Regulation of Jumonji-domain containing histone demethylases by hypoxia inducible factor (HIF) 1-alpha

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Synopsis

The transcription factor hypoxia inducible factor (HIF) mediates a highly pleiotrophic response to hypoxia. Many recent studies have focused on defining the extent of this transcriptional response. Here we have analysed regulation by hypoxia amongst transcripts encoding human Fe(II) and 2-oxoglutarate dependent oxygenases. Our results show that many of these genes are regulated by hypoxia and define two groups of Jumonji-domain containing histone demethylases as new classes of hypoxia regulated gene. Patterns of induction were consistent across a range of cell lines with JMJD1A and JMD2B demonstrating robust, and JMJD2C more modest, upregulation by hypoxia. Functional genetic and chromatin immunoprecipitation studies demonstrated the importance of HIF-1alpha in mediating these responses. Given the importance of histone methylation status in defining patterns of gene expression under different physiological and pathophysiological conditions these findings predict a role for the HIF system in epigenetic regulation.

Introduction

Hypoxia is a major component of many human diseases and induces a range of cellular and systemic responses that function to restore oxygen homeostasis or better adapt cells to the hypoxic environment. At the cellular level these responses include regulation of fundamental physiological processes such as energy metabolism, proliferation, apoptosis, motility, and differentiation (for review, see [1, 2]).

Important insights into these processes have been gained though the definition of hypoxia signaling pathways that regulate gene expression in accordance with cellular oxygen availability. Hypoxia inducible factor (HIF) is an alpha/beta heterodimeric transcription factor that binds DNA at hypoxia response elements (HREs) associated with its transcriptional target genes [1]. HIF itself is regulated by post-translational hydroxylation of specific residues in its alpha subunits by a set of Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenases [3, 4]. In the presence of oxygen, hydroxylation (catalysed by three closely related prolyl hydroxylase domain enzymes, PHD1, 2 and 3) at two prolyl-residues promotes binding of HIF-alpha subunits to the von Hippel-Lindau tumor suppressor (pVHL) E3 ubiquitin ligase leading to proteasomal degradation (for review see [5]). In hypoxia, these reactions are suppressed allowing the assembly of an active HIF complex and induction of a range of genes that respond directly or indirectly to HIF.

Recent work has sought to better define the extent of this transcriptional cascade, with studies demonstrating both an extensive set of direct HIF transcriptional target genes, and indirect effects through the induction of regulatory molecules that themselves influence gene expression [6, 7]. Pan-genomic assays of transcript abundance have emphasized the overall importance of the HIF hydroxylase system in defining patterns of hypoxia inducible gene expression and have revealed many new and important hypoxia-inducible genes [8-10]. In the main, such analyses have focused on defining statistically robust induction of specific genes rather than interrogating patterns of response among specific families encoding functionally or structurally related proteins.

Of particular interest in this respect is the 2OG dependent oxygenase superfamily itself. In addition to 2OG dependent oxygenases that function in cellular oxygen sensing, the family encompasses members with diverse functions including post-translational modification of proteins (e.g. collagen stabilization), fatty acid metabolism and DNA repair [11-13]. Since all 2OG dependent oxygenases have an obligatory requirement for dioxygen as co-substrate, the activity of some of these enzymes in cells may be affected by the extracellular oxygen status. Several 2OG dependent oxygenases, including two of the HIF hydroxylases have been identified as hypoxia-inducible gene products [14-17].

Bioinformatic analyses have predicted that the human genome encodes in excess of sixty 2OG dependent dioxygenases Though the functions of many remain unassigned, a number have recently been demonstrated to function in histone modification, particularly histone lysine and arginyl demethylation [18, 19].

To investigate patterns of induction by hypoxia we assembled a refined compilation of human 2OG-dependent oxygenases, reanalyzed microarray-based transcriptome profiling of the MCF7 breast cancer cell line in normoxic and hypoxic conditions [10], and tested the role of the HIF system in such responses using a series of genetic interventions on the HIF pathway. The results define genes encoding the JmjC-domain-containing histone demethylases (JMJDs) as a new class of gene regulated by the HIF system and demonstrate consistent patterns of induction by HIF-1alpha amongst different family members.

Experimental

Cell culture

Human oesteosarcoma (U2OS), human breast cancer (MCF7) and human cervical carcinoma (HeLa) cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) (Sigma). Human neuroblastoma cells (IMR 32) were cultured in Eagle's minimal essential medium (Invitrogen) with 10% (v/v) FCS, 2mM glutamine (Invitrogen) and non-essential amino acids. Human promyelocytic leukemia (HL60) cells were grown in RPMI (Invitrogen) with 15% (v/v) FCS. All cells were maintained in a humidified atmosphere of 5% CO_2 and 21% O_2. Both R38 (human renal carcinoma (RCC4) cells that express a tetracycline-inducible hemaglutinin (HA)-tagged VHL transgene) and C29 (U2OS cells expressing tetracycline inducible HIF-1alpha) have been previously described [20, 21] and were cultured in DMEM with 10% (v/v) tetracycline-free FCS (BD Bioscience) supplemented with 1µg/ml G418 and 5µg/ml Blasticidin (Sigma). In the experimental work, cells were cultured under normoxic conditions (5% CO_2, 21% O_2) or in hypoxia (16 hours at 5% CO_2, 0.5% O_2). Where specified, cells were treated with dimethyloxalylglycine (DMOG) (1 mM for 16 hours) or desferrioxamine (DFO) (100µM for 16 hours).

siRNA Treatment of Cells

MCF7 and U2OS cells were seeded at 30% confluency and grown in normoxic conditions. Cells were transfected with siRNA (20nM) using Oligofectamine (Invitrogen) according to the manufacturer's protocol. After 24 hours the cells were transfected again and exposed to hypoxia for 16 hours after which they were washed, lysed and RNA and protein was extracted. The siRNA oligonucleotide sequences used were as previously described [22].

RNA Extraction and cDNA synthesis

Total RNA was extracted using the Sigma Total RNA kit (Sigma) according to the manufacturer's protocol. First strand cDNA synthesis was generated from 5µg of total RNA (GE Healthcare).

Protein Extraction and Immunoblot Analysis

Preparation of cell extracts and immunoblot analyses were performed as described previously [23]. Primary antibodies used were mouse anti-HIF-1alpha (BD Transduction Laboratories), rabbit anti-HIF-2alpha (Novus Biologicals), rabbit anti-JMJD1A (Abcam), mouse horseradish peroxidase (HRP)-conjugated anti-HA (Dako) and mouse HRP-conjugated anti-actin (Abcam).

Chromatin Immunoprecipitation (ChIP) Assays

Chromatin immunoprecipitation assays were performed using a modified version of the Upstate Protocol (Millipore). In brief, MCF7 cells were incubated with 2mM DMOG for 16 hours to increase HIF-alpha levels. DNA-binding proteins were cross-linked to DNA using formaldehyde at a final concentration of 1% (w/v) for 10 minutes at 25°C, followed by treatment with glycine (125mM) for a further 5 minutes. Cells were washed in PBS, lysed in SDS lysis buffer, and sonicated (Sonics & Materials, VCX 500). The supernatant was collected by centrifugation and pre-cleared with protein A agarose beads (Millipore). Chromatin was then incubated overnight with rabbit polyclonal anti-sera to HIF-1alpha (PM14) and HIF-2alpha (PM9) [24] before adding protein A agarose for a further hour. Pre-immune serum was used as a negative control. The beads were washed and immunoprecipitated complexes were eluted into 1% SDS, 0.1 M sodium bicarbonate elution buffer. Cross-linking was reversed by overnight incubation at 65°C and followed by protein digestion with proteinase K. DNA was recovered by phenol/chloroform extraction and ethanol precipitation.

Real-time quantitative PCR

RT-Q-PCR for mRNA quantification employed Taqman gene expression assays on a StepOne thermocycler (Applied Biosystems). Normalization was to β-actin mRNA and relative gene expression was calculated using the ΔΔCT method. 50 ng of cDNA template per reaction was used and 3 biological replicates, each in triplicate, were performed for each experiment. For quantification of HIF binding sites, ChIP DNA (5 ng) from each of input, preimmune sera, PM14 and PM9 was subject to RT-Q-PCR using oligonucleotides designed to amplify putative HRE consensus sequences (supplementary data). Fold enrichment of each HRE in the DMOG treated cells was determined by the ΔΔCT method.

Statistical analysis

Statistically comparisons were performed using Student's unpaired t-test.

Results

The human genome encodes multiple sub-groups of 2-OG dependent oxygenases that are regulated by hypoxia

Using structurally informed sequence analysis coupled to functional knowledge, we grouped known or predicted 2OG dependent oxygenases into subfamilies, and defined the existence or otherwise of regulation by hypoxia by reference to comparative whole genome expression arrays [10] conducted on normoxic and hypoxic MCF7 cells (Table 1). The data indicated that some, but not all transcripts are substantially up-regulated in hypoxic cells. Reported patterns of induction by hypoxia were confirmed for several subfamilies of enzyme, including the HIF prolyl hydroxylases (PHDs), the pro-collagen prolyl hydroxylases (C-P4Hs) and the pro-collagen lysyl hydroxylases (PLODs) [4, 14-17]. In addition several other classes of 2OG dependent oxygenases contained members that manifest significant upregulation by hypoxia. Particularly striking was the upregulation of transcripts encoding the Jumonji domain containing proteins JMJD1A and JMJD2B. These proteins belong to the JMJD1 (otherwise known as JHDM2 and containing JmjC and modified zinc-finger domains) and JMJD2 (otherwise known as JHDM3 and containing JmjC, JmjN, plant homology domains and Tudor domains) subfamilies of 2OG dependent oxygenase that have recently been demonstrated to possess histone lysyl demethylase (HDM) activity [18, 25, 26]. To better define this new class of hypoxia-regulated gene we performed further experiments on seven members (JMJD1A-C; JMJD2A-D) of these families that have been definitively assigned as possessing HDM activity.

First, to verify the results of the micro-array analysis and to determine whether regulation by hypoxia was manifested in different cell types, expression of all seven transcripts was analysed by real-time quantitative PCR (RT-Q-PCR) in a panel of cell lines. MCF7, U2OS, HeLa, IMR32 and HL60 cells were exposed to hypoxia (0.5% O_2 for 16 hours). Consistent patterns of induction by hypoxia were observed across all five cell lines (Figure 1a). Much the most striking induction by hypoxia was observed for JMJD1A (ranging from 2.6-4.7 fold) and JMJD2B mRNA (ranging from 2.5-4.6 fold) (Figure 1a). Other family members did not manifest induction by hypoxia, with the exception of JMJD2C which manifested modest (up to 2-fold) induction in some cell types.

Regulation of genes encoding Jumonji domain containing proteins JMJD1 and JMJD2 by the HIF hydroxylase pathway

Inspection of the microarray data indicated that genes manifesting induction by hypoxia were also commonly induced by dimethyloxalylglycine (DMOG) a cell-penetrating competitive inhibitor of many 2OG oxygenases including the hydroxylases that regulate HIF [4]. To pursue the potential role of HIF hydroxylase pathways in the regulation of genes encoding JMJD1 and JMJD2 family members, we extended this analysis to other cell types, and tested responses to both DMOG (1mM for 16 hours) and desferrioxamine (DFO, 100μM for 16 hours), another agent that upregulates HIF by inhibition of 2OG oxygenases including the HIF hydroxylases [4]. Results are shown for U2OS cells (Figure 1 b and c). As expected we observed robust observed robust upregulation of HIF-1alpha by immunoblot analysis that equalled or exceeded that achieved by the hypoxic exposure (Figure 1b). RT-Q-PCR analysis of JMJD1 and JMJD2 transcripts demonstrated substantial upregulation, the pattern being closely similar to that observed for hypoxia (Figure 1c). JMJD1A, JMJD2B and JMJD2C were again the most strongly induced genes, with responses to DMOG exceeding 20-fold induction for JMJD2B (Figure 1c). Assay of effects on endogenous JMJD protein levels is limited by the availability of appropriate antibodies. Nevertheless we were able to confirm that JMJD1A protein levels were increased significantly; both under hypoxia and with the addition of DMOG (Figure 1d).

To test the involvement of HIF in these responses we next analysed cells bearing different genetic manipulations of the HIF system. First, we analysed changes in expression in response to normoxic overexpression of HIF-1alpha, using the C29 cell line, a stable U2OS transfectant bearing a doxycycline-inducible HIF-1alpha transgene [20]. Exposure of normoxic C29 cells to doxycycline resulted in sustained induction of HIF-1alpha (Figure 2a) that was associated with sustained, though more moderate induction of JMJD1A, JMJD2B and JMJD2C over the 48 hour period of doxycycline exposure (Figure 2b).

We next analysed responses in R38 cells, a VHL-defective RCC (RCC4) transfectant that re-expresses a doxycycline-inducible HA-tagged wild-type VHL transgene [21]. In keeping with base-line VHL-defective status, R38 cells expressed increased levels of HIF-1alpha sub-units in normoxia, whereas treatment with doxycycline over a 48 hour period resulted in sustained expression of HA-VHL and reduction of HIF-1alpha (Figure 2c). Consistent with a role for HIF in the regulation of JMJD1 and JMJD2 gene families, downregulation of HIF-1alpha was associated with downregulation of JMJD1 and JMJD2 transcripts in a pattern that was the inverse of that observed for hypoxia and induction of HIF-1alpha in that JMJD1A, JMJD2B and JMJD2C showed the greatest reduction in expression (Figure 2d).

Finally we tested the role of HIF-1alpha and HIF-2alpha isoforms using siRNA mediated knockdown in hypoxic MCF7 cells. As indicated in Figure 2e MCF7 cells express both HIF-1alpha and HIF-2alpha; siRNAs directed against each HIF-alpha isoform, but not control siRNA, resulted in specific knock-down of the target. Analysis of JMJD transcripts from hypoxic cells indicated that siRNA directed against HIF-1alpha substantially reduced expression of JMJD1A, JMJD2B and JMJD2C, whereas siRNA directed against HIF-2alpha had essentially no effect (Figure 2f).

Binding of HIF-alpha subunits to the promoters of JMJD1A and JMJD2B

Taken together, these results indicate that induction of JMJD1A, JMJD2B and JMJD2C by hypoxia is mediated directly or indirectly by HIF-1alpha. Direct transcriptional activation at HIF target gene loci is mediated by binding of the HIF complex to hypoxia response elements containing the core motif RCGTG [1]. To identify such sites at loci encoding JMJD1 and JMJD2 family members, we searched the March 2006 Human Genome assembly (http://genome.ucsc.edu/) *in silico* for HIF binding sites that were highly conserved amongst mammalian species, within the regions (-5 to + 0.5kB) of the assigned transcriptional start site. Three potential HIF binding sites within these regions were identified in the three members that were demonstrated to be induced in hypoxia by HIF-1alpha (JMJD1A and JMJD2B, C) and no such sites in the remaining four members (JMJD1B, C and JMJD2A ,D), suggesting that the three hypoxia inducible genes might be direct target genes.

To test whether these loci did indeed bind HIF-alpha subunits we performed ChIP assays using anti-HIF-1alpha antibodies (PM14) or anti-HIF-2alpha antibodies (PM9) or control pre-immune sera, and carried out RT-Q-PCR analysis of precipitated DNA using primers designed to amplify each of the predicted HREs. To provide further controls, primers were also designed to a region of the JMJD1A promoter region that does contain an HRE and to the JMJD2A promoter that does not contain an HRE and did not exhibit regulation by HIF or hypoxia in the transcript analyses. Significant and reproducible enrichment of DNA containing HREs at the JMJD1A and JMJD2B loci was observed with both anti-HIF-1alpha and anti-HIF-2alpha antibodies (Table 2). More moderate enrichment was observed at the JMJD2C locus with anti HIF-1alpha but not anti HIF-2alpha. No enrichment of the non-HRE containing amplicons was detected with either antibody.

Discussion

This study has defined genes encoding Jumonji-domain containing 2OG dependent oxygenases, with histone demethylase activity, as a new class of HIF-responsive hypoxia-inducible genes. We focused our analysis on two groups of closely related genes; those encoding JMJD1A-C proteins that contain only the catalytic JmjC domain, and JMJD2A-D that contain both JmjN and JmjC domains (as well Tudor and plant homology domains in the case of JMJD2A-C) [18]. Among these families, we observed consistent patterns of regulation with genes encoding JMJD1A, JMJD2B and JMJD2C, but not other members being inducible by hypoxia. Though JMJD1A and JMJD2B showed particularly striking regulation by hypoxia, prompting us to focus on the JMJD groups, a range of other genes encoding human 2OG dependent oxygenases showed hypoxia-inducible behaviour in the MCF7 cell gene expression array (Table1). Consistent with previous reports regulation by hypoxia was observed for the HIF hydroxylases PHD2 and PHD3 [4, 16, 17], and for the procollagen prolyl and lysyl hydroxylases (C-P4H and PLOD groups) [14, 15]. However, and also consistent with reported data [4, 20], PHD1 and FIH (factor inhibiting HIF, an asparaginyl hydroxylase, which like PHD2 and PHD3 are proposed to act as sensing enzymes in the HIF system, are not, or much less significantly, regulated by hypoxia. Modest regulation by hypoxia was also noted in the MCF7 cell gene expression array for other classes of Jumonji-domain containing proteins including members of the JARID and JTR groups. Several of these proteins also have histone demethylase activity so that it is possible that regulation by hypoxia extends more widely across this group of enzymes.

Dynamic control of histone methylation status is proposed to regulate chromatin assembly and gene expression [18, 26]. Histone lysyl methylation is currently known to occur on histone 3, (H3), at K4, K9, K27, K36 and K79, and on histone 4 (H4) at K20; each site potentially existing as mono-(me1), di-(me2), or tri-(me3) methylated forms associated with distinct biological functions [18, 26-28]. Though the patterns of substrate specificity are as yet incompletely defined, some HDMs are reported to be highly site specific. For instance JMJD1A has so far been shown to exhibit demethylation activity on H3K9me1 and H3K9me2 but not H3K9me3 [29]; JMJD2B exhibits poor enzyme activity *in vitro* but has been demonstrated to specifically reduce H3K9me3 when overexpressed in cells [30]; JMJD2C exhibits demethylation activity on both H3K9me3 and H3K36me3 [31]. Interestingly specific HDMs have been associated with the regulation of particular transcriptional pathways. For instance JMJD1A is associated with the androgen receptor (AR) and has been shown to positively regulate certain AR target genes [29, 32]. In embryonic stem cells JMJD1A and JMJD2C enhance the expression of genes associated with self-renewal and are targets of the transcription factor Oct4 [33]. This suggests that expression levels of specific histone demethylases may themselves be regulated as component of physiological control systems directing patterns of gene expression. Interestingly, Oct-4 has itself been identified as gene responding specifically to HIF-2alpha in embryonic stems cells [34].

Our studies, in a range of different cells lines, have identified a different and more direct connection between histone demethylases and the HIF system. Pharmacological and genetic analyses demonstrated functional control of particular JMJD-encoding genes by HIF, specifically by HIF-1alpha, whilst ChIP assays defined HIF-alpha binding sites in the promoters of these hypoxia-inducible genes. Interestingly, whilst ChIP assays indicated that both HIF-1alpha and HIF-2alpha bound to promoter sequences, siRNA-mediated suppression suggested that HIF-1alpha, but not HIF-2alpha, was responsible for induction of JMJD1A and JMJD2B in hypoxia, even though both HIF-alpha isoforms were induced under the experimental conditions. Such behaviour has been noted at certain other HIF target gene loci [22, 35], and is consistent with recent data indicating that HIF-alpha isoform transcriptional selectivity is mediated by post-DNA binding mechanisms conveyed by sequences distal to the DNA binding and dimerization domains [24, 35]. The nature of these mechanisms, together with the reasons why endogenous HIF-2alpha appears transcriptionally inactive at many loci, is incompletely understood, though evidence has been provided for the existence of a titratable repressor that limits HIF-2alpha activity in many cell types [35].

Although hypoxia has long been recognized to have major effects on genome integrity, gene expression and cell differentiation that likely involve epigenetic controls, detailed analyses of hypoxic effects on epigenetic modification of DNA and histones have so far been limited. Hypoxia, has,

however been noted to alter levels of both histone acetylation and histone methylation at certain sites [36, 37]. A recent study demonstrated that changes in both histone methyl transferase and histone demethylase activity contributed to changes in global H3K9 methylation status in response to hypoxic stress and focused on the upregulation of histone methyl transferase G9a in hypoxia by both HIF-dependent and HIF-independent mechanisms [38].

The current evidence for direct regulation of genes encoding specific histone demethylases by HIF-1alpha suggests another means by which activation of the HIF pathway may contribute to the modulation of histone marks and gene expression profiles in hypoxic cells. Given the existence of several different mechanisms by which HIF and hypoxia itself may alter histone methylation, the difficulty of separating these effects accurately, and uncertainties regarding the extent to which transcriptional upregulation of JMJDs is reflected in biological activity, it is currently difficult to predict effects of our findings on overall cellular demethylase activity. Since the JMJD1 and JMJD2 enzymes are 2OG dependent oxygenases that have an absolute requirement for dioxygen as co-substrate it could be argued that any increases in enzyme abundance in hypoxia simply compensate for reduced activity. However, given the specificity of the HIF-mediated hypoxia-inducible response for particular histone lysyl demethylases with different methyl-lysyl substrate preferences, and different biological effects at particular transcriptional loci, this appears unlikely. Rather, we propose that this new class of HIF transcriptional target adds another level of control that shapes the transcriptional response to hypoxia. Our findings raise interesting questions as to whether the hypoxia-inducible histone demethylases are specifically associated with the HIF transcriptional complex, or with specific classes of hypoxia-inducible target gene loci, and if so how they function to modulate patterns of gene expression under conditions of varying oxygen availability.

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References:

1 Semenza, G. L. (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer **3**, 721-732

2 Simon, M. C. and Keith, B. (2008) The role of oxygen availability in embryonic development and stem cell function. Nat Rev Mol Cell Biol **9**, 285-296

3 Bruick, R. K. and McKnight, S. L. (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. Science **294**, 1337-1340

4 Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J. and Ratcliffe, P. J. (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell **107**, 43-54

5 Kaelin, W. G., Jr. and Ratcliffe, P. J. (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell **30**, 393-402

6 Yun, Z., Maecker, H. L., Johnson, R. S. and Giaccia, A. J. (2002) Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. Dev Cell **2**, 331-341

7 Kulshreshtha, R., Ferracin, M., Wojcik, S. E., Garzon, R., Alder, H., Agosto-Perez, F. J., Davuluri, R., Liu, C. G., Croce, C. M., Negrini, M., Calin, G. A. and Ivan, M. (2007) A microRNA signature of hypoxia. Mol Cell Biol **27**, 1859-1867

8 Hu, C. J., Wang, L. Y., Chodosh, L. A., Keith, B. and Simon, M. C. (2003) Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol **23**, 9361-9374

9 Manalo, D. J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B. D., Ye, S. Q., Garcia, J. G. and Semenza, G. L. (2005) Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood **105**, 659-669

10 Elvidge, G. P., Glenny, L., Appelhoff, R. J., Ratcliffe, P. J., Ragoussis, J. and Gleadle, J. M. (2006) Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1alpha, HIF-2alpha, and other pathways. J Biol Chem **281**, 15215-15226

11 Aravind, L. and Koonin, E. V. (2001) The DNA-repair protein AlkB, EGL-9, and leprecan define new families of 2-oxoglutarate- and iron-dependent dioxygenases. Genome Biol **2**, RESEARCH0007

12 Clifton, I. J., McDonough, M. A., Ehrismann, D., Kershaw, N. J., Granatino, N. and Schofield, C. J. (2006) Structural studies on 2-oxoglutarate oxygenases and related double-stranded beta-helix fold proteins. J Inorg Biochem **100**, 644-669

13 Takeuchi, T., Watanabe, Y., Takano-Shimizu, T. and Kondo, S. (2006) Roles of jumonji and jumonji family genes in chromatin regulation and development. Dev Dyn **235**, 2449-2459

14 Takahashi, Y., Takahashi, S., Shiga, Y., Yoshimi, T. and Miura, T. (2000) Hypoxic induction of prolyl 4-hydroxylase alpha (I) in cultured cells. J Biol Chem **275**, 14139-14146

15 Hofbauer, K. H., Gess, B., Lohaus, C., Meyer, H. E., Katschinski, D. and Kurtz, A. (2003) Oxygen tension regulates the expression of a group of procollagen hydroxylases. Eur J Biochem **270**, 4515-4522

16 Pescador, N., Cuevas, Y., Naranjo, S., Alcaide, M., Villar, D., Landazuri, M. O. and Del Peso, L. (2005) Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. Biochem J **390**, 189-197

17 Metzen, E., Stiehl, D. P., Doege, K., Marxsen, J. H., Hellwig-Burgel, T. and Jelkmann, W. (2005) Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene: identification of a functional hypoxia-responsive element. Biochem J **387**, 711-717

18 Shi, Y. and Whetstine, J. R. (2007) Dynamic regulation of histone lysine methylation by demethylases. Mol Cell **25**, 1-14

19 Chang, B., Chen, Y., Zhao, Y. and Bruick, R. K. (2007) JMJD6 is a histone arginine demethylase. Science **318**, 444-447

20 Stolze, I. P., Tian, Y. M., Appelhoff, R. J., Turley, H., Wykoff, C. C., Gleadle, J. M. and Ratcliffe, P. J. (2004) Genetic analysis of the role of the asparaginyl hydroxylase factor inhibiting hypoxia-inducible factor (HIF) in regulating HIF transcriptional target genes. J Biol Chem **279**, 42719-42725

21 Wykoff, C. C., Sotiriou, C., Cockman, M. E., Ratcliffe, P. J., Maxwell, P., Liu, E. and Harris, A. L. (2004) Gene array of VHL mutation and hypoxia shows novel hypoxia-induced genes and that cyclin D1 is a VHL target gene. Br J Cancer **90**, 1235-1243

22 Sowter, H. M., Raval, R. R., Moore, J. W., Ratcliffe, P. J. and Harris, A. L. (2003) Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. Cancer Res **63**, 6130-6134

23 Wiesener, M. S., Turley, H., Allen, W. E., William, C., Eckardt, K.-U., Talks, K. L., Wood, S. M., Gatter, K. C., Harris, A. L., Pugh, C. W., Ratcliffe, P. J. and Maxwell, P. H. (1998) Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1α. Blood **92**, 2260-2268

24 Lau, K. W., Tian, Y. M., Raval, R. R., Ratcliffe, P. J. and Pugh, C. W. (2007) Target gene selectivity of hypoxia-inducible factor-alpha in renal cancer cells is conveyed by post-DNA-binding mechanisms. Br J Cancer **96**, 1284-1292

25 Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M. E., Borchers, C. H., Tempst, P. and Zhang, Y. (2006) Histone demethylation by a family of JmjC domain-containing proteins. Nature **439**, 811-816

26 Klose, R. J., Kallin, E. M. and Zhang, Y. (2006) JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet **7**, 715-727

27 Shi, Y. (2007) Histone lysine demethylases: emerging roles in development, physiology and disease. Nat Rev Genet **8**, 829-833

28 Loenarz, C. and Schofield, C. J. (2008) Expanding chemical biology of 2-oxoglutarate oxygenases. Nat Chem Biol **4**, 152-156

29 Yamane, K., Toumazou, C., Tsukada, Y., Erdjument-Bromage, H., Tempst, P., Wong, J. and Zhang, Y. (2006) JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell **125**, 483-495

30 Fodor, B. D., Kubicek, S., Yonezawa, M., O'Sullivan, R. J., Sengupta, R., Perez-Burgos, L., Opravil, S., Mechtler, K., Schotta, G. and Jenuwein, T. (2006) Jmjd2b antagonizes H3K9 trimethylation at pericentric heterochromatin in mammalian cells. Genes Dev **20**, 1557-1562

31 Whetstine, J. R., Nottke, A., Lan, F., Huarte, M., Smolikov, S., Chen, Z., Spooner, E., Li, E., Zhang, G., Colaiacovo, M. and Shi, Y. (2006) Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. Cell **125**, 467-481

32 Okada, Y., Scott, G., Ray, M. K., Mishina, Y. and Zhang, Y. (2007) Histone demethylase JHDM2A is critical for Tnp1 and Prm1 transcription and spermatogenesis. Nature **450**, 119-123

33 Loh, Y. H., Zhang, W., Chen, X., George, J. and Ng, H. H. (2007) Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev **21**, 2545-2557

34 Covello, K. L., Kehler, J., Yu, H., Gordan, J. D., Arsham, A. M., Hu, C. J., Labosky, P. A., Simon, M. C. and Keith, B. (2006) HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev **20**, 557-570

35 Hu, C. J., Sataur, A., Wang, L., Chen, H. and Simon, M. C. (2007) The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. Mol Biol Cell **18**, 4528-4542

36 Johnson, A. B. and Barton, M. C. (2007) Hypoxia-induced and stress-specific changes in chromatin structure and function. Mutat Res **618**, 149-162

37 Johnson, A. B., Denko, N. and Barton, M. C. (2008) Hypoxia induces a novel signature of chromatin modifications and global repression of transcription. Mutat Res **640**, 174-179

38 Chen, H., Yan, Y., Davidson, T. L., Shinkai, Y. and Costa, M. (2006) Hypoxic stress induces dimethylated histone H3 lysine 9 through histone methyltransferase G9a in mammalian cells. Cancer Res **66**, 9009-9016

Table Legends

Table 1. Hypoxia up-regulates mRNA expression of selected known and potential human 2OG-dependent oxygenases.

Relative changes in expression levels upon exposure to hypoxia or treatment with DMOG (mean of 3 biological replicates) compiled from data deposited in association with [10] at http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE3188. 2OG-dependent oxygenases are grouped by homology, and assigned according to HGNC (HUGO Gene Nomenclature Committee). NCBI GeneIDs are also given. For known catalytic functions and substrates see references in [28]; note the correct physiological substrate(s) may differ from those with which activity has been shown *in vitro*. Names given to each group reflect known or proposed functional assignments associated with the 2OG oxygenase and, in some cases, other distinct domains. Abbreviations are as follows: EGFH, epidermal growth factor aspartate/asparaginyl hydroxylase; C-P3H, collagen prolyl 3-hydroxylase; PHD, prolyl hydroxylase domain; OGFOD, 2OG and iron-dependent oxygenase domain; C-P4H, pro-collagen prolyl 4-hydroxylase; PLOD, pro-collagen lysyl oxygenase domain; JmjC, C-terminal Jumonji-domain; JTR, Jumonji and tetratricopeptide repeat containing; JARID, Jumonji, AT rich interactive domain; JMJD1/2, Jumonji domain containing 1/2; JFBXL, Jumonji and F-box and leucine-rich repeat containing; JPHF, Jumonji and PHD finger containing; ALKBH, alkB alkylation repair homolog (*E .coli*); FTO, fat mass and obesity associated; PHYH (PAHX), phytanoyl-CoA 2-hydroxylase; CAS-like, clavaminic acid synthase-like. HGNC official full names of genes are given (common names italicized in brackets); abbreviations as above or as follows: ASPH, aspartate beta-hydroxylase; ASPHD1/2, aspartate beta-hydroxylase domain containing 1/2; LEPRE, leucine proline-enriched proteoglycan (leprecan); LEPREL1/2, leprecan-like 1/2; EGLN1/2/3, egl nine homolog 1/2/3 (*C. elegans*); P4H-TM, prolyl 4-hydroxylase transmembrane domain; P4HA1/2/3, procollagen-proline, 2OG 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I/II/III; JMJD4/5/6, jumonji domain containing 4/5/6; HSPBAP1, HSPB (heat shock 27kDa) associated protein 1; HIF1AN, hypoxia-inducible factor 1, alpha subunit inhibitor (FIH, factor inhibiting HIF); C2orf60, chromosome 2 open reading frame 60; MINA, MYC induced nuclear antigen; C14orf169, chromosome 14 open reading frame 169; UTX, ubiquitously transcribed tetratricopeptide repeat, X chromosome; UTY, ubiquitously transcribed tetratricopeptide repeat gene, Y-linked; JMJD3, Jumonji domain containing 3; HR, hairless homolog (mouse); JHDM2/3, Jumonji C domain containing histone demethylase 2/3; FBXL10/11, F-box and leucine-rich repeat protein 10/11; PHF2/8, PHD finger protein 2/8; JHDM1D, Jumonji C domain containing histone demethylase 1 homolog D (*S. cerevisiae*); TMLHE, trimethyllysine hydroxylase, epsilon; BBOX1 (GBBH), butyrobetaine (gamma), 2OG dioxygenase (gamma-butyrobetaine hydroxylase) 1; *, genes not assigned by HGNC. Other abbreviations; ARD, ankyrin repeat domain; me, methylated; meA, methyladenosine; meC, methylcytidine; meT, methylthymidine.

Table 2: ChIP assays of DNA at hypoxia responsive promoters.

The core sequence and position related to the transcriptional start site is given for each HRE. Data are the mean (range) of the fold enrichment of chromatin precipitated DNA. The means and ranges were determined from 3 biological replicates, each assayed in triplicate.

*significant enrichment $p<0.01$

#significant enrichment $p<0.05$

Figure 1:

A: Hypoxic induction of JMJD1A-C and JMJD2A-D mRNA in U2OS, MCF7, HeLa, IMR32 and HL60 cells. Data is the fold increase in mRNA in cells cultured in hypoxia (0.5% O_2) related to parallel cultures in normoxia (21% O_2). JMJD1A and 2B are significantly upregulated ($p<0.01$) in each cell type. Significant hypoxic induction of JMJD2C ($p<0.01$) was observed in U2OS, HeLa and IMR32 cells. Y-error bars indicate one standard deviation calculated from 3 biological replicates, each assayed in triplicate.

B: Immunoblot demonstrating increase of HIF-1alpha protein accumulation under hypoxia (lane 2), or with addition of either DFO (lane 4) or DMOG (lane 5). Protein loading is indicated by β-actin (bottom panel).

C: Effect of DFO and DMOG treatment on expression of JMJD1 and JMJD2 transcripts in U2OS cells. JMJD1A, 2B and 2C mRNAs are significantly elevated by both stimuli ($p<0.01$); JMJD1C mRNA was significantly increased by DMOG treatment ($p<0.01$) but not after DFO treatment. Y-error bars indicate one standard deviation calculated from 3 biological replicates, each assayed in triplicate.

D: Effect of hypoxia and DMOG on expression of JMJD1A protein in U2OS cells. Immunoblot demonstrates increase of HIF-1 alpha (middle panel) and JMJD1A (top panel) protein under hypoxia (lane 2) or with the addition of DMOG (lane 3). Protein loading is indicated by β-actin (bottom panel).

Figure 2:

A and B: Inducible HIF-1alpha overexpression in U2OS cells. Immunoblot (A) demonstrating increased expression of HIF-1alpha protein at 24 hours (lane 2) and 48 hours (lane 3) after doxycycline administration. RT-Q-PCR analysis (B) shows a significant ($p<0.01$) increase in JMJD1A, 2B and 2C mRNA after doxycycline administration.

C and D Expression of VHL in RCC4 cells. Immunoblot (C) demonstrates reduction of HIF-1alpha protein at 24 hours (lane 2) and absence of HIF-1alpha protein at 48 hours (lane 3). RT-Q-PCR analysis (D) shows significant ($p<0.01$) decrease in JMJD1A, 2B and 2C mRNA by 48 hours after doxycycline administration.

E and F Effect of HIF-1alpha and HIF-2alpha siRNA. Immunoblot (E) demonstrates effects on HIF-alpha proteins. siRNA against *Drosophila* HIF (dH, lane 1) had no effect on HIF-1alpha or HIF-2alpha protein levels, whereas siRNA to HIF-1alpha and HIF-2alpha each abolish protein levels of their specific target genes (lanes 2-3). β-actin was used as a control to assay gel loading (bottom panel). RT-Q-PCR analysis (F) shows significant ($p<0.01$) reduction in hypoxic induction of JMJD1A, 2B and 2C mRNAs by HIF-1alpha directed siRNA.

Y-error bars indicate one standard deviation calculated from 3 biological replicates, each assayed in triplicate.

Table 1:

Group	Symbol	Hypoxia	DMOG	Catalytic function (proposed) & substrate	GeneID
EGFH	ASPH *(EGFH)*	3.04	3.22	Asn/Asp 3*R*-hydroxylation (EGF-domains)	444
	ASPHD1 *(EGFHD1)*	1.34	0.98		253982
	ASPHD2 *(EGFHD2)*	0.87	1.03		57168
C-P3H	LEPRE1	1.20	1.12	Pro 3*S*-hydroxylation (collagen)	64175
	LEPREL1	1.04	1.00		55214
	LEPREL2	1.15	1.01		10536
	* *(FLJ22222)*	1.06	1.09		79701
PHD	EGLN3 *(PHD3)*	15.29	19.47	Pro 4*R*-hydroxylation (HIF-alpha)	112399
	EGLN1 *(PHD2)*	3.66	5.95		54583
	EGLN2 *(PHD1)*	0.69	0.86		112398
OGFOD-only	* *(P4H-TM)*	1.16	0.90		54681
	OGFOD1	0.56	0.65		55239
C-P4H	P4HA1	5.36	7.56	Pro 4*R*-hydroxylation (pro-collagen)	5033
	P4HA2	7.47	9.85		8974
	P4HA3	0.96	0.98		283208
PLOD	PLOD1	2.19	1.49	Lys 5*R*-hydroxylation (pro-collagen)	5351
	PLOD2	2.63	3.40		5352
	PLOD3	1.27	0.90		8985
JmjC-domain	JMJD4	0.91	0.91		65094
	JMJD5	0.97	0.95		79831
	JMJD6	1.19	1.45	Arg(me) demethylation (histones H3R2/H4R3)	23210
	HSPBAP1	1.09	0.90		79663
	HIF1AN *(FIH)*	0.91	0.96	Asn 3*S*-hydroxylation (HIF-alpha/ARDs)	55662
	C2orf60	0.70	0.81		129450
	* *(LOC8681)*	0.68	1.08		8681
	MINA	0.55	0.58		84864
	C14orf169	0.37	0.40		79697
JTR	UTX	1.36	1.01	Lys(me) demethylation (histone H3K27)	7403
	UTY	1.62	1.18		7404
	JMJD3	1.64	1.58		23135
JARID	JARID1A	1.53	1.55	Lys(me) demethylation (histone H3K4)	5927
	JARID1B	1.72	1.85		10765
	JARID1C	1.21	1.39		8242
	JARID1D	1.03	1.02		8284
	JARID2	1.20	1.03		3720
JMJD1	HR	1.50	1.65		55806
	JMJD1A *(JHDM2A)*	7.01	9.80	Lys(me) demethylation (histone H3K9)	55818
	JMJD1B *(JHDM2B)*	1.07	1.08		51780
	JMJD1C *(JHDM2C)*	1.96	1.93		221037
JMJD2	JMJD2A *(JHDM3A)*	1.14	0.91	Lys(me) demethylation (histone H3K9/K36)	9682
	JMJD2B *(JHDM3B)*	3.04	2.62		23030
	JMJD2C *(JHDM3C)*	1.40	1.44		23081
	JMJD2D *(JHDM3D)*	1.23	1.01		55693
JFBXL	FBXL11	1.49	1.33	Lys(me) demethylation (histone H3K36)	22992
	FBXL10	0.93	0.83		84678
JPHF	PHF2	0.79	0.77		5253
	PHF8	1.05	1.01		23133
	JHDM1D	1.38	1.89		80853
ALKBH	ALKBH1	0.76	0.99		8846
	ALKBH2	0.95	0.75	1-meA/3-meC demethylation (DNA/RNA)	121642
	ALKBH3	0.98	0.94		221120
	ALKBH4	0.96	1.15		54784
	ALKBH5	1.3	1.48		54890
	ALKBH6	1.04	0.67		84964
	ALKBH7	1.07	0.95		84266
	ALKBH8	0.89	0.82		91801
FTO	FTO	1.06	0.81	3-meT/1-meA/3-meC demethylation (DNA/RNA)	79068
PHYH	PHYH *(PAHX)*	1.26	1.04	*threo* 2-hydroxylation (phytanoyl-CoA)	5264
CAS-like domain	TMLHE	1.03	1.01	5*R*-hydroxylation (trimethyllysine)	55217
	BBOX1 *(GBBH)*	1.04	0.96	3*S*-hydroxylation (γ-butyrobetaine)	8424

Table 2:

Gene	HRE (base pairs from mRNA start)	Enhancement (mean/range)		
		anti-HIF-1alpha	anti-HIF-2alpha	pre-immune
JMJD1A	-598 ACGTG -594	*135 (113-161)	*44 (26-76)	2.7 (1.1-3.1)
JMJD1A	---	2.3 (0.8-3.7)	1.8 (1.1-3-1)	1.5 (0.5-2.9)
JMJD2B	-248 ACGTG -244	*72 (37-140)	*117 (38-188)	1.9 (0.7-4.2)
JMJD2C	+290 ACGTG +294	#11.8 (3.0-46.2)	0.7 (0.2-2.1)	0.9 (0.3-2.6)
JMJD2A	---	1.8 (1.4-2.3)	1.2 (0.5-3.2)	1.1 (0.5-1,8)

Figure 1

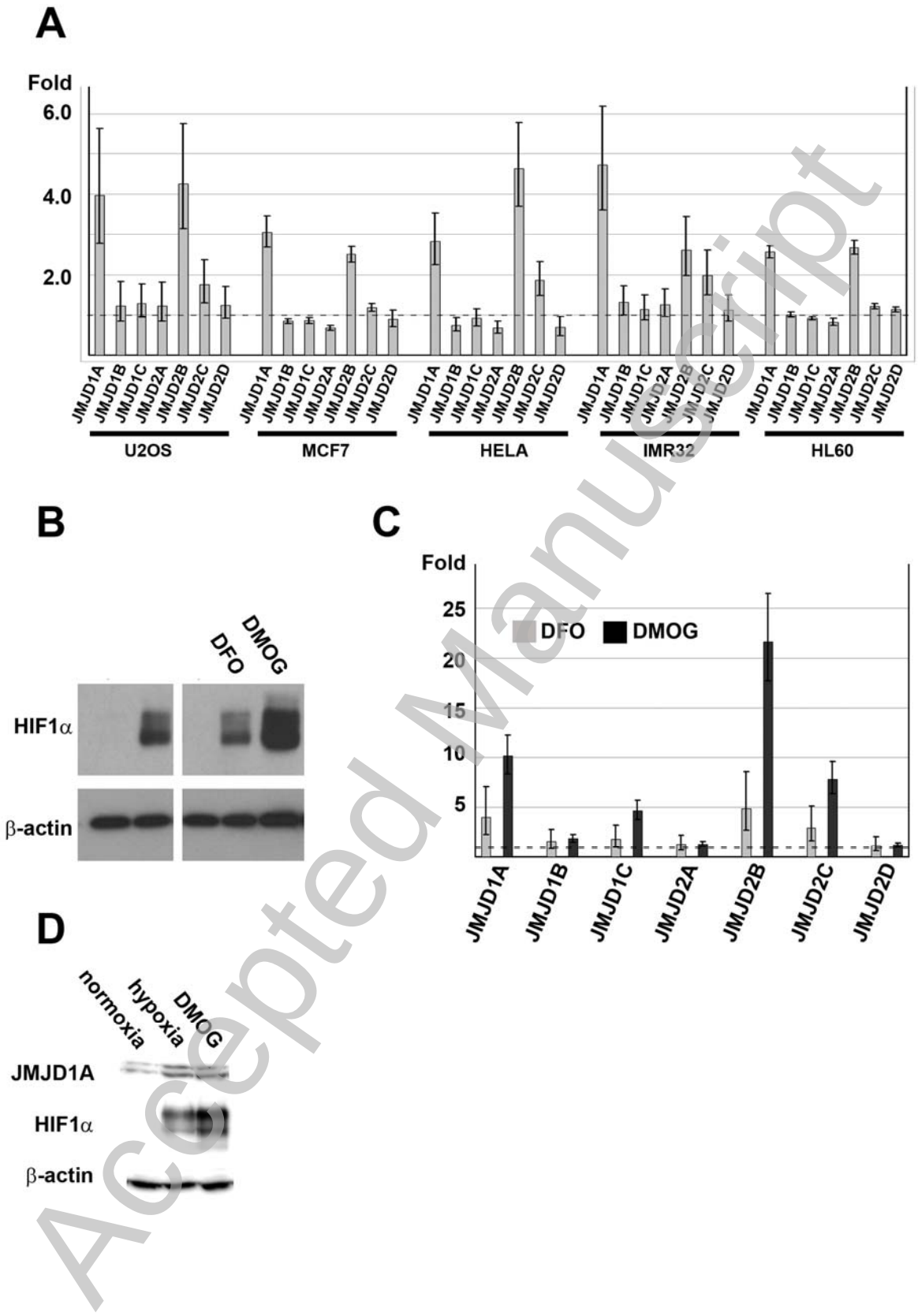

Figure 2