoxia and hypoxia (Fig. 3A). We identified 821 genes induced >1.5-fold by hypoxia in cells transfected with control siRNA (Fig. 3B, left circle), a result similar to those in earlier reports (34). Of these genes, 53 were dependent on *JMJD1A* for full hypoxic induction (region of overlap in Fig. 3B). Included in this data set were a number of known HIF targets such as *ADM*, *EDN1*, *SERPINE1*, *PLAUR*, and

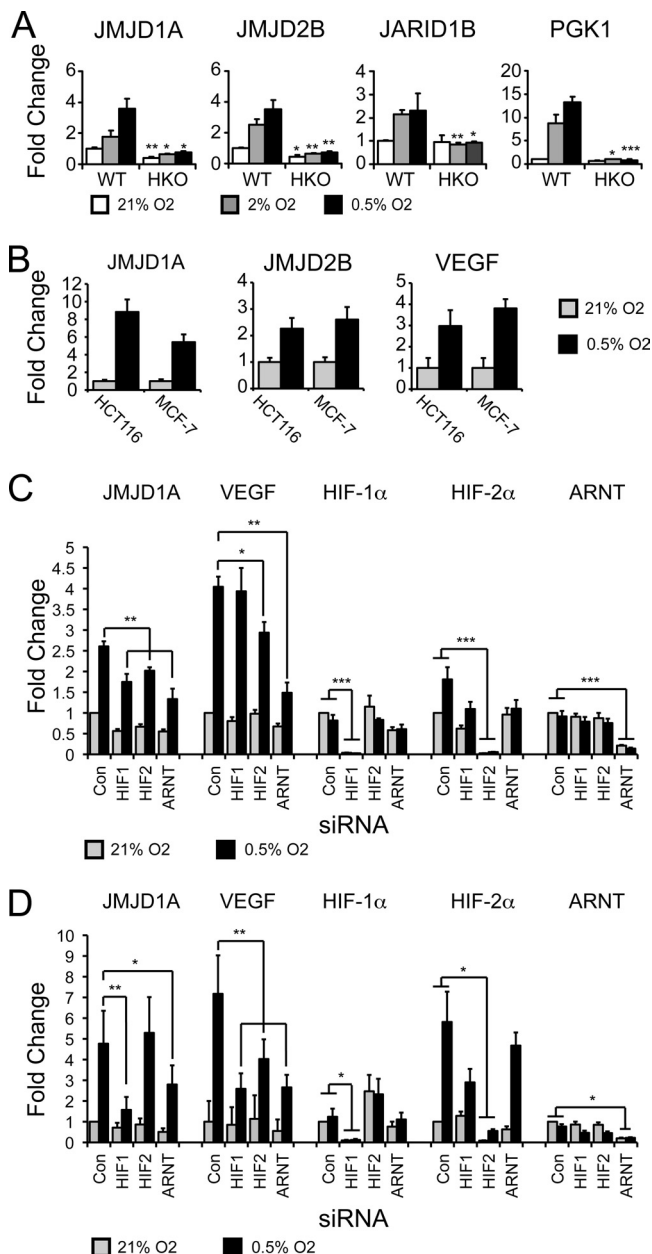


FIG. 2. Jumonji domain histone demethylases are hypoxia inducible in a HIF-dependent manner. (A) QRT-PCR measurement of murine *Jmjd1a*, *Jmjd2b*, and *Jarid1b* in wild-type and HIF-1 α knockout MEFs (HKO) exposed to 21%, 2%, and 0.5% O₂ for 20 h. *Pgk1* serves as a control for a HIF-1 α -specific gene. Statistical significance was calculated using Student's *t* test: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. Asterisks refer to comparisons within the respective oxygen concentrations. (B) *JMJD1A*, *JMJD2B*, and *VEGF* expression in HCT116 and MCF-7 cells exposed to 21% or 0.5% oxygen for 20 h. (C) QRT-PCR measurement of hypoxic expression of *JMJD1A* and *VEGF* in RCC4+VHL cells after transfection with siRNAs to HIF-1 α , HIF-2 α , and ARNT. Data represent the averages from six independent experiments. Statistical significance was calculated using Student's *t* test: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. (D) QRT-PCR measurement of hypoxic expression of *JMJD1A* and *VEGF* in MCF-7 cells transfected as for panel C. Data represent the averages from three independent experiments, measured in triplicate and normalized to 18S rRNA. Error bars indicate standard errors of the means. Statistical significance was calculated using Student's *t* test: *, *P* < 0.05; **, *P* < 0.01.

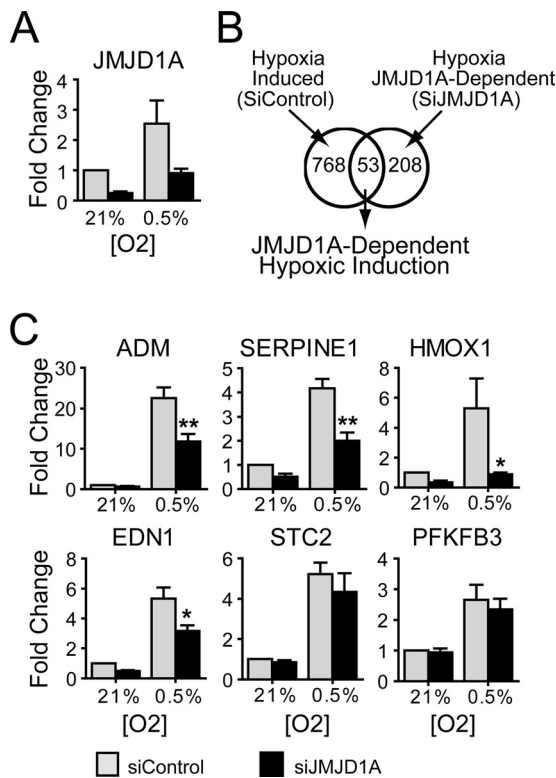


FIG. 3. *JMJD1A* regulates hypoxic induction of a subset of hypoxia-regulated genes in RCC4+VHL cells. (A) Quantitative RT-PCR of *JMJD1A* expression in RCC4+VHL cells transfected with siRNA to *JMJD1A* (siD1A) and exposed to 21% or 0.5% oxygen for 16 h. (B) Venn diagram graphically showing the intersection between genes induced greater than 1.5-fold by hypoxia in the siControl-transfected cells (left circle) and genes reduced at least 1.5-fold under hypoxia with siJMJD1A compared to the hypoxic siControl (right circle). Genes dependent on *JMJD1A* for hypoxic induction are depicted in the overlap. (C) RCC4+VHL cells were transfected as for panel A, and the expression of genes identified in panel B (*ADM*, *SERPINE1*, *EDN1*, and *HMOX1*) was measured by QRT-PCR. *STC2* and *PFKFB3* serve as examples of two *JMJD1A*-independent hypoxia inducible genes. Data are from six experiments measured in triplicate (\pm standard errors of the means) and normalized to the normoxic control siRNA (SiCon). Statistical significance was calculated using Student's *t* test: *, $P < 0.05$; **, $P < 0.01$.

HMOX1 (complete lists are in Table S2 in the supplemental material). We also identified 208 genes with decreased hypoxic expression with loss of *JMJD1A* but were not induced in the control cell line, implying that hypoxic induction of *JMJD1A* plays a broader role in maintaining gene expression during hypoxia (Fig. 3B, right circle; see Table S2 in the supplemental material).

Validation of *JMJD1A*-dependent expression was confirmed using QRT-PCR (Fig. 3C). *ADM*, *SERPINE1* (*PAII*), *EDN1*, and *HMOX1* had statistically significant dependence on *JMJD1A* for hypoxic induction. These genes were chosen for further analysis because they were known HIF targets and had known functions, making them more fruitful targets for later functional analysis. Though not statistically significant, *JMJD1A* increased hypoxic expression of *SERPINE8*, *EDN2*, and *GDF15* (data not shown), confirming a trend of regulation by *JMJD1A*. The majority of hypoxia-induced genes, including

STC2 and *PFKFB3*, showed little or no dependence on *JMJD1A* (Fig. 3C; see Table S2 in the supplemental material). The specific regulation of some but not all hypoxia-induced genes by *JMJD1A* suggests that multiple mechanisms regulate hypoxic gene expression, highlighting the importance of promoter-specific recruitment of transcription regulators.

Because of the clear link between VHL loss, HIF activation, and progression of renal cell carcinoma, cell lines like RCC4 are ideal systems for the study of HIF activity and hypoxic transcription phenomena. A significant disadvantage of using RCC-derived cells is the generally poor rate of tumor initiation in xenograft experiments, making it difficult to evaluate the functional aspects of gene expression. We found that *JMJD1A* is also robustly induced in HCT116 colon carcinoma cells (Fig. 2B), which grow well in tumor xenograft experiments, making them a better system for studying *in vivo* function of *JMJD1A*. In HCT116 cells transfected with siRNA to *JMJD1A*, there was a robust knockdown of *JMJD1A* RNA and protein (Fig. 4A and B). Accordingly, the hypoxic induction of *ADM* and *GDF15* was robustly and significantly dependent on hypoxic *JMJD1A* expression (Fig. 4C and D). For use in longer-term studies, we also established two HCT116-derived cell lines expressing shRNA to *JMJD1A*. Compared to a line expressing a nonsilencing control construct, *JMJD1A* mRNA was reduced approximately 2- to 3-fold in both cell lines in normoxia (Fig. 4E). While both shJMJD1A-1 and shJMJD1A-2 significantly suppressed hypoxic induction of *ADM* by approximately 25 to 30% compared to the nonsilencing control (Fig. 4E), cells expressing shJMJD1A-2 maintained effective knockdown more consistently over time, making it a more useful line for further experiments (data not shown). These results demonstrate that hypoxic induction of *JMJD1A* is crucial for optimal hypoxic expression of targets and not solely a result of reduced expression under normoxia.

Transcriptional regulation by *JMJD1A* during hypoxia is due to changes in histone methylation of target promoters. Since *JMJD1A* induces gene expression by specifically reducing the methylation of histone H3K9me2 and H3K9me1 to the unmethylated state *in vitro* and *in vivo* (56), reduction of *JMJD1A* expression would be expected to increase H3K9 dimethylation on target promoters such as *ADM* and *GDF15*. HCT116 cells expressing shJMJD1A-2 shRNA or a nonsilencing control construct were exposed to hypoxia for 4 or 16 h and fixed with formaldehyde for chromatin immunoprecipitation. Cells grown in normoxia were fixed at time zero as a control for hypoxic changes. Using chromatin immunoprecipitation followed by QPCR analysis, we measured the ratio of H3K9me1, H3K9me2, and H3K9me3 to bulk histone H3 on regions approximately 200 bp downstream of the transcription start sites of *ADM* and *GDF15*. A representative ChIP on the *ADM* promoter is depicted in Fig. 5A, demonstrating robust enrichment over the IgG control for all histone antibodies tested. After 16 h of hypoxia, H3K9me2 was approximately two times higher in the shJMJD1A-2 cells than in the nonsilencing control, proportional to the reduction of *JMJD1A* (Fig. 4C). This result was confirmed by calculating the fold change in histone methylation compared to the normoxic, nonsilencing control cells for four independent experiments and averaging the data at each time point (Fig. 5C). While there was no statistically significant difference in H3K9me1 or H3K9me3 for either

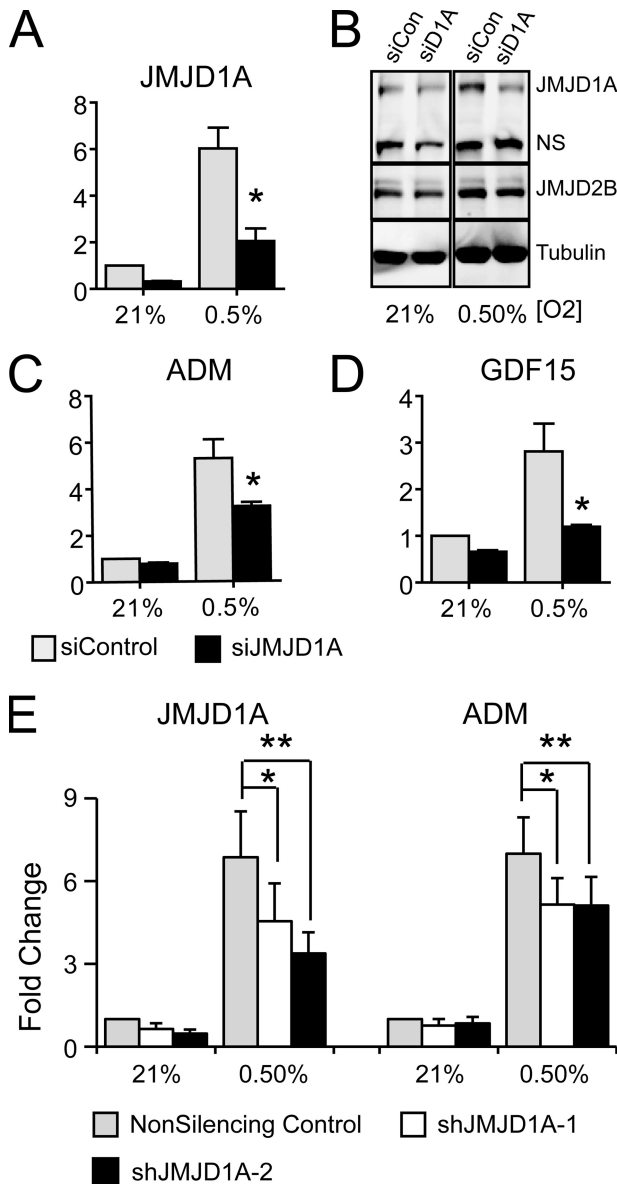


FIG. 4. JMJD1A regulates *ADM* and *GDF15* in HCT116 cells. (A) Quantitative RT-PCR of *JMJD1A* expression in HCT116 cells transfected with siRNA to *JMJD1A* (siD1A) and exposed to 21% or 0.5% oxygen for 16 h. Data are the averages from three experiments measured in triplicate (\pm standard errors of the means), normalized to the normoxic siControl-transfected cells. Statistical significance was calculated using Student's *t* test: *, $P < 0.05$. (B) Western blot analysis of whole-cell lysates harvested from HCT116 colon carcinoma cells transfected with siRNA to *JMJD1A* and exposed to normoxia (21% O₂) and hypoxia (0.5% O₂). Both a nonspecific band (NS) and tubulin serve as loading controls. (C and D) QRT-PCR of *ADM* (C) and *GDF15* (D) expression in HCT116 cells transfected as for panel A. Data are the averages from three experiments measured in triplicate (\pm standard errors of the means), normalized to the normoxic siControl-transfected cells. Statistical significance was calculated using Student's *t* test: *, $P < 0.05$. (E) HCT116 cells stably expressing two different shRNA constructs and a nonsilencing control were exposed to 0.5% or 21% oxygen for 16 h, and expression of *JMJD1A* and *ADM* was analyzed by QRT-PCR. Fold change was calculated relative to normoxic nonsilencing control cells. Data represent average fold changes for five independent replicates measured in triplicate \pm standard errors of the means. Expression normalized to statistical significance was calculated using Student's *t* test: *, $P < 0.05$; **, $P < 0.01$.

ADM or *GDF15*, H3K9me2 was increased in a statistically significant manner after 4 h and 16 h for *GDF15* ($P < 0.05$) and *ADM* ($P < 0.01$), respectively (Fig. 5C). On the *MLH1* promoter, a gene previously shown to be repressed by hypoxic activation of G9a and regulated independently of *JMJD1A* (reference 8 and data not shown), H3K9me2 increased with hypoxia in both the nonsilencing control and shJMJD1A-2 cells, but there was no significant difference between the two cell lines.

Because individual variation in cell density, hypoxic treatments, and ChIP procedure created some variability in the fold changes measured (see H3K9me2 for *GDF15* at 16 h), we also calculated the individual ratio of histone methylation between the two cell lines at each time point and averaged the data for the same four independent replicates (Fig. 5D). This analysis highlights the overall trends in JMJD1A-dependent methylation during hypoxia, confirming the results obtained by comparing fold change. With reduced JMJD1A expression, there is a clear relative increase in H3K9me2 in hypoxia for *ADM* and *GDF15*, but not for *MLH1*, while there are no statistically significant differences for H3K9me1. For H3K9me3, there appears to be a relative decrease in H3K9me3 for *ADM* with loss of *JMJD1A*, but it was not statistically significant because of high variability in the ratios at time zero.

H3K9 trimethylation showed little dependence on *JMJD1A* expression, but did show some differences between the different promoters. In the nonsilencing control cells, H3K9me3 was induced higher on *ADM* than on *GDF15* or *MLH1*, suggesting that H3K9 methylation on *ADM* is regulated by a methylase with terminal trimethylation activity such as ESET, while *GDF15* may be regulated by a methyltransferase that terminates with H3K9me2 such as G9a (Fig. 5B). *MLH1* has previously been shown to be regulated by G9a under hypoxia (8). Reduction of *JMJD1A* in the knockdown line also reduced JMJD1A binding on the *ADM* and *GDF15* promoters, but not *MLH1* (Fig. 5B), confirming that *ADM* and *GDF15* are direct targets of demethylation by *JMJD1A*.

JMJD1A enhances tumor growth *in vivo*. We have demonstrated *in vitro* that in both clear-cell renal cell carcinoma and colon carcinoma cell lines, *JMJD1A* regulates genes implicated in cancer cell growth, invasion, and survival (Fig. 3 and 4; see Table S2 in the supplemental material). Subcutaneous injection of nonsilencing control and shJMJD1A-2 HCT116 cells into the flanks of SCID mice resulted in a significantly reduced rate of tumor growth for the shJMJD1A line (Fig. 6A). Tumor xenografts are hypoxic (1), and the reduction in tumor growth resulting from reduced JMJD1A expression is consistent with the reduced hypoxic expression of protumorigenic factors such as *ADM* and *GDF15* *in vitro*. In contrast, culturing HCT116 cells in monolayers in 2% oxygen reduced the growth rate compared to normoxia (21% oxygen), but loss of *JMJD1A* expression had little to no effect (Fig. 6B), suggesting that JMJD1A influences tumor growth within the context of the tumor microenvironment. In HCT116 cells, *JMJD1A* is robustly induced during hypoxia in a HIF-1 α -dependent manner (Fig. 6C), consistent with a report that colorectal carcinomas have a hypoxic signature (13). *JMJD1A* expression is also significantly higher in renal cell carcinoma (14, 45), a cancer type associated with loss of the VHL tumor suppressor and constitutive activation of HIF and its transcriptional targets (Fig.

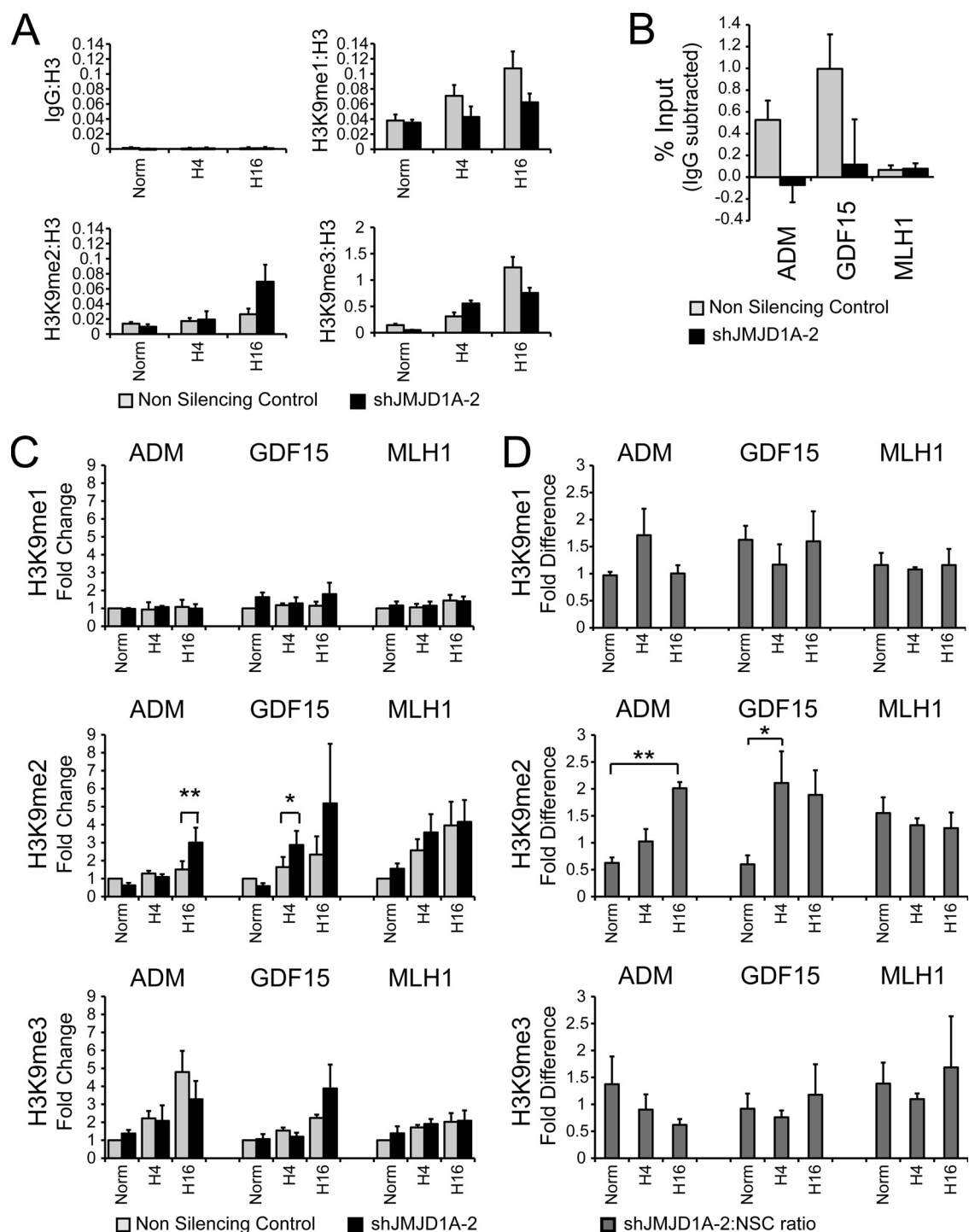


FIG. 5. *JMJD1A* binds and demethylates H3K9 on target promoters. HCT116 cells stably expressing shRNA to *JMJD1A* (shJMJD1A-2) or a nonsilencing control sequence were exposed to 0.5% oxygen for 0 (Norm), 4 (H4), and 16 (16H) hours; fixed in formaldehyde; and interrogated by chromatin immunoprecipitation with antibodies against histone H3K9me1, H3K9me2, and H3K9me3. (A) Representative ChIP of region spanning 250 bp downstream of the *ADM* transcription start site, demonstrating significant enrichment compared to the IgG control. Data are for a single experiment measured in triplicate and normalized to histone H3 as described in Materials and Methods. (B) Chromatin immunoprecipitated from HCT116 shJMJD1A-2 and nonsilencing control cells (black and gray bars, respectively) with anti-JMJD1A antibody and enrichment on target promoters measured by QPCR. Data represents the average enrichment for three independent experiments after subtraction of IgG signal. (C) Average fold change of H3K9me1, H3K9me2, and H3K9me3 downstream of the *ADM*, *GDF15*, and *MLH1* transcription start sites. Data represents the averages for four independent ChIP experiments measured in triplicate and normalized to the normoxic nonsilencing control \pm standard errors of the means. Statistical significance was calculated using Student's *t* test: *, $P < 0.05$; **, $P < 0.01$. (D). Average fold difference of H3K9 methylation for the experiments of panel C (calculation is described in Materials and Methods). Data represent averages from four independent experiments measured in triplicate \pm standard errors of the means. Statistical significance was calculated using Student's *t* test: *, $P < 0.05$; **, $P < 0.01$.

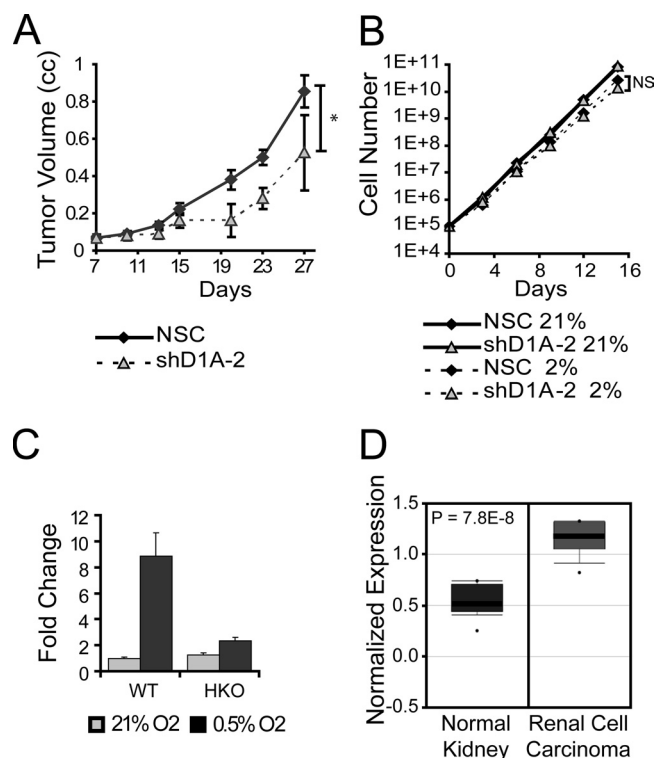


FIG. 6. *JMJD1A* promotes tumor growth. (A) Loss of *JMJD1A* reduces tumor growth rate. A total of 3×10^6 HCT116 cells expressing shRNA to *JMJD1A* (shD1A-2; $n = 3$) or a nonsilencing control (NSC; $n = 5$) were injected into the flanks of SCID mice and the volume measured at the indicated intervals. Data are the averages of tumor volumes \pm standard errors of the means. *, $P < 0.05$ as determined by nonlinear regression. (B) Loss of *JMJD1A* has no effect on *in vitro* growth rate. A total of 10^5 HCT116 cells expressing shRNA to *JMJD1A* or a nonsilencing control were grown as described in Materials and Methods in 21% oxygen or 0.5% oxygen. NS indicate a nonsignificant difference in growth rate as measured by nonlinear regression. (C) Hypoxic *JMJD1A* expression is abolished in HIF-1 α knockout (HKO) HCT116 cells exposed to 21% and 0.5% O₂ for 16 h. Expression is normalized to that in normoxic wild-type cells (WT). (D) Box-and-whisker plot of *JMJD1A* expression in renal cell carcinoma. The plot is a scalable vector graphic derived from a microarray analysis by Gumz et al. (14) downloaded from Oncomine (<http://www.oncomine.org/>) with text labels modified for clarity.

6D). *JMJD1A* therefore plays an important part in mediating the protumorigenic effects of the hypoxic microenvironment and HIF activation by demethylating the chromatin near key target promoters (Fig. 7).

DISCUSSION

When cells are exposed to hypoxic conditions, they undergo a fundamental shift in gene expression patterns that permits adaptation and survival (27). The hypoxia-inducible factors (HIFs) are key regulators of the hypoxic response, regulating transcription of genes involved in metabolism, angiogenesis, differentiation, invasion, and metastasis. In this report, we identify HIF-1 α -dependent expression of three known JHDM genes: *JMJD1A*, *JMJD2B*, and *JARID1B*. Recent studies have also shown that *JMJD1A*, *JMJD2B*, and *JARID1B* are direct HIF targets; however, the roles of JHDMs in the cellular

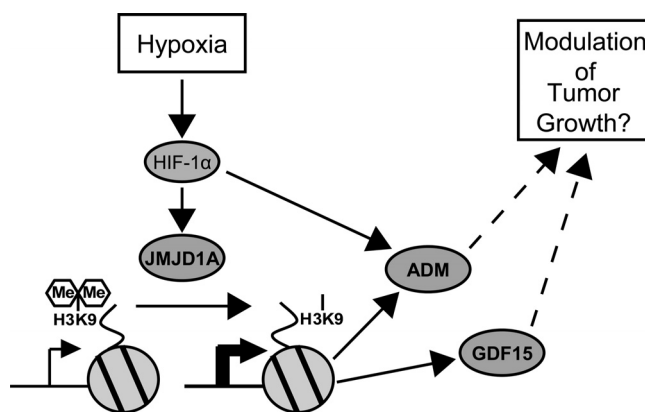


FIG. 7. Model for *JMJD1A* action during hypoxia. Cellular hypoxia stabilizes HIF-1 α , which induces several genes, including *JMJD1A*. Increased *JMJD1A* expression decreases H3K9 methylation on the promoters of target genes such as *ADM* and *GDF15*, enhancing hypoxic expression and possibly modulating tumor growth.

response to hypoxia have not been identified (3, 43, 53, 55). In this report, we demonstrate that *JMJD1A* regulates a subset of hypoxia-induced genes, including *ADM*, *EDN1*, *HMOX1*, and *GDF15*, and that *JMJD1A* is important for tumor growth in the hypoxic microenvironment of tumor xenografts.

All of the Jumonji domain histone demethylases (JHDMs) currently described utilize 2-oxoglutarate and molecular oxygen to hydroxylate and remove the methyl groups from lysine residues of modified histones (25, 39, 46). Consistent with the observed mechanism of JHDMs, hypoxia has been reported to increase bulk histone methylation, presumably through inhibition of JHDMs or increased methyltransferase activity (8, 21). However, in these same studies, reduced methylation is maintained on the promoters of hypoxia-inducible genes, implying that something is actively demethylating H3 at those loci or is blocking the activity of histone methyltransferases. In this report, we demonstrate that *JMJD1A* is important for increasing or maintaining the expression of specific genes under hypoxia by maintaining a lower level of H3K9 dimethylation. Reduced *JMJD1A* expression resulted in increased H3K9me2 directly downstream of the *ADM* and *GDF15* promoters during hypoxia, demonstrating that *JMJD1A* is enzymatically active in cells even under restrictive oxygen concentrations. Hypoxic induction of *JMJD1A* may increase the concentration of histone demethylase activity to promoters to compensate for reduced oxygen during hypoxia. shRNA-mediated knockdown of *JARID1B* resulted in global increase in H3K4me3 in HepG2 cells treated with hypoxia, providing another demonstration that JHDMs can function despite reduced oxygen concentrations (55).

Our results are consistent with the observation that exogenous overexpression of *JMJD1A* in hypoxia does not affect global H3K9 methylation (3). While the transcriptional mechanisms inhibited by H3K9 dimethylation during hypoxia remain to be elucidated, *JMJD1A*-dependent demethylation on specific promoters is very consistent with the more selective regulation by *JMJD1A* observed in other cell systems (31, 38, 48, 56). Our observation that hypoxic regulation of *JMJD1A* has specific effects on histone methylation and gene expression

provides a direct and specific mechanism for maintaining a derepressed pattern of histone methylation on target promoters during hypoxia. More comprehensive analysis of histone methylation phenomena during hypoxia will be important for determining the extent of hypoxic JMJD1A localization and activity. Additionally, identifying the functional overlap and interactions between the other hypoxia-regulated JHDMs will be crucial for understanding how global regulation of histone demethylation mediates hypoxic gene expression.

Many of the genes that we identified as *JMJD1A* targets were factors involved in vascular dilation (*ADM*) (23), iron metabolism (*GDF15*) (47), and angiogenesis (*ADM*, *EDN1*, *SERPINE1*, and *HMOX1*) (27). This is consistent with the finding that loss of *JMJD1A* had an effect on tumor xenograft growth but not *in vitro* cell growth. Regulation of *JMJD1A* by HIF-1 α may represent a feed-forward mechanism for minimizing the energy required to reactivate or maintain expression transcriptional targets after exposure to hypoxia. Additionally, hypoxia and *JMJD1A* have separately been shown to maintain stem cell populations (9, 31). While the effects of *JMJD1A* had relatively modest effects on tumor growth, hypoxic regulation of *JMJD1A* may help maintain the undifferentiated phenotype characteristic of cancer cells.

A key goal for future studies will be to understand the mechanism that targets *JMJD1A* to specific hypoxia-inducible targets and not others. *JMJD1A* has recently been shown to regulate expression of adipogenic genes by facilitating peroxisome proliferator-activated receptor gamma (PPAR γ) binding and transcriptional activity on the *Ucp1* promoter (48). *JMJD1A* also acts as a coactivator for the androgen receptors (56), presumably through an LXXLL motif common to many coactivators for nuclear hormone receptors (15). While the estrogen-related receptors (ERRs) have been shown to cooperate with HIF (2), ERR- α was shown to bind the promoters of glycolytic genes, which are not *JMJD1A* targets in our analysis (see Table S2 in the supplemental material). It remains to be determined what proportion of hypoxic expression depends on combinatorial action of nuclear hormone receptors and HIFs. Although the PAS domain of ARNT has recently been shown to interact with coactivators containing LXXLL motifs (41), the selective regulation of hypoxic targets by *JMJD1A* suggests that it is not generally recruited through HIFs or ARNT. A recent study has also demonstrated recruitment of *JMJD1A* to myocardin factors (30), suggesting that multiple regulatory pathways can recruit *JMJD1A* to target promoters. The specific recruitment of *JMJD1A* to target promoters during hypoxia therefore remains an open question and one of active study.

In conclusion, we have dissected a pathway linking oxygen sensing to induction of JHDM expression and alterations in chromatin modification during hypoxia (modeled in Fig. 7). In hypoxia, stabilized HIF-1 α induces expression of *JMJD1A*, reducing H3K9 dimethylation on specific hypoxia-responsive promoters, including *ADM*, *EDN1*, *HMOX1*, and *GDF15*, resulting in increased expression and subsequent adaptive responses favoring tumor cell growth. Since *JMJD1A* also regulates normoxic expression of target genes, hypoxic induction may feed forward to maintain expression of key hypoxia-regulated genes after an initial exposure to reduced oxygen. Hypoxic regulation of *JMJD1A* likely serves as a "signal amplifier"

to enhance and prolong the protumorigenic phenotype of cells exposed to a hypoxic microenvironment.

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