KDM4A regulates HIF-1 levels through H3K9me3

Grzegorz Dobrynin1, Tom E. McAllister1,3, Katarzyna B. Leszcynska1, Shalini Ramachandran1, Adam J. Krieg4, Akane Kawamura2,3 & Ester M. Hammond1

Regions of hypoxia (low oxygen) occur in most solid tumours and cells in these areas are the most aggressive and therapy resistant. In response to decreased oxygen, extensive changes in gene expression mediated by Hypoxia-Inducible Factors (HIFs) contribute significantly to the aggressive hypoxic tumour phenotype. In addition to HIFs, multiple histone demethylases are altered in their expression and activity, providing a secondary mechanism to extend the hypoxic signalling response. In this study, we demonstrate that the levels of HIF-1α are directly controlled by the repressive chromatin mark, H3K9me3. In conditions where the histone demethylase KDM4A is depleted or inactive, H3K9me3 accumulates at the HIF-1α locus, leading to a decrease in HIF-1α mRNA and a reduction in HIF-1α stabilisation. Loss of KDM4A in hypoxic conditions leads to a decreased HIF-1α mediated transcriptional response and correlates with a reduction in the characteristics associated with tumour aggressiveness, including invasion, migration, and oxygen consumption. The contribution of KDM4A to the regulation of HIF-1α is most robust in conditions of mild hypoxia. This suggests that KDM4A can enhance the function of HIF-1α by increasing the total available protein to counteract any residual activity of prolyl hydroxylases.

Hypoxia occurs in most solid tumours as a consequence of the rapid proliferation of cancer cells and an inadequate/inefficient vasculature. Most importantly, the degree of tumour hypoxia has been shown to correlate with poor patient survival in numerous tumour types1. The predominant transcriptional response to hypoxia is mediated by the hypoxia inducible factors (HIF), which include HIF-1, 2 and 3. Each HIF is composed of the same constitutively expressed β subunit (HIF-1β) and an oxygen labile α subunit (HIF-1α, 2α or 3α). Under normoxic conditions, HIF-1α is hydroxylated by the prolyl hydroxylases (PHDs) within the N- and C-terminal oxygen dependent degradation domains (NODD and CODD)4. Once hydroxylated, HIF-1α interacts with the tumour suppressor, von-Hippel-Lindau complex (VHL), is ubiquitinated and targeted for proteosomal degradation. The PHDs are Fe(II) and 2-oxoglutarate (2-OG) dependent oxygenases and do not function in hypoxic conditions where insufficient oxygen is available, therefore allowing the stabilisation of HIF-α subunits and dimerisation with HIF-1β. HIF-1α function is also regulated by another 2-OG oxygenase, factor inhibiting HIF (FIH). FIH hydroxylates an asparagine residue in the C-terminal transactivation domain of HIF1-α which prevents complex formation with p300, thus inhibiting transcriptional activation6. In addition to HIF-mediated changes in gene expression, the epigenetic landscape is also altered in response to hypoxia7,8. KDM4A (JMJD2A) is a member of the histone lysine demethylase (KDM) family of enzymes that catalyse the removal of methyl groups from lysine residues and are involved in transcriptional regulation of gene expression9–11. Like the PHDs, the enzymatic activity of KDMs relies on 2-OG, Fe(II) and the presence of molecular oxygen as essential cofactors12–15. KDM4A expression has been reported to be increased in several cancer types including colorectal, this combined with a number of functional studies have suggested KDM4A is an attractive target for cancer therapy16–20. In this study, we describe a novel role for KDM4A in the regulation of HIF-1α mRNA expression and identify inhibition of KDM4A as a unique strategy to decrease HIF signalling. These findings strongly support the hypothesis that KDM4A is a potential therapeutic target for improving the treatment response of radioresistant, hypoxic solid tumours.

1Cancer Research UK and Medical Research Council Oxford Institute for Radiation Oncology, Department of Oncology, The University of Oxford, Oxford, OX3 7DQ, UK. 2Division of Cardiovascular Medicine, Radcliffe Department of Medicine, Wellcome Trust Centre of Human Genetics, Roosevelt Drive, The University of Oxford, Oxford, OX3 7BN, UK. 3Department of Chemistry, Chemistry Research Laboratory, The University of Oxford, Mansfield Road, Oxford, OX1 3TA, UK. 4Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, Oregon, USA. Correspondence and requests for materials should be addressed to E.M.H. (email: Ester.Hammond@oncology.ox.ac.uk)
Results

KDM4A as a therapeutic target. KDM4A has been described as over-expressed in a range of cancer types although the underlying mechanism is unclear. As both KDM4B and KDM4C have been demonstrated to be targets of HIF-1, we investigated the possibility that KDM4A is also hypoxia regulated by correlating expression with a previously described hypoxia signature\(^21\)-\(^23\). Using the TCGA colorectal adenocarcinoma data set, we found no significant correlation between KDM4A expression and the hypoxia signature. In contrast, KDM4B expression did positively correlate with the hypoxia signature (Supplementary Figure S1A,B). In support of this finding there was no increase in KDM4A mRNA expression after exposure to hypoxia (2% or <0.1% O\(_2\)) (Supplementary Figure S1C). However, in agreement with previous reports suggesting KDM4A has a prolonged half-life in hypoxia, there was a clear increase in KDM4A protein levels in RKO cells exposed to either 2% or <0.1% O\(_2\), (Fig. 1A)\(^24\),\(^25\). As expected, the levels of H3K9me3 and H3K36me3 increased in response to <0.1% O\(_2\) (Fig. 1B)\(^26\),\(^27\). Interestingly, although the effect of depletion of KDM4A on the total levels of H3K9me3 and H3K36me3 was slight, this was most pronounced at 2% O\(_2\) (Fig. 1B and Supplementary Figure S1D). Since KDM4A has been described as having the potential to act as an oxygen sensor and is highly sensitive to oxygen concentration, it is likely that while KDM4A would retain activity in our mild hypoxic conditions (2% \(\text{O}_2\)), it would be inactive at the more severe level (<0.1% \(\text{O}_2\))\(^28\). To evaluate KDM4A as a potential therapeutic target we examined the KDM4A-dependent contribution to key biological processes associated with cancer aggressiveness. Depletion of KDM4A significantly decreased the extracellular acidification rate (ECAR) in RKO cells exposed to hypoxia, which is
Depletion of KDM4A decreases the HIF-1 response in hypoxia. In contrast to ML324/JIB-04 treatment, depletion of KDM4A using siRNA led to a significant decrease in HIF-1α levels in hypoxia. This decrease in HIF-1α was more significant in cells exposed to 2% O2 compared to <0.1% O2. Furthermore, KDM4A depletion led to a decrease in cell metabolism and migration (Fig. 3A, B). The levels of CAIX and Glut1, both HIF-1 targets, were also decreased in a KDM4A-dependent manner. Decreased HIF-1α after siRNA-mediated depletion of KDM4A was validated using additional siRNAs to validate that this was not due to an off-target effect and was also verified in additional cell lines (MDA-MB-231 and HCT116) (Supplementary Figure S3A, B, and C). Interestingly, a decrease in HIF-2α was also observed in HCT16 cells exposed to hypoxia (2% O2) upon depletion of KDM4A (Supplementary Figure S3C). Decreased HIF-1 activity in hypoxia after KDM4A depletion was further confirmed using a reporter assay (Fig. 3B) and by measuring the expression of specific HIF target genes; Glut1 (Supplementary Figure S3D), CAIX (Supplementary Figure S3E), SLC2A3/Glut3, which regulates metabolism (Fig. 3C) and TWIST1, which has a role in cell migration (Fig. 3D). In each case loss of KDM4A leads to decreased induction of the HIF-target gene in response to hypoxia (2% O2). Loss of KDM4A did not impact gene expression in <0.1% O2 and we attribute this to the oxygen dependency of KDM4A and suggest that as the demethylase would be unable to function at <0.1% O2, there was no added effect of reducing the level of KDM4A protein. To ensure that loss of KDM4A had not reduced transcription in general we measured the expression of a non-hypoxia responsive gene, OA21, and found this to be unaffected by KDM4A loss (Supplementary Figure S3F). Interestingly, recent reports describe a novel mechanism in which KDM4A interacts with and co-activates the transcription factors E2F1 and ETV1, which have roles in regulating cell metabolism and migration30, 31. ZEB2 is a known target of E2F1 and HIF-1 and so we investigated the KDM4A-dependent effects on expression in both normoxia and hypoxia32. ZEB2 was not induced in response to hypoxia in the RKO cells used, however loss of KDM4A significantly decreased ZEB2 expression in both normoxia and hypoxia (2% O2) (Fig. 3E). Loss of KDM4A had a similar effect on SNAI1 expression, which is regulated by ETV1 and HIF-1 (Fig. 3F). It is likely that the KDM4A-dependent effects on ZEB2 and SNAI1 expression observed in normoxia could be attributed to decreased activity of E2F1 and ETV1 respectively, whilst the effects of KDM4A as a therapeutic target.
seen in hypoxia may result from a combination of repressed E2F1/ETV1 and HIF-1 activity. Again, there was no significant impact of depleting KDM4A on gene expression in the more severe hypoxia (<0.1% O2).

HIF-1α mRNA expression is regulated by H3K9me3. HIF-1α stability is known to be controlled via post-translational modifications involving hydroxylation, acetylation, ubiquitination and phosphorylation36. To determine the mechanism behind the reduced levels of HIF-1α protein after KDM4A depletion, we

Figure 2. ML324 stabilises HIF-1α protein levels in normoxic conditions (21% O2). (A) RKO cells were treated with ML324 (10 μM) for 48 h in either 21% or 2% O2. Western blotting was then carried out. (B) RKO cells were treated as in (A) with JIB-04 (5 μM). Uncropped blots are shown in Figure S6A and B. (C) RKO cells co-transfected with HRE (HIF-1 responsive element)-Firefly Luciferase and Renilla Luciferase were treated with ML324 (10 μM) for 24 h, incubated in 21% O2 or 2% O2 for an additional 24 h and the intensity of Firefly Luciferase was measured relative to the levels of Renilla Luciferase. (D) The relative motility of MCF-7 cells treated with ML324 was determined by xCELLigence assay. (E) Oxygen consumption rate (OCR) of RKO cells treated with ML324 (10 μM) for the indicated periods of time was measured. (F) Extracellular acidification rate (ECAR) of RKO cells treated with ML324 (10 μM) for the indicated periods of time was measured. (G) IC50 determination of inhibitors against PHD2 and FIH using mass spectrometry assay. Italicised IC50 values are reported values from literature30–32. The recombinant proteins used were PHD2 181-42643 and recombinant FIH as previously described44. Dose response curves were generated as shown in Supplementary Figure S2G and H and used to calculate the IC50 values shown.
began by analysing the levels of HIF-1α mRNA in cells lacking KDM4A exposed to hypoxia (2% O₂) or <0.1% O₂. Surprisingly, we found that loss of KDM4A led to a significant decrease in HIF-1α mRNA in both normoxia and the milder hypoxic conditions (2% O₂) (Fig. 4A). A significant decrease in HIF-2α mRNA levels were also observed in cells depleted of KDM4A in hypoxic (2% O₂) conditions (Supplementary Figure S4A). We did not see a similar effect on HIF-1α mRNA levels when KDM4B or C were depleted (Supplementary Figure S4B,C,D). These data also highlight that HIF-1α mRNA expression is reduced in severely hypoxic conditions (<0.1% O₂) and that this was not further reduced by loss of KDM4A. A natural antisense of HIF-1α transcript (aHIF-1α), which is complementary to the 3′-untranslated region of the HIF-1α mRNA, has been described to serve as an additional level of HIF-1α transcription regulation. We asked whether KDM4A-depletion could influence the levels of aHIF-1α. We observed that the expression of aHIF-1α increased in response to hypoxia (2% and <0.1% O₂), but depletion of KDM4A did not have significant effect (Fig. 4B). In addition, depletion of KDM4A did not have a significant effect on the levels of key transcription factors known to play a role in HIF-1α mRNA expression. Using the UCSC genome browser (GRCh37/hg19 genome assembly), we found regions of moderate enrichment of the H3K9me3 chromatin mark along the HIF-1α gene (Supplementary Figure S4F). Therefore, we carried out a ChIP assay for H3K9me3 in KDM4A-depleted cells which were incubated in normoxia or hypoxia (2% and <0.1% O₂) (Fig. 4C). This analysis provided a number of key findings.

Figure 3. Depletion of KDM4A leads to decrease of HIF-1α activity in hypoxia (2% O₂). (A) RKO cells were treated with siKDM4A and then incubated in 21%, 2% or <0.1% O₂ for 24 h. Western blotting was carried out. Uncropped blots are shown in Figure S7. (B) FaDuHRE-Luc cells were treated as in part (A) and the relative intensity of Firefly Luciferase was measured relative to the number of cells in the respective conditions. RKO cells were treated as in part (A) and then mRNA levels were determined; (C) Glut3, (D) TWIST1, (E) ZEB2 and (F) SNAI1.
Firstly, we noticed that H3K9me3 accumulates at the 
HIF-1A
gene in response to severe hypoxia (<0.1% O2), offering a potential explanation for why HIF-1α mRNA levels decrease in these conditions. Secondly, whilst H3K9me3 did not accumulate at the 
HIF-1A
gene in response to 2% O2, this was significantly increased by the loss of KDM4A. Finally, loss of KDM4A did not alter the levels of H3K9me3 on the 
HIF-1A
gene in severe hypoxia (<0.1% O2) and this is consistent with the demethylase being inactive in these conditions (Fig. 4D,E). In addition, we observed that depletion of KDM4A led to increased levels of H3K9me3 on 4 other regions (Supplementary Figure S4G).

**Discussion**

This study describes a novel role of the KDM4A histone demethylase in regulating HIF-1α expression and activity. Most interestingly, our study describes a novel transcriptional method of HIF-1 regulation. In normal conditions a balance of methyl transferase and demethylase activity regulates repressive marks such as H3K9me3 and
therefore gene expression. Here, we showed that when the activity of one such demethylase, KDM4A, is restricted due to depletion of the protein or lack of an essential co-factor, such as oxygen, H3K9me3 accumulates along the HIF-1A gene (Fig. 4E). This in turn leads to decreased levels of HIF-1α mRNA and reduced protein stabilisation/ activity.

Although inhibition of KDM4A has not undergone clinical testing yet in cancer patients it is widely considered an attractive strategy. Caution is warranted however, as the likely off-target effects of active-site metal-chelating KDM inhibitors include stabilising HIF through direct or indirect (e.g. via altering Fe(II) availability in cells) inhibition of related 2OG oxygenases PHD/FIH. Interestingly, a recent report demonstrated that LSD1 regulates HIF-1α, highlighting further overlap between the histone demethylases activity and HIF40. Most importantly, our data suggests that specific KDM4A inhibitors become available they may well have the previously unforeseen benefit of reducing HIF activity and therefore significantly impact tumour aggressiveness and therapy resistance.

Methods

Cell lines, transfections and drug treatments. RKO, MCF7, MDA-MB-231, HCT116 and FaDuHIF-RE-luc were cultured in Dulbecco’s modified Eagle’s medium (D-MEM) supplemented with 10% FBS and 1% penicillin/streptomycin. For siRNA experiments, cells were transfected with 10 nM siRNA using Lipofectamine RNAiMAX in OptiMEM minimal medium (Invitrogen). As a negative control, ON-TARGETplus Non-targetting Control Pool siRNA (Dharmacon, D-001810-10) was used. siKDM4A S1 (Ambion, ID: 148456), siKDM4A Q1-Q4 (Qiagen, 10274116), siKDM4B (Ambion, ID: 148507), siKDM4C (Ambion, ID: 106664), siKDM4D (Dharmacon, siGENOME SMARTpool D-027097-01 to 04). ML324 (Sigma-Aldrich, SML0741), JIB-04 (Sigma-Aldrich, SML0808) and IOX1 (Sigma Aldrich, SML0067).

Hypoxia treatment. Hypoxic treatments at 2% O2 were carried out in a Don Whitley H35 Hypoxystation and at <0.1% O2 in a Bactron chamber (Shel Lab). Radiation of cells in hypoxic conditions was performed as described previously45. Values are presented as mean ± SEM of three independent experiments.

Immunoblotting. Cells were lysed in UTB (9 M urea, 75 mM Tris-HCl pH 7.5, 0.15 M (3-mercaptoethanol) and briefly sonicated. Primary antibodies used: HIF-1α (BD Transduction Labs., 610959), KDM4A (Abcam, ab24545), CAIX (Biotechne, AB1001), Glut1 (Abcam, ab14683), Actin (Santa Cruz, sc-69879), H3K9me3 (Millipore, 07-422), H3K36me3 (Abcam, ab9050), H3 (Cell Signaling, 36385), NFκB p52 (Millipore, 05-361), Sp1 (Millipore, 07-645), E2F-1 (Cell Signaling, 3742S), HIF-2α (Novus Biologicals, NB100-122). Secondary antibodies were IRDye® 680RD Goat anti-Mouse IgG (H+L), IRDye® 800CW Donkey anti-Mouse IgG (H+L) and IRDye® 800CW Donkey anti-Rabbit IgG (H+L) from LI-COR Biosciences. Odyssey IR imaging technology (LI-COR Biosciences) was used for imaging.

qRT-PCR. mRNA was prepared using TRI reagent (Sigma). A NanoDrop was used for quantification. cDNA was synthesised using the Verso cDNA Enzyme kit (Life Technologies). qPCR was carried out with SYBR mix using a Step One Plus Real-time PCR Detection System (Applied Biosystems). All mRNA expression levels are normalised to siCtrl 18S mRNA. Primer sequences are available in the SI. Values are presented as mean ± SEM of three independent experiments.

HIF reporter assay. Luciferase was measured with Dual-Glo® Luciferase Assay System (Promega). Readings of RKO cells were normalised to Renilla Luciferase signal whereas for FaDuHIF-RE-luc they were normalised to the number of cells in each condition. Values are presented as mean ± SEM of three independent experiments.

ChIP. As previously described28, IgG (Cell Signaling), H3 (Abcam, ab1791), H3K9me3 (Abcam, ab8898) antibodies were used. Primers are listed in SI. Values are presented as mean ± SEM of three independent experiments.

Measurement of ECAR and OCR. ECAR was measured using a Seahorse Bioscience XF96 Extracellular Flux Analyzer (Agilent Technologies). RKO cells (16000 cells/well) were seeded on polystyrene Seahorse XF Cell Culture Microplates and depleted of KDM4A for 48 h. 24 h before the experiment, cells were incubated in 2% O2 for 24 h. The medium was changed to physiological medium (XF Assay Medium with 5 mM Glucose, 5 mM Pyruvate, 4 mM Glutamine, pH 7.4) and measurements immediately acquired. ECAR was calculated by the Seahorse XF96 software and subsequently normalised to cell number, which was quantified by staining the cells with Hoechst and measuring the intensity with POLARstar Omega microplate reader Spectrophotometer (BMG Labtech). Values are presented as mean ± SEM of three independent experiments.

xCELLigence assay. The xCELLigence Real-Time Cell Analyser (RTCA) DP Instrument equipped with a CIM-plate 16 (Roche) was used as previously described. For quantification, the cell index at indicated time points was averaged from three independent experiments.

Statistical analysis. Statistical significance was calculated using GraphPad Prism software. For qRT-PCR, HIF reporter assay, ChIP and Seahorse results unpaired, two-tailed t-test was used, whereas for xCELLigence and colony formation experiment Two-way ANOVA with Alpha 0.05 was employed.

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**Author Contributions**

E.H., A.J.K., A.K. and G.D. wrote the main manuscript text. G.D. wrote the supplementary text. G.D. conducted the majority of the experiments with help from K.B.L. and S.H. TEM carried out the PHD/FIH inhibition assays with supervision from A.K. All authors read and approved the manuscript.

**Additional Information**

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