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Hypoxia upregulates the histone demethylase JMJD1A via HIF-1

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ABSTRACT

The histone demethylase Jumonji domain containing 1A (JMJD1A) demethylates H3K9 residues and thereby transactivates distinct target genes. Investigating the effect of hypoxia on JMJD1A expression, we found increased *JMJD1A* mRNA in different organs of rats exposed to normobaric hypoxia (8% O_2). Compared to adult samples, *JMJD1A* was increased in most tissues of human fetuses in whom oxygen supply is low compared to postnatal levels. Upregulation of JMJD1A mRNA and protein in cultured human cells exposed to hypoxia or iron scavengers *in vitro* was abrogated when hypoxia-inducible factor-1 (HIF-1) signaling was blocked by siRNAs. A single pivotal hypoxia responsive element (HRE) in the promoter of the human *JMJD1A* gene was identified that mediates *JMJD1A* upregulation by hypoxia, iron scavengers, and HIF-1. These findings demonstrate that JMJD1A can be stimulated by hypoxia both *in vitro* and *in vivo* involving binding of HIF-1 to a specific HRE in the *JMJD1A* promoter.

Hypoxia affects cellular maintenance on various biological levels including modulations of chromatin structures. While a variety of prolyl and asparaginyl hydroxylases work as cellular oxygen sensors, the heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1) acts as an effector by transactivating a panoply of genes including those encoding erythropoetin, vascular endothelial growth factor A (VEGFA) and glucose transporter-1 [1]. The HIF-1 heterodimer is composed of a constitutively expressed β-subunit and an oxygen-responsive α-subunit, both are members of the basic helix–loop–helix PAS protein family [1].

In an attempt to identify hypoxia-inducible genes, we had performed gene expression analyses comparing the global genomic response to reduced oxygen tension in a HIF-1-deficient and a HIF-1-competent lymphoblastic cell line [2]. In the HIF-1-competent cell line we found a new candidate gene, JMJD1A, which was upregulated by hypoxia alongside several known HIF-1 target genes. JMJD1A (jumonji domain containing 1A also known as jumonji C domain containing histone demethylase 2A, JHDM2A) is a member of the fast growing group of histone demethylases that belongs with the prolyl and asparaginyl hydroxylases to the Fe(II)- and 2-oxoglutarate-dependent family of dioxygenases [1,3]. JMJD1A specifically and reversibly demethylates H3K9 residues and thereby transactivates distinct target genes, notably in testis [4,5] and in embryonic stem cells, contributing to perpetuation of pluripotency [6].

In the experiments described here, we prove that JMJD1A is upregulated in response to low oxygen both *in vitro* and *in vivo*, show that this involves the HIF-1 transcription factor, and identify the *JMJD1A* promoter region involved in the interaction with HIF-1.

Materials and methods

Cell culture, hypoxia, and tissue panels. Human embryonic kidney cells (HEK-293, DSMZ, Braunschweig, Germany) and human microvascular endothelial cells (HMEC-1) were obtained and cultured as described previously [2,7]. Normoxia was defined as 20% O_2 and 5% CO_2, in hypoxia experiments, O_2 was tightly regulated at 0.5% as described previously [2]. In some experiments, HIF-1α of cells kept in normoxia was targeted by incubation with the transition metal chelator 2,2α-dipyridyl (DIP), the iron chelator desferoxamine (DFO) or cobalt chloride ($CoCl_2$) all from Sigma–Aldrich (Buchs, Switzerland) and used at a final concentration of 100 μM.

The human Multiple Tissue cDNA (MTC) panels, human I, human II, and human fetal include altogether 24 tissue cDNA samples and were purchased from Clontech (Saint-Germain-en-Laye, France). Each tissue sample of the human I and II MTC panels is pooled from several healthy Caucasians while the fetal MTC panels

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are pooled from several spontaneously aborted Caucasian fetuses with a gestational age of 16–37 weeks.

Various tissues including brain, heart, liver, and kidney from male Sprague–Dawley rats, weighing 200–280 g, which have been exposed to either normobaric hypoxia (8% O_2/92% N_2) for 6 or 12 h or normoxia (n = 2–3 for each time point and experimental condition) as described previously [8] were kindly provided by Dr. W.M. Bernhardt (Department of Nephrology and Hypertension, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany). Upon isolation, the tissues were frozen in liquid nitrogen and stored at −80 °C until analyzed.

Plasmids DNA. Preparation of human genomic DNA and PCR were performed as described previously [2]. For amplification of different human *JMJD1A* promoter fragments, one common reverse (R) and different forward (F) primers were used. The distance of each primers first 5′ nucleotide relative to the transcription initiation site serves as primer identification. After indication of each F-primer, the length of the resulting promoter (p) fragment is given in brackets: R+149 5′-GCTCTTGAACCAAGTCAGCG, F−923 5′-TTCCA CGCTTGTAAAATGGG (p1072), F−555 5′-TTTATCCTTCAAAATGGC GG (p704), F−484 5′-ATTTCGCCCAGCAGCCTAA (p633), F−444 5′-CGAGCCTCCAGAGCACTATC (p593), F−350 5′-CCTCACCCTTTCC TGTGAGA (p499). Mutation of the putative HIF-1 consensus binding site (hypoxia response element, HRE) within the promoter fragment p593 (CACG, in negative orientation) was achieved by use of a 3′ extended F−444 primer containing the mutated HRE (underlined), F−444-mut 5′-CGAGCCTCCAGAGCACTATCGCTCCT CCCTCTAGCTGAGTACGCCTGAAACCGATGCTTCTCTTCCCT. For construction of the different reporter constructs, extension of the forward primers with the sequence of the KpnI restriction enzyme and the reverse primer with that of NheI enabled direct ligation of the PCR products into the KpnI/NheI sites of the pGL2basic plasmid (Invitrogen, Basel, Switzerland). Sequences were verified by sequencing of both strands.

Cell transfections and reporter gene assays. Transfection experiments were done in HEK-293 cells and transfectants were lysed after 24 h with subsequent determination of luciferase and β-galactosidase activities as published previously [9]. Values are given as relative light units normalized to β-galactosidase activities for internal control of transfection efficiencies. Results shown are averages of at least three transfection experiments, each performed in triplicates.

Stable repression of HIF-1α by siRNA. HMEC-1 cells with stable downregulation of HIF-1α propagated of stable transfectants by use of the psiRNA-hH1neo G2 vector containing a short hairpin (si) RNA directed against HIF-1α were generated previously and cultured as described [7]. The control cell line was transfected with empty psiRNA-hH1 neoscr plasmid.

Reverse transcription (RT)-PCR. RNA preparation and RT were performed as described previously [2]. For quantitation, cDNA was amplified by use of SYBR GreenER qPCR SuperMix Universal (Invitrogen) and real-time PCR was carried out on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) as previously described [2].

For normalization of *JMJD1A* expression in the MTC panels we applied two different reference genes (*myosin phosphatase-Rho interacting protein*, *M-RIP*, and *polymerase (RNA) II (DNA directed) polypeptide F, POLR2F*) which were recently shown in a comprehensive gene expression analyses to be well suited for normalization within different tissues including fetal and adult tissues [10]. For normalization of *JMJD1A* and *VEGFA* expression in human cell culture experiments and in rat tissues oligonucleotides against *β2-microglobulin* (*B2M*) and ribosomal highly basic 23 kDa protein (RPL13A), respectively, were used as previously published [2].

The following forward (F) and reverse (R) oligonucleotides were used:

JMJD1A human	F 5′-GAGCTGTTTCCCACACCGA, R 5′-TGCTCTCCTTAGAAGGCTGTAGAC
JMJD1A rat	F 5′-GAGCTGTTTCTCACACTGA, R 5′-TGCTCTTCTTAGAAGGCTGTAGA
VEGFA human	F 5′-CCCTGATGAGATCGAGTACATCTT, R 5′-CCCTGATGAGATCGAGTACATCTT
VEGFA rat	F 5′-TTACTGCTGTACCTCCACC, R 5′-ACAGGACGGCTTGAAGATA
β2-microglobulin (*B2M*) human	F 5′-GATGAGTATGCCTGCCGTGTG, R 5′-TCCAATCCAAATGCGGCATCT
ribosomal highly basic 23 kDa protein (RPL13A)rat	F 5′-GCGGATGAACACCAACCC, R 5′-GTAGGCTTCAGCCGCACAAC
M-RIP human	F 5′-ATCTCAGCCATCGAAGCCAT, R 5′-TGGCTCTTCTCCAGCTCCC
POLR2F human	F 5′-CCCGAAAGATCCCCATCAT, R 5′-CACCCCCCAGTCTTCATAGC

Protein extraction and Western blotting. Proteins were extracted using the TransFactor Whole Cell Extraction Kit as recommended (Clontech), were separated by SDS–PAGE and transferred to PVDF membrane. The membrane was probed with either polyclonal anti-Jmjd1a (ab32440; Abcam, Cambridge, UK) or monoclonal anti-HIF-1α antibody (Transduction Laboratories, Lexington, KY) [2]. Anti-β-actin (Sigma–Aldrich) was used as loading control. Bound antibodies were detected as described previously [2].

Statistical analysis. Data are presented as means ± SD and analyzed using unpaired Student's t-test. p Values <0.05 were considered statistically significant.

Results

Expression pattern of JMJD1A within fetal and adult tissues

JMJD1A was described for the first time when a testis cDNA library was assessed [11], and *JMJD1A* was subsequently named *Testis-specific gene A* (*TSGA*) as *TSGA* was detected only in male germ cells [11]. Our survey of various tissues corroborate high expression in testis, irrespective whether the *JMJD1A* expression was normalized to *POLR2F* (see Fig. 1) or to *M-RIP* used as a second reference gene. Our survey, however, revealed high *JMJD1A* mRNA levels also in leukocytes and fetal thymus, comparable to those found in testis.

We included in our survey eight samples from pooled tissues of fetal origin because during embryogenesis the development of the fetus occurs in a relative hypoxic surrounding. Arterial pO_2 in the fetus is about 30 mmHg, which corresponds to about one third of levels in healthy adult humans. Within the eight pairs of corresponding fetal and adult tissues, *JMJD1A* expression was found to be significantly higher in five fetal tissues compared to adults, in two there was no significant difference, and only in lung tissue *JMJD1A* expression was found to be significantly lower in the fetal counterpart (see Fig. 1). Normalization against *M-RIP* revealed the same results with almost identical empirical level of significance (data not shown).

Hypoxia increases expression of JMJD1A in vivo

Hypoxic exposure for 6 h of rats increased significantly *JMJD1A* mRNA levels in brain, heart, lung, and kidney on average 1.8–7.7-fold (Table 1). The smallest increase in response to hypoxia (8% O_2), compared with rats maintained in normoxia (20% O_2), was seen in kidneys. When hypoxia was extended to

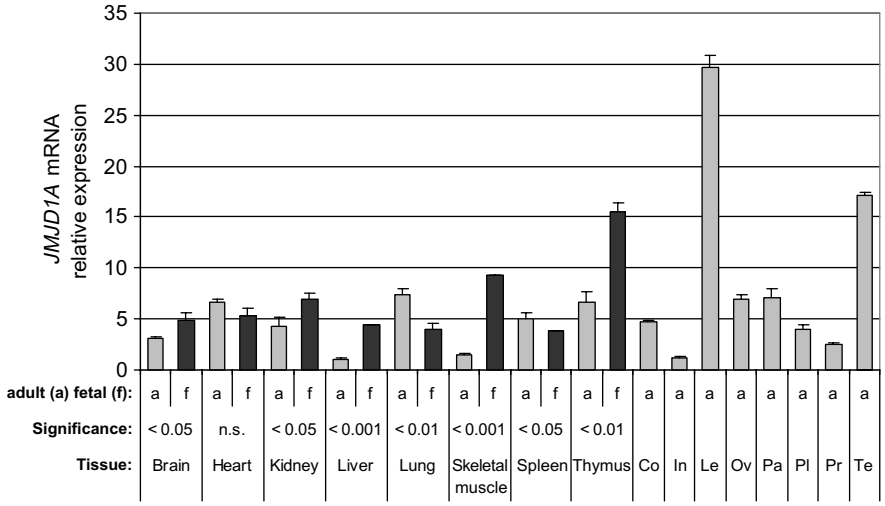

Fig. 1. Expression of *JMJD1A* mRNA in various fetal (f) and adult (a) tissues. Tissue designations: Co, colon; In, intestine; Le, leukocytes; Ov, ovary; Pa, pancreas; Pl, placenta; Pr, prostate; Te, testis. *JMJD1A* mRNA levels were normalized to mRNA levels of *polymerase (RNA) II (DNA directed) polypeptide F, POLR2F*, and are expressed as percentage to *POLR2F* mRNA levels. Values represent means ± SD of quadruplicates. *p*-Values of corresponding fetal vs. adult samples are given.

Table 1
JMJD1A and *VEGFA* mRNA expression *in vivo* after exposure of rats to hypoxia

	Hypoxia [h]	*JMJD1A*			*VEGFA*		
		Mean	Fold	*p*	Mean	Fold	*p*
mRNA expression of target gene relative to reference gene							
Brain	0	16.5	—	—	4.3	—	—
	6	38.3	2.3	0.00006	31.0	7.3	0.0007
	12	17.8	1.1	0.7	15.3	3.6	0.005
Heart	0	7.2	—	—	15.6	—	—
	6	23.6	3.3	0.01	56.5	3.6	0.04
	12	25.4	3.5	0.08	22.8	1.5	0.1
Kidney	0	11.0	—	—	11.7	—	—
	6	19.5	1.8	0.11	24.4	2.1	0.02
	12	9.6	0.9	0.5	7.0	0.6	0.1
Liver	0	6.3	—	—	3.5	—	—
	6	49.0	7.7	0.03	43.7	12.4	0.006
	12	26.5	4.2	0.02	19.9	5.7	0.004

This table shows mRNA expression of *JMJD1A* and *VEGFA* in homogenized tissues including brain, heart, kidney, and liver of rats, which have been exposed to either normobaric hypoxia (8% O_2/92% N_2) for 6 or 12 h or normoxia (0 h hypoxia), $n = 2–3$ for each time point and experimental condition. Results are given as a ratio of copies of target gene (*JMJD1A* or *VEGFA*) to copies of the reference gene (*RPL13A*). Fold change of mRNA expression in the different experiment samples compared with the corresponding normoxic samples are given and *p* values were analyzed using unpaired Student's *t*-test.

12 h, elevated *JMJD1A* mRNA expression persisted in the heart, partially decreased in the liver, and reverted to normoxic levels in brain and kidney. *VEGFA*, a known HIF-1 target gene serving as control, depicted similar expression characteristics (Table 1).

Hypoxia stimulates JMJD1A mRNA and protein expression in cultured cells and downregulation of HIF-1α by siRNA ameliorates this effect

Incubating HEK-293 for 6 h at 0.5% O_2 increased the number of *JMJD1A* transcript copies approximately 2.5-fold compared with cells that were grown at 20% O_2 (Fig. 2A). Hypoxic induction of JMJD1A mRNA and protein could be mimicked by treatment of HEK-293 cells with $CoCl_2$, DIP, and DFO (100 μM each) (Fig. 2B). Similar findings were made with HMEC-1, which also expressed *JMJD1A* at low ambient oxygen (Fig. 2C). This increase occurred within the same time course of the known HIF-1 target gene *VEGFA*, serving as positive control (Fig. 2C). In HMEC-1 cells with stable downregulation of HIF-1α by use of siRNA expression vectors, the hypoxia-induced upregulation of both, *JMJD1A* and *VEGFA* was significantly impaired, further indicating the implication of HIF-1α in the oxygen-dependent regulation of *JMJD1A* expression (Fig. 2C).

Hypoxic induction of the JMJD1A promoter is dependent on a HIF-1 consensus binding site

Eight putative HIF-1 consensus binding sites, also known as hypoxia response element (HRE) were identified in the proximal promoter (approximately first 1000 bp) of the human *JMJD1A* gene either in forward (F, CGTG) or reverse (R, CACG) orientation: 1, R−920; 2, R−569; 3, R−522; 4, R−510; 5, F−508; 6, R−446; 7, R−391; and 8, F+116, the position is given relative to the transcription initiation site (Fig. 3A). Transient transfections employing a series of *JMJD1A* promoter deletion constructs containing progressively reduced numbers of these putative HREs with a heterologous reporter were carried out as depicted in Fig. 3A. Normalized luciferase activities were enhanced up to 7-fold in cells transfected with all promoter constructs including HRE7 and treated with DIP to mimic hypoxic induction (Fig. 3A). Neither the promoter construct p499 containing only HRE8 nor the "empty" pGL2basic plasmid depicted enhanced luciferase activity when transfected cells were exposed to DIP or hypoxia (Fig. 3B). Thus, HRE7 at position-391 appears as the most likely candidate of binding HIF-1. Therefore site directed mutagenesis was used to destroy this potential HIF-binding site within the *JMJD1A* promoter construct p593. As shown in Fig. 3B, deletion of HRE7 abrogated completely the sensitivity of the *JMJD1A* promoter to hypoxia and DIP.

Overexpression of exogenous HIF-1α directly increases JMJD1A promoter activity

To investigate whether HIF-1α is capable of transactivating reporter activity directly through HRE7, experiments with transient cotransfection of different *JMJD1A* promoter constructs and a HIF-1α expression construct were performed under normoxic conditions. As illustrated in Fig. 3B, overexpression of exogenous HIF-1α resulted in significant induction of *JMJD1A* promoter activity. This HIF-1α-mediated induction was totally blunted when HRE7 was destroyed by site directed mutagenesis or the shorter construct

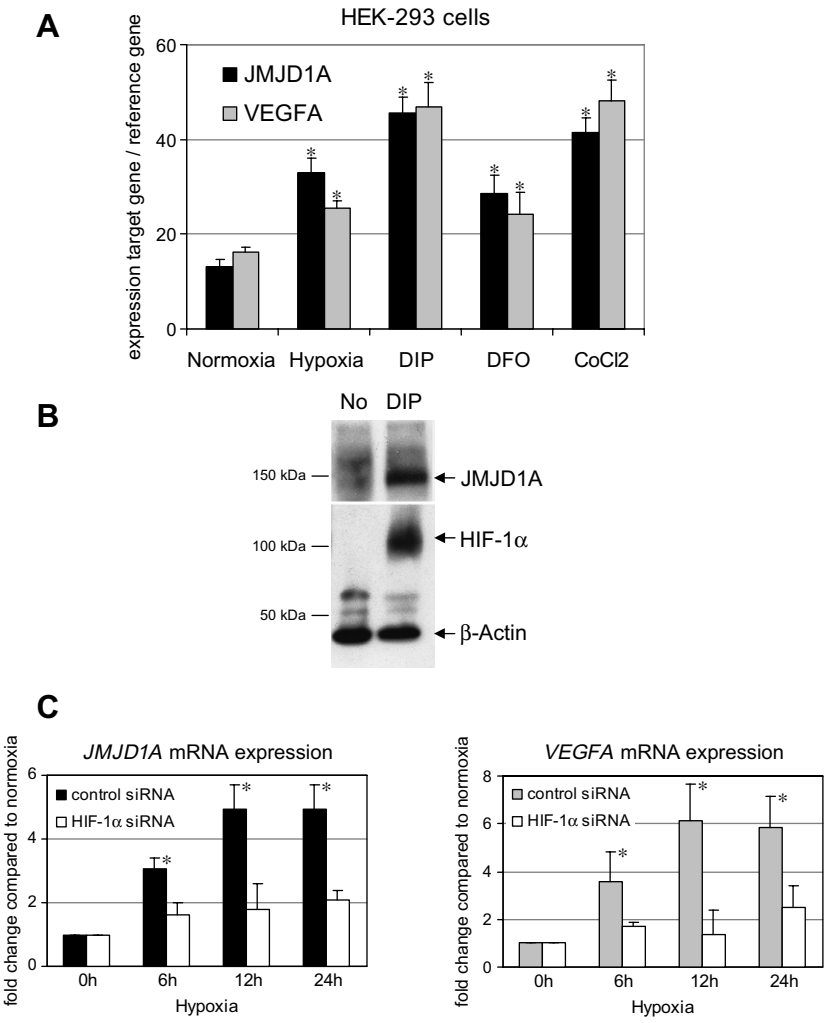

Fig. 2. JMJD1A mRNA and protein expression is induced by hypoxia as well as iron scavengers in HEK-293 (A, B) and HMEC-1 cells (C), and is abrogated when HIF-1α-signaling was blocked by specific siRNA (C). *JMJD1A* and *VEGFA* mRNA levels were normalized to *B2M* mRNA levels and are expressed as relative amounts (A), or fold change of normoxia (C), respectively. *VEGFA* is a known HIF-1 target gene [1] and served as control. Values represent means ± SD of $n = 3$ different experiments. $^{*}p < 0.05$ was considered statistically significant vs. controls at 20% O_2. (A) HEK-293 cells were exposed for 6 h to either normoxia, hypoxia (0.5% oxygen), 2,2α-dipyridyl (DIP), desferoxamine (DFO), or cobalt chloride ($CoCl_2$). (B) HEK-293 cells were exposed for 18 h to either normoxia (No) or DIP and immunoblotting was done for JMJD1A, HIF-1α, and β-actin. (C) HMEC-1 cells were stably transfected with a plasmid expressing HIF-1α siRNA (open squares) or a plasmid containing a control siRNA (filled squares). Cells were exposed to hypoxia (0.5% oxygen) for various hours as indicated.

without HRE7 was used (Fig. 3B). These results support the notion that HIF-1α directly transactivates *JMJD1A* promoter by interacting with a single HRE at position-391.

Discussion

In the experiments described here, we demonstrate that JMJD1A is regulated in an oxygen-dependent manner *in vitro* and *in vivo*. Stimulation of JMJD1A transcription and translation by low oxygen tension apparently involves HIF-1, based on the following three observations: First, JMJD1A upregulation in response to hypoxia is attenuated when HIF-1 signaling is blocked by siRNA targeting HIF-1α. Second, the promoter of the *JMJD1A* gene confers sensitivity to hypoxia and iron chelators that mimic hypoxia by blocking the prolyl hydroxylase that hydroxylates HIF-1α at proline residues 402 and 564 under normoxic conditions [1]. Third, deletion of a hypoxia responsive consensus element (HRE) from the proximal *JMJD1A* promoter abrogates induction by HIF-1α.

Our screening of *JMJD1A* expression in a fetal and adult tissue panel corroborated the known predominant expression of *JMJD1A* in testis [4,11,12]. In addition, leukocytes and to a lesser extend fetal thymus depicted also high *JMJD1A* mRNA levels irrespective of the reference genes used. Therefore, JMJD1A may play a role in the ontogeny of white blood cells. Few years ago, we stumbled upon JMJD1A when we analyzed two related lymphoblastic cell line for hypoxia sensitive gene expression [2]. Similar experiments were done by other groups to identify the response of the transcriptome to hypoxia. Indeed, *JMJD1A* occurs as a putative hypoxic response gene in various published gene lists based on the use of various cell types supporting our findings [13–18]. In this study we present for the first time experimental evidence that *JMJD1A* expression can be stimulated by hypoxia both *in vitro* and *in vivo*, involving binding of HIF-1 to a specific HRE in the *JMJD1A* promoter.

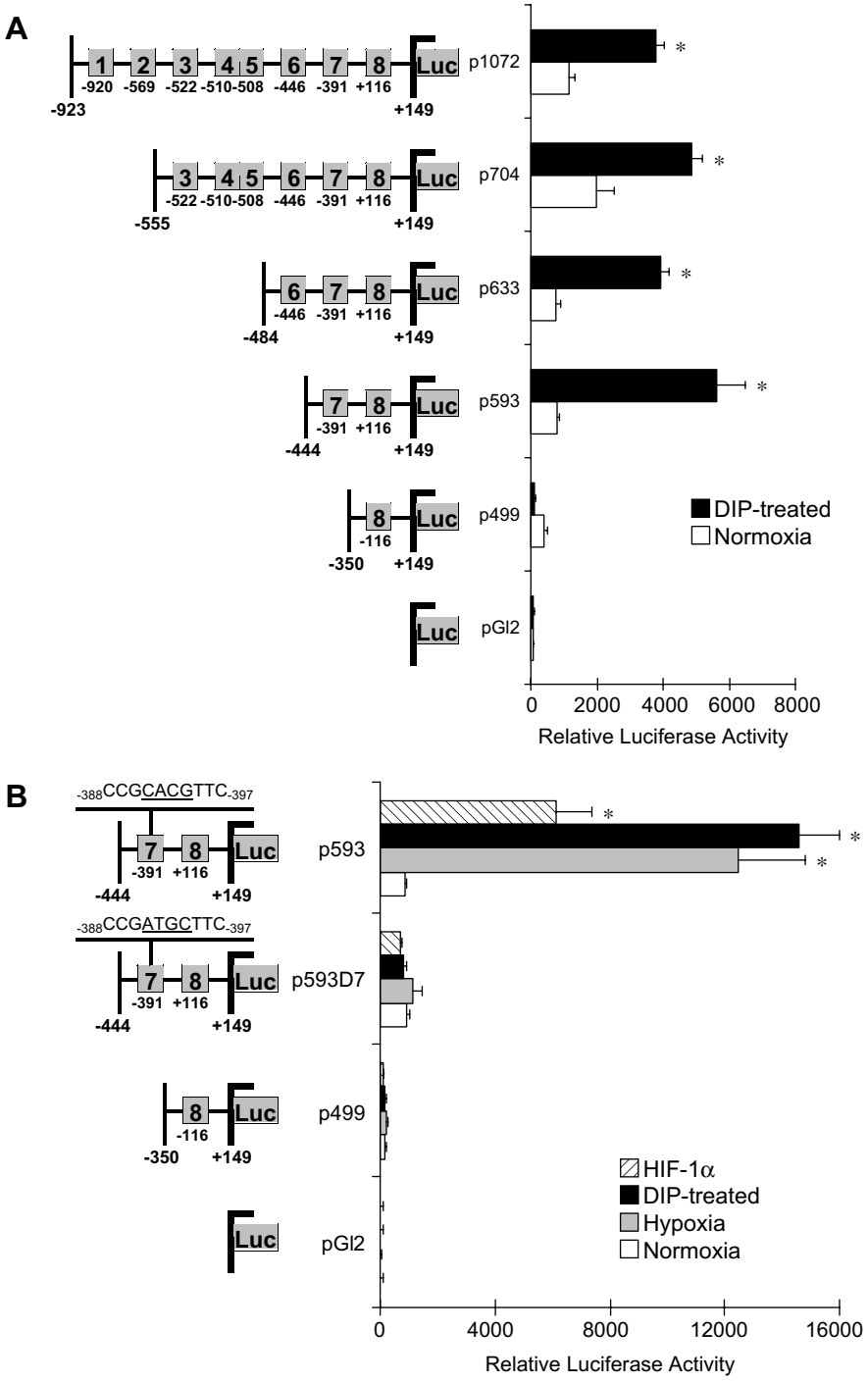

Fig. 3. The promoter of the JMJD1A gene mediates hypoxic induction of a luciferase reporter. Map showing all cloned *JMJD1A* constructs, identified by annotation of each sequence length in bp with full length *JMJD1A* construct (p1072) or indicated 5′ truncations, p704, p633, p593, p499, respectively. Relative positions of each clone to the transcription start site are annotated. Putative HIF-1 consensus binding sites are numbered and are also shown relative to the transcription start site. HMEC-1 monolayers were transfected with plasmids expressing various *JMJD1A* sequences as well as "empty" pGL2basic vector serving as control. Luciferase activities were normalized to β-galactosidase in each sample. (A) Transfected cells harboring full length JMJD1A construct or 5′ truncated constructs were treated with 2,2α-dipyridyl (DIP) or vehicle alone (normoxia) for 18 h as indicated. (B) Site-directed mutagenesis of HRE7 resulted in a loss of sensitivity of the *JMJD1A* promoter to DIP, hypoxia (0.5% O_2) and HIF-1α overexpression. Data are means ± SD from three separate experiments (*significant difference from normoxia, $p < 0.01$).

To date, at least seven different histone demethylases including JMJD1A have been described that demethylate the K9 lysine residue within the N-terminal tail of histone H3 [3]. JMJD1A is specific for demethylation of mono- or dimethylated H3K9 [4,5] and in embryonic stem (ES) cells, JMJD1A is involved in maintaining pluripotency by preventing the promoter regions of distinct pluripotency-associated genes from H3K9 dimethylation [6]. Moreover, JMJD1A is thought to play a key role in maintenance of ES cell self-renewal as JMJD1A is a direct target of Oct4 signaling [6].

Hypoxia is considered as a prerequisite for maintaining a niche of stem cells [19]. In line with this notion, various main players of controlling stem cell self-renewal and multipotency had been identified recently as HIF targets, including Oct4 [20]. Given that Oct4 acts in a hypoxic context and that Oct4 recruits JMJD1A, it

is an open question how JMJD1A is able to catalyze demethylation in a hypoxic environment when oxygen is an essential substrate for the oxidative demethylation reaction [1]. In the light of our findings, we propose that in a moderate hypoxic range, JMJD1A activity is perpetuated or induced directly via HIF-1 binding to the *JMJD1A* proximal promoter and indirectly via Oct4 binding resulting in JMJD1A overexpression. However, when oxygen tensions decline below a critical threshold, JMJD1A overexpression might become insufficient to compensate for a loss of enzymatic activity due to a loss of oxygen as a substrate.

Histone methylation regulates gene expression by creating binding sites for specific proteins and potentially antagonizes or augments the effects that other histone modifications have on gene expression. Hypoxia has been reported to increase global dimethylation of H3K9, temporally correlated with an increase in histone methyltransferase G9a protein and enzyme activity, in several mammalian cell lines [21] and in fetal lung cells, associated with suppression surfactant protein A gene transcription [22]. However, in contrast to the global increase of histone methylation under hypoxic conditions, distinct HIF-target genes (*EGR1* and *VEGF*) that are upregulated by hypoxia display a decrease in total, quantified H3K9 dimethylation at their promoters during hypoxia [23]. It is tempting to speculate that this complex regulation may involve JMJD1A, serving to demethylate select promoters to reduce nucleosomal occupancy and facilitate HIF binding. Upregulation of JMJD1A in response to hypoxia may help to balance the reduced availability of O_2 as a substrate. When designing experiments aimed at elucidating the sequence of events linking hypoxia, altered histone methylation, and gene transcription patterns, JMJD1A should be considered as an important player.

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