Repression of hypoxia-inducible factor α signaling by Set7-mediated methylation

Xing Liu[1,2], Zhu Chen[2,3], Chenxi Xu[1,2], Xiaoqian Leng[1,2], Hong Cao[1,2], Gang Ouyang[2] and Wuhan Xiao[1,2,*]

[1]State Key Laboratory of Freshwater Ecology and Biotechnology Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, P. R. China, [2]The Key Laboratory of Aquatic Biodiversity and Conservation, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, P. R. China and [3]Department of Reproduction, Maternal and Child Health Hospital of Hubei Province, Wuhan, 430070, P. R. China

ABSTRACT

Hypoxia-inducible factor (HIF)-1α and HIF-2α are the main regulators of cellular responses to hypoxia. Post-translational modifications of HIF-1α and 2α are necessary to modulate their functions. The methylation of non-histone proteins by Set7, an SET domain-containing lysine methyltransferase, is a novel regulatory mechanism to control cell protein function in response to various cellular stresses. In this study, we show that Set7 methylates HIF-1α at lysine 32 and HIF-2α at lysine K29; this methylation inhibits the expression of HIF-1α/2α targets by impairing the occupancy of HIF-α on hypoxia response element of HIF target gene promoter. *Set7*-null fibroblasts and the cells with shRNA-knocked down Set7 exhibit upregulated HIF target genes. Set7 inhibitor blocks HIF-1α/2α methylation to enhance HIF target gene expression. *Set7*-null fibroblasts and the cells with shRNA-knocked down Set7 or inhibition of Set7 by the inhibitor subjected to hypoxia display an increased glucose uptake and intracellular adenosine triphosphate levels. These findings define a novel modification of HIF-1α/2α and demonstrate that Set7-medited lysine methylation negatively regulates HIF-α transcriptional activity and HIF-1α-mediated glucose homeostasis.

INTRODUCTION

Lysine methylation in non-histone proteins acts as a novel regulatory mechanism to control protein functions (1–8). Set7, also known as Setd7, Set9 and Set7/9, was originally identified as a monomethylase of histone H3 lysine 4; thus, Set7 is involved in gene activation (9,10). Set7 monomethylates various non-histone proteins, including p53, TAF10, DNMT1, E2F1, AR, ERα, RelA, PCAF, Stat3, Yap, FOXO3a, Suv39h1 and pRb (11–28). This methylation produces varied results. For instance, the methylation of p53, TAF10, AR, ERα and pRb enhances their corresponding activities by improving protein stability or via other mechanisms (11,12,15,16,18–20); by contrast, the methylation of DNMT1, Stat3, Yap, Foxo3a and Suv39h1 impairs their corresponding activities (13,24–28). However, contradictory results have been observed in E2F1 and RelA methylation (14,17,21,22,29). Furthermore, the *in vivo* function of p53 methylation by Set7 remains debatable (16,30,31). In most cases, the significance of non-histone methylation by Set7 should be investigated. Set7 substrates and underlying biological consequences should be detected to elucidate the physiological relevance of Set7 in catalyzing the monomethylation of non-histone proteins.

Oxygen homeostasis is critical for normal functioning and development of aerobic organisms (32–36). Low ambient oxygen stimulates hypoxic responses, an ancient stress response controlled by hypoxia-inducible transcription factors (such as HIF-1 and HIF-2). The α subunit of HIF-1 or HIF-2 is rapidly degraded by the pVHL E3 ubiquitin ligase complex under normoxia; conversely, this subunit is stabilized when O_2-dependent prolyl hydroxylase that targets the O_2-dependent degradation domain of HIF-α is inhibited under hypoxia (37–43). HIF activation under hypoxia induces numerous genes involved in energy metabolism and promotes angiogenesis to maintain tissue integrity/homeostasis; thus, organisms can adapt to cellular hypoxia (44–47).

HIF-α is mainly regulated post-translationally; post-translational modifications, including ubiquitination, sumoylation, phosphorylation and acetylation, significantly contribute to the biological functions of HIF-α (48,49). However, converse effects may be observed in some cases. HIF-1α sumoylation can stabilize HIF-1α or de-stabilize HIF-1α (50–53). MAPK-induced phosphorylation of Ser-641/643 and CDK1-induced phosphorylation of Ser-668 enhance the transcriptional activity of HIF-1α

*To whom correspondence should be addressed. Tel: +86 27 68780087; Fax: +86 27 68780087; Email: w-xiao@ihb.ac.cn

(54,55), conversely, casein kinase 1-induced phosphorylation of Ser-247 and Plk3-induced phosphorylation of Ser-576/657 impair HIF-1α activity (56,57). The reversible lysine acetylation/deacetylation of HIF-1α/2α positively or negatively regulates their transcriptional activity (48,49,58,59). Acetylation at Lys-532 in HIF-1α leads to VHL-dependent HIF-1α degradation, whereas acetylation at Lys-674 is beneficial for HIF-1α transcriptional activity (48,60). Further investigations can reveal post-translational modifications of HIF-1α/2α and underlying biological consequences.

Lysine methylation of non-histone proteins is involved in stress responses. FOXO3a methylation regulates oxidative stress-induced neuronal cell death (17). Set7 is also a critical regulator of E2F1 activity in response to genotoxic stresses (29). Under oxidative stress, Set7 methylates ARTD1 to enhance poly-ADP-ribose formation (61). Despite these studies, whether Set7 is involved in hypoxia stress by methylating the main regulators, namely, HIF-1α and HIF-2α, of the hypoxia signaling pathway remains unknown.

In this study, HIF-1α and HIF-2α were identified as novel substrates of Set7. Set7 monomethylates HIF-1α at lysine 32 and HIF-2α at lysine 29; as a result, HIF-1α/2α transcriptional activity is inhibited. Set7 further inhibits HIF-1α-mediated metabolic reprogramming and functions as a hypoxia-suppressive gene. Our finding reveals a novel function of Set7 in the hypoxia signaling pathway.

MATERIALS AND METHODS

Cell line and culture conditions

HEK293T, HepG2, HCT116, H1299, HT29 and 786-O cells were obtained from ATCC. RCC4 cells were provided by Peter J. Ratcliffe. HEK293T, HepG2 and RCC4 cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) (HyClone) with 10% fetal bovine serum (FBS). 786-O and H1299 cells were cultured in RPMI 1640 (HyClone) with 10% FBS. HCT116 and HT29 cells were cultured in Mc-Coy5A (HyClone) with 10% FBS. Set7 wild-type and Set7-null mouse embryo fibroblasts (MEFs) were maintained in DMEM supplemented with sodium pyruvate (110 mg/l), 10% FBS, 1× nonessential amino acids (Sigma) and 1% penicillin–streptomycin. The cells were grown at 37°C in a humidified incubator containing 5% CO_2. The cells were cultured under hypoxic condition (1% O_2) by using an incubator with O_2 control, filled with 5% CO_2, and balanced with N_2 (NBS Galaxy 48R).

Plasmid construction

The p2.1 reporter was purchased from ATCC. VEGF promoter luciferase reporter, EPO promoter luciferase reporter, BNIP3 promoter luciferase reporter, hypoxia response element (HRE) reporter and PAI-1 promoter luciferase reporter were provided by Amato Giaccia, Eric Huang, Spencer Gibson, Navdeep Chandel and Xin-Hua Feng. Wild-type human Set7 gene (originally obtained from Zengqiang Yuan) and its enzymatically dead mutant from H297A were PCR amplified and subcloned into the pCMV-Myc vector and the lentivirus vector pHAGE-CMV-MCS-IZsGreen. Wild-type human HIF-1α and K32R mutant

were subcloned into the pCMV-HA vector; wild-type human HIF-2α and its K29R mutant were subcloned into the pCMV-Tag2C vector (Stratagene). Human HIF-1α domains were subcloned into pGEX-2B, and human Set7 gene was cloned into pET-32α. Set7 short hairpin RNAs (shRNAs) and control shRNA (GFP) were cloned into pSuper-Retro-Puro vector. HIF-1α shRNAs, Set7-shRNAs and control shRNA (luciferase) were cloned into the lentivirus vector lentiLox3.7.

Antibodies and chemical reagents

Anti-Myc (9E10), anti-His (H-15), anti-GAPDH (0411) and anti-HIF-1α (H206) antibodies were purchased from Santa Cruz. Anti-hemagglutinin (anti-HA) antibody was purchased from Covance. Anti-flag (F1804) antibody was purchased from Sigma. Anti-α-tubulin (EPR1333) and anti-Set7 (EPR5574) antibodies were purchased from Epitomics. Anti-HIF-1α (NB100–105) was purchased from Novus Biologicals. Anti-SOD2 (EPR2560Y) antibody was purchased from GeneTex. Anti-PAI-1 (612025) antibody was purchased from BD Transduction Laboratories. Anti-Histone H3 [(D1H2)XP] antibody was purchased from Cell Signaling. Anti-HIF-1α(CH₃)-K32 polyclonal antibody was obtained at Abmart with the HIF-1α-K32-me1 peptide (Shanghai, China). The active Set7 protein (14–469) was purchased from Millipore, and S-adenosylmethionine (SAM) (B903S) was purchased from New England Biolabs (NEB). 2ME2 (S1233) was purchased from Selleck, and (R)-PFI-2 was obtained from Tocris. Glucose assay kit was purchased from BioVision, and adenosine triphosphate (ATP) assay kit from Beyotime. 6-[*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose(2-NBDG), a glucose analog, was purchased from Invitrogen.

Virus production

Lentiviruses for wild-type Set7, its H297A mutant and control (empty vector) were generated by transfecting HEK293T cells with the transducing vector and packaging vectors, including pMD.2 and pspAX. Lentiviruses used to knock down Set7, HIF-1α and control (targeting luciferase) were generated by transfecting HEK293T cells with a transducing vector and packaging vectors, including VSVG, RSV-REV and pMDL g/p RRE. After transfection was performed for 48 h, virus particles in the medium were harvested, filtered and transduced into target cells. The following shRNA target sequences were used: control shRNA (luciferase shRNA), 5′-GTTGGCACCAGCAGCGCAC-3′,

Set7 shRNA-1, 5′-GGGAGTTTACACTTACGAA-3′,
Set7 shRNA-2, 5′-GCCTTGTAGGAGAAGTAAA-3′,
HIF-1α shRNA-1, 5′-GAGCTTGCTCATCAGTTGC-3′,
HIF-1α shRNA-2, 5′-GGGTTGAAACTCAAGCAAC-3′,

Luciferase reporter assays

The cells were grown in 24-well plates and transfected with various amounts of plasmids by VigoFect (Vigorous

Biotech, Beijing, China). pTK-*Renilla* was used as an internal control. After the cells were transfected for 18–24 h, luciferase activity was determined using a dual-luciferase reporter assay system (Promega). For luciferase reporter assays in HCT116 cells with knocked down Set7, shRNA targeting green fluorescent protein (GFP; targeting sequence: 5′-GCAAGCTGACCCTGAAGTTCAT-3′) in pSuper was used as control. Transient transfection was conducted for subsequent assays.

Data were normalized to *Renilla* luciferase. Data were reported as mean ± standard error of the means (SEM) from three independent experiments performed in triplicates. Statistical analysis was performed using Graph Pad Prism 5 (unpaired *t*-test; GraphPad Software Inc., San Diego, CA, USA).

Immunoprecipitation and western blot

Coimmunoprecipitation and western blot analysis were performed as previously described (62). Anti-Myc antibody-conjugated agarose beads were purchased from Sigma. Protein A/G-Sepharose beads were purchased from GE Company. Fuji Film LAS4000 mini-luminescent image analyzer was used to photograph the blots. Multi Gauge V3.0 was used to quantify protein levels based on the band density obtained by western blot analysis.

In vitro methylation assay

The samples were incubated at 30°C for 60 min in a reaction buffer containing 50 mM Tris-HCl (pH 8.5), 5 mM MgCl$_2$, 4 mM DTT, active Set7 protein and 1 μM SAM (NEB). HIF-1α K32 peptide or GST–HIF-1α (amino acids 1–80) fusion proteins (1 μg) were used as substrates. The total volume of the reaction mixture was adjusted to 10 μl. The samples were subjected to dot blot or separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis; western blot analysis was conducted to detect methylation with an anti-HIF-1α(CH$_3$)-K32 antibody.

Chromatin immunoprecipitation

The following primers were used to amplify the LDHA gene promoter region: 5′-TTGGAGGGCAGCACCTTACTTAGA-3′ and 5′-GCCTTAAGTGGAACAGCTATGCTGAC-3′. The following primers were used to amplify the RPL13A promoter region were 5′-GAGGCGAGGGTGATAGAG-3′ and 5′-ACACACAAGGGTCCAATTC-3′. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (63).

Semi-quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized using a first-strand cDNA synthesis kit (Fermentas). The following primers were used for human Set7: 5′-TTCACTCCAAACTGCATCTACGA-3′ and 5′-GGGTGCGGATGCATTTG-3′. The following primers were used for internal control 18SrRNA: 5′-TCAACTTCGATGGTAGTCGCCGT-3′ and 5′-TCCTTGGATGTGGTAGCCGTTCT-3′. Other primers were synthesized, as described in previous reports (64,65).

Glucose uptake assays

RCC4 cells were transduced with a lentivirus encoding control shRNA or Set7 shRNAs. Intracellular glucose level was determined with a fluorescence-based glucose assay kit (BioVision).

2-NBDG glucose uptake assays were also performed for MEFs and RCC4 cells. In brief, the cells were grown under specified conditions with or without 100 μM 2-NBDG (Invitrogen) in the media for 1 h. Glucose uptake was quantified by flow cytometry (FACS), as previously described (64,66).

ATP concentration assays

RCC4 cells were transduced with lentivirus encoding control shRNA or Set7 shRNAs, in the absence or presence of (R)-PFI-2 (1 μM, 24 h), and ATP concentration was measured by ATP assay kit (Beyotime) following manufacturer instruction.

Nucleus and cytoplasm separation

Nucleus and cytoplasm separation was conducted using the Nucl-Cyto-Mem Preparation Kit (P1201, Apply Gen.). Briefly, HEK293T cells transfected with indicated plasmids were washed with phosphate buffered saline and lysed by sonicating for 40 times in 500 μl CER buffer provided by the kit. The homogenate was centrifuged at 800 × *g* for 5 min and the pellet was saved as the crude nuclei, which was then washed twice with 500 μl NER buffer provided by the kit.

RESULTS

HIF-1α is methylated by a lysine-specific methyltransferase Set7 at lysine 32 *in vitro* and *in vivo*

To determine the involvement of Set7 in the hypoxia signaling pathway, we analyzed whether the HIF-1α protein sequence harbors a conserved domain. The three 'RSK' residues in the bHLH domain (amino acids 30–32) of HIF-1α (Figure 1A1) are identical to the core local recognition sequence of Set7, namely, (K/R) (S/T) K. This core sequence is identified in Set7-targeted proteins, such as histone H3, p53, E2F1, estrogen receptor-α and LIN28A (9,11,17,19,20,67,68). These three residues are also evolutionarily conserved among human, macaque, cow, pig, dog, mouse and rat (Figure 1A1). Thus, HIF-1α is a potential substrate of Set7.

To determine the role of Set7 on HIF-1α methylation *in vivo*, we generated a polyclonal antibody that specifically recognizes monomethylated HIF-1α-K32. We initially screened rabbit antibodies against HIF-1α peptides harboring K32 with different degrees of methylation. An antibody that specifically recognized a monomethylated peptide but failed to detect an unmodified peptide or equivalent peptides that were di- or tri-methylated was selected by dot-blot assays [anti-HIF-1α(CH$_3$)-K32] (Figure 1A2).

To determine whether HIF-1α is a novel substrate of Set7, we overexpressed wild-type Set7 or enzymatically dead mutant Set7 (H297A) with the wild-type HIF-1α. We
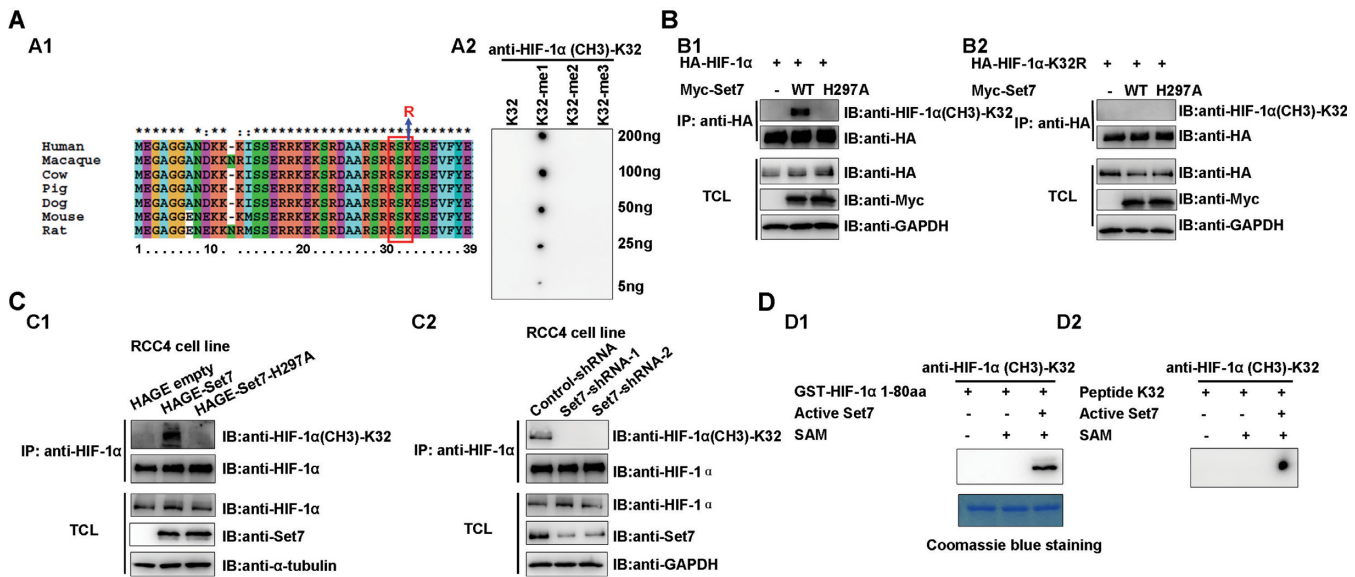
Figure 1. Set7 methylates HIF-1α. (**A**) (A1) Sequence alignment of partial HIF-1α proteins (1–39 amino acids) of human, macaque, cow, pig, dog, mouse and rat. The red box indicates a conserved consensus motif (R/K-S/T-K) methylated by Set7; the blue arrow indicates the conserved lysine (K32) mutated to arginine in methylation assays. The amino acid is numbered based on human HIF-1α amino acid sequence. (A2) Dot-blot assay for HIF-1α peptides containing various degrees of methylation. Equal amounts of unmodified (K32), mono-(K32-me1), di-(K32-me2) and tri-(K32-me3) methylated HIF-1α were immunoblotted with monomethyl HIF-1α antibody [anti-HIF-1α(CH₃)-K32]. (**B**) (B1) Western blot analysis of HIF-1α methylated by the wild-type Set7 or mutant Set7 (H297A) in transfected cells with monomethyl HIF-1α antibody [anti-HIF-1α(CH₃)-K32]. Anti-HA agarose beads were used for immunoprecipitation. (B2) Western blot analysis of mutant HIF-1α (K32R) methylated by the wild-type or mutant Set7 (H297A) in transfected cells with monomethyl HIF-1α antibody [anti-HIF-1α(CH₃)-K32]. (**C**) (C1) Detection of endogenous methylated HIF-1α from whole-cell extracts of RCC4 stable cell lines overexpressing the wild-type or mutant Set7 (H297A). Methylated HIF-1α was detected by western blot analysis with anti-HIF-1αK32me antibody [anti-HIF-1α(CH₃)-K32] (top). Western blot signals obtained with anti-HIF-1α antibody as loading controls. Anti-HIF-1α conjugated agarose beads were used for immunoprecipitation. (C2) Detection of endogenous methylated HIF-1α from whole-cell extracts of RCC4 stable cell lines with knockdown of endogenous Set7 by Set7-shRNA-1 and Set7-shRNA-2. (**D**) (D1) Western blot analysis of recombinant partial HIF-1α peptide (1–80 amino acids) methylated by Set7 (Millipore) *in vitro* with monomethyl HIF-1α antibody [anti-HIF-1α(CH₃)-K32] (top). Coomassie-blue staining of the peptide (1–80 amino acids) as loading control (bottom). (D2) Western blot analysis of the synthesized HIF-1α peptide (ARSRRSKESEVF) methylated by Set7 (Millipore) *in vitro* with monomethyl HIF-1α antibody [anti-HIF-1α(CH₃)-K32].

then examined the methylation status of HIF-1α by using anti-HIF-1α(CH₃)-K32 antibody. Wild-type Set7, not mutant Set7 (H297A), could methylate HIF-1α (Figure 1B1). After the K32 residue in HIF-1α was mutated to arginine, anti-HIF-1α(CH₃)-K32 antibody could not detect the methylation of HIF-1α mutant, HIF-1α(K32R) (Figure 1B2). We concluded that an antibody specifically recognized K32-methylated HIF-1α and HIF-1α may be a novel substrate of Set7.

To further determine whether HIF-1α is methylated by Set7 *in vivo*, we used RCC4 cell, a pVHL-deficient clear cell renal carcinoma cell line with high HIF-α under normoxic condition (69). We transduced RCC4 cells with lentiviruses to overexpress or knock down Set7. The overexpression of the wild-type Set7 enhanced endogenous HIF-1α methylation, as revealed by coimmunoprecipitation assays; by contrast, the overexpression of the mutant Set7 (H297A) did not improve (Figure 1C1). The knockdown of Set7 by two Set7 shRNAs, namely, Set7-shRNA-1 and Set7-shRNA-2, decreased the endogenous methylation level of HIF-1α to lower than the basal level of the control (Figure 1C2). To determine whether Set7 can directly methylate HIF-1α, we performed *in vitro* methylation assays. The active Set7 protein (14–469) (Millipore) could effectively methylate the bacterial-purified HIF-1α fragment (amino acids 1–80) harboring the core local reorganization sequence of

Set7 (Figure 1D1). The active Set7 protein could also effectively methylate the synthesized unmodified peptide (NH₂-RSRRSKESEVF-COOH), as revealed by dot-blot assays (Figure 1D2).

These data suggest that Set7 methylates HIF-1α at K32 *in vitro* and *in vivo*.

HIF-1α interacts with Set7 *in vitro* and *in vivo*

We examined the interaction between HIF-1α and Set7 to determine the mechanism of Set7-mediated methylation. We transfected Myc-tagged Set7 (Myc-Set7) with HA-tagged HIF-1α (HA-HIF-1α) or HA empty vector (Figure 2A1), as well as HA-HIF-1α with Myc-tagged Set7 (Myc-Set7) or Myc empty vector, into HEK293T cells; we then performed coimmunoprecipitation assays. HA-HIF-1α or Myc-Set7 could efficiently pull down Myc-Set7 (Figure 2A1) or HA-HIF-1α (Figure 2A2), respectively; this result indicated that HIF-1α interacts with Set7. To determine whether these proteins interact endogenously, we performed coimmunoprecipitation assays in RCC4 cells by using a monoclonal antibody against HIF-1α. The results showed that endogenous HIF-1α could interact with endogenous Set7 (Figure 2B).

To determine whether Set7 can directly bind to HIF-1α and to which domains of HIF-1α, we performed GST
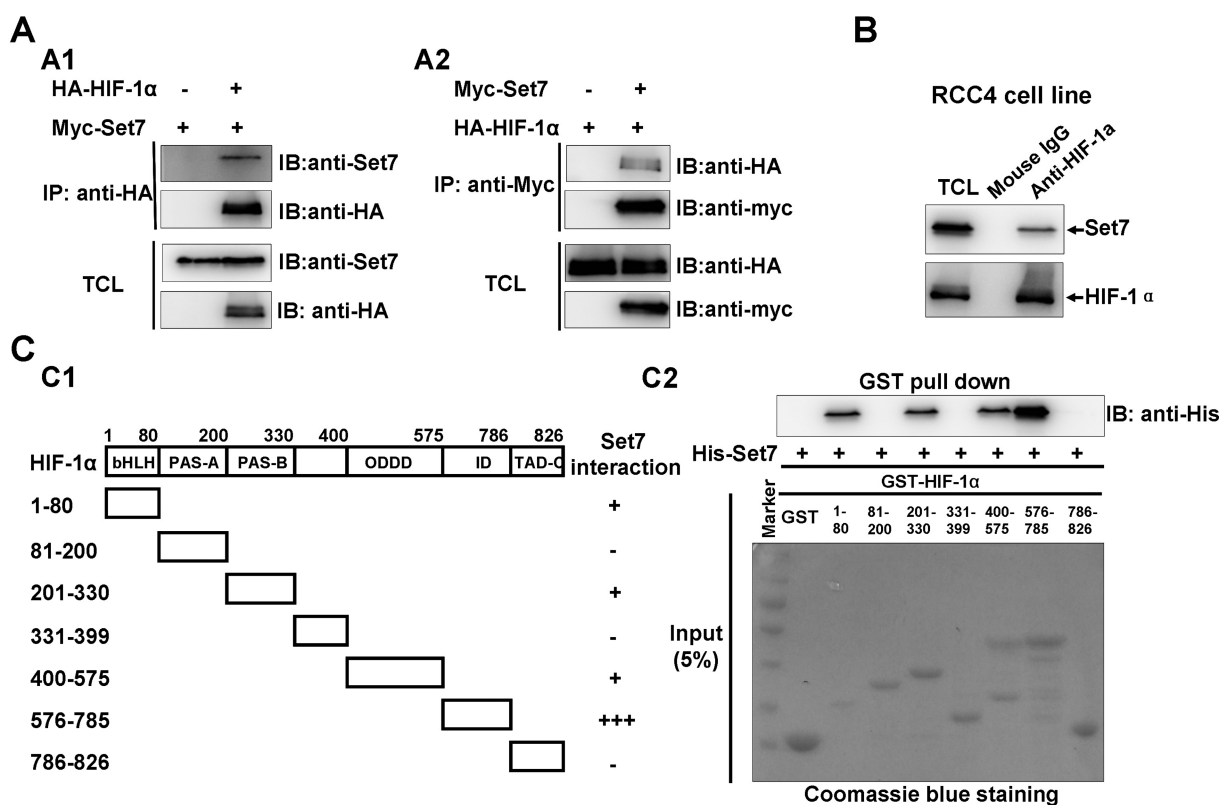
Figure 2. Set7 interacts with HIF-1α *in vitro* and *in vivo*. (**A**) Coimmunoprecipitation of Myc-tagged Set7 with HA-tagged HIF-1α and vice versa. Myc-tagged Set7 was transfected with the HA empty vector or HA-tagged HIF-1α into HEK293T cells, and anti-HA antibody conjugated agarose beads were used for immunoprecipitation (A1); HA-tagged HIF-1α was transfected with the Myc empty vector or Myc-tagged Set7 into HEK293T cells, and anti-Myc antibody conjugated agarose beads were used for immunoprecipitation (A2). (**B**) Endogenous interaction between HIF-1α and Set7. Western blot analysis of Set7 after coimmunoprecipitation with endogenous HIF-1α or mouse IgG control. (**C**) (C1) Schematic of the HIF-1α domains. The extent of the interaction between Set7 and HIF-1α domains is indicated by the number of plus signs (+). (C2) GST-pull down assay for GST-tagged HIF-1α domains and His-tagged Set7. The association of the GST-HIF-1α domains with His-Set7 was detected by immunoblotting with anti-His antibody. GST and GST fusion proteins were stained with Coomassie blue.

pull down assays. His-tagged Set7 and GST-tagged HIF-1α domains were bacterially expressed and purified. His-Set7 is strongly associated with GST-HIF-1α-(amino acids 576–785) and relatively weakly associated with GST-HIF-1α-(amino acids 1–80), (amino acids 201–330) and (amino acids 400–575). Nevertheless, the associations of His-Set7 with GST-HIF-1α-(amino acids 81–200), (amino acids 331–399) and (amino acids 786–826) were barely detected (Figure 2C). We concluded that Set7 directly interacts with HIF-1α to catalyze HIF-1α methylation.

### Set7 negatively regulates HIF-1α transcriptional activity by catalyzing HIF-1α methylation

To assess the functional importance of HIF-1α methylation by Set7 on HIF-1 transcriptional activity, we examined the effect of Set7 on the activation of five well-defined luciferase reporters, including BNIP3-luciferase (BNIP3-luc), EPO-luciferase (EPO-luc), VEGF-luciferase (VEGF-luc), p2.1-reporter and multiple HRE repeat reporters (70–74). The overexpression of Set7 in HEK293T cells inhibited the activity of these reporters under normoxic and hypoxic conditions (Figure 3A–E). The knockdown of Set7 in HEK293T cells by Set7-shRNA-1 and Set7-shRNA-2 significantly in-

creased the activity of p2.1 and EPO-luc reporters (Figure 4A1 and A2).

We further compared the reporter activity of BNIP3-luc and EPO-luc between the wild-type Set7 MEF cells (Set7$^{+/+}$) and Set7-null MEF cells (Set7$^{-/-}$). The activities of BNIP3-luc and EPO-luc were lower in the wild-type Set7 MEFs than those in Set7-null MEFs under hypoxia (Figure 4B1 and B2). The wild-type Set7 was recovered in Set7-null MEF cells (Set7$^{-/-}$) by overexpression; as a result, BNIP3-luc and p2.1-reporter activities were inhibited. Conversely, the overexpression of the enzymatically dead mutant Set7 (H297A) failed to inhibit the activities of BNIP3-luc and p2.1-reporter (Figure 4C1 and C2). These data suggest that Set7 represses HIF-1 transcriptional activity, and this repressive role may depend on the methyltransferase activity of Set7.

We directly examined the role of Set7 on HIF-1α target gene expression by semi-quantitative real-time PCR assays. The overexpression of Set7 in HepG2 cells significantly decreased *LDHA* expression under hypoxic condition (Figure 5A). The efficiency of Set7-shRNA-1 and Set7-shRNA-2 mediated knockdown of *Set7* in HCT116 cells was confirmed by semi-quantitative real-time PCR assays (Figure 5B1). The knockdown of Set7 by Set7-shRNA-1
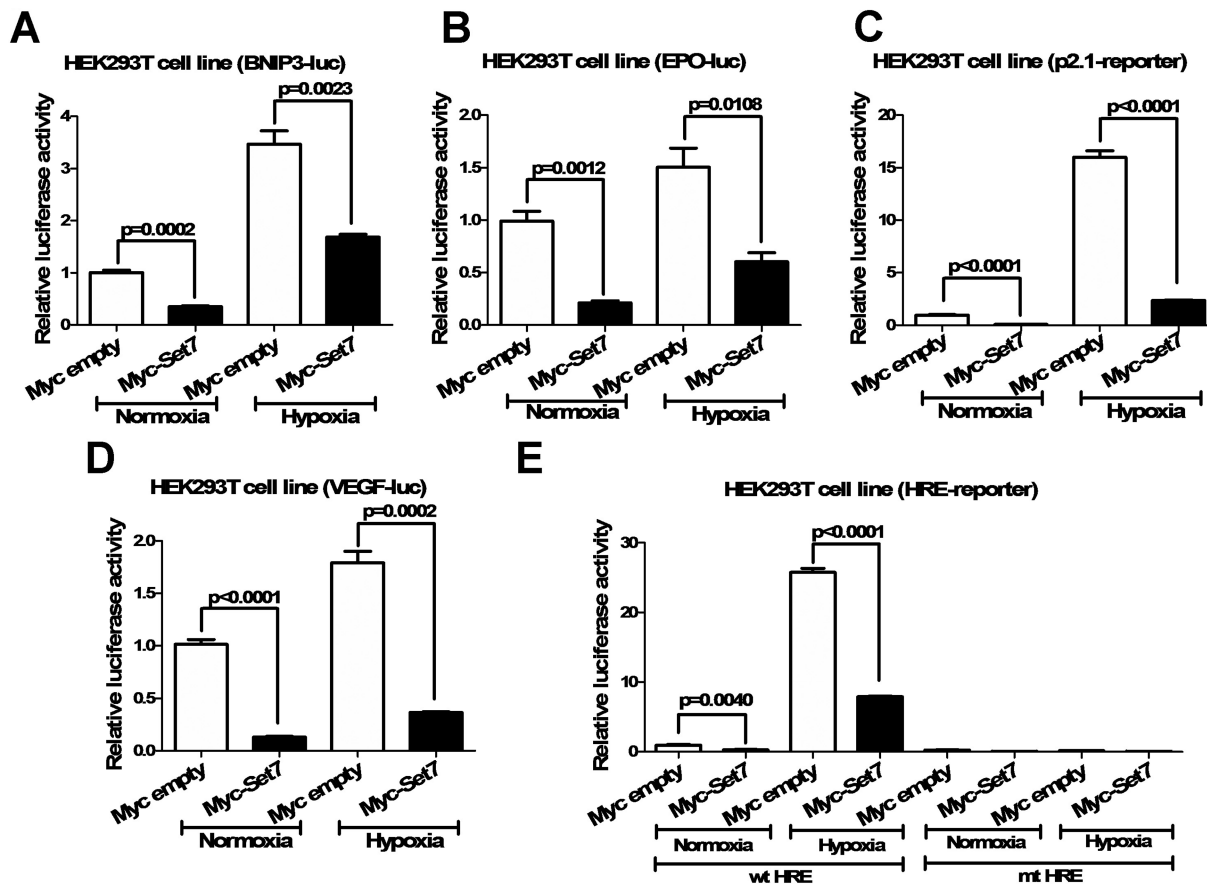
Figure 3. Set7 inhibits HIF-1α transcriptional activity. (**A**) The overexpression of Set7 inhibits the activity of BNIP3-luc, a well-defined hypoxia-induced reporter, in HEK293T cells under normoxia (20% O_2) or hypoxia (1% O_2) conditions. (**B**) The overexpression of Set7 inhibited the activity of EPO-luc, a well-defined hypoxia-induced reporter, in HEK293T cells under normoxia (20% O_2) or hypoxia (1% O_2) conditions. (**C**) The overexpression of Set7 inhibited the activity of p2.1 reporter, a well-defined hypoxia-induced reporter, in HEK293T cells under normoxia (20% O_2) or hypoxia (1% O_2) conditions. (**D**) The overexpression of Set7 inhibited the activity of VEGF-luc, a well-defined hypoxia-induced reporter, in HEK293T cells under normoxia (20% O_2) or hypoxia (1% O_2) conditions. (**E**) The overexpression of Set7 inhibited the activity of HRE-luc but not the mutant HRE in HEK293T cells under normoxic (20% O_2) or hypoxic (1% O_2) conditions. Luciferase activity was determined at 24 h post transfection. HRE, hypoxia responsible element; wt, wide type; mt, mutant type. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired *t*-test).

and Set7-shRNA-2 in HCT116 cells considerably increased the mRNA levels of *HK1* (Figure 5B2). We also compared the expression of HIF-1α target genes in wild-type Set7 MEFs and Set7-null MEFs. The mRNA levels of *Slc2a1*, *Pgk1* and *Vegfa* were higher in the Set7-null MEFs than in the wild-type Set7 MEFs (Figure 5C). In addition, the knockdown of *Set7* by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells significantly induced *LDHA* and *PDK1* expression (Figure 5D).

We also examined whether Set7 can directly suppress HIF-1α transcriptional activity. Under normoxic condition, the overexpression of HIF-1α increased the reporter activity of VEGF-luc and p2.1-reporter, but this increase was inhibited by co-transfection of Set7 (Figure 6A1 and A2). The transactivity of the HIF-1α mutant (K32R) was enhanced compared with that of the wild-type HIF-1α, as indicated by the activities of BNIP3-luc and HRE-reporter in HEK293T cells (Figure 6A3 and A4). This finding indicated that the endogenous Set7 lost its inhibitory role on the mutant; thus, transactivity was enhanced when the Set7-methylated site of HIF-α underwent mutation (K32R).

To determine whether the effect of Set7 on the hypoxia signaling is mediated by HIF-1α, we used the potent and specific inhibitor of HIF-α, namely, 2ME2 (75). As shown in Figure 6B, treatment with 2ME2 eliminated the effect of Set7 knockdown on the expression of HIF-1α target genes, including *SLC2A1*, *PDK1*, *EPO* and *PGK1* (Figure 6B1 to B4). Hence, the effect of Set7 on hypoxia signaling depends on the activity of endogenous HIF-α.

To gain insights into the mechanisms of Set7 on affecting the expression of HIF-1α downstream targets, we conducted ChIP assays. The knockdown of Set7 in RCC4 cells significantly enhanced HIF-1α occupancy at the HRE region of the *LDHA* promoter (Figure 6C). By contrast, the knockdown of Set7 did not affect the HIF-1α occupancy in the *RPL13A* promoter (Figure 6C2), which does not contain the HRE region. Therefore, the methylation of HIF-1α by Set7 may diminish HIF-1α occupancy on the HRE region of HIF-1 targets, resulting in gene downregulation.

Next, we examined whether the suppression of Set7 on HIF-1α transcriptional activity was due to the effect of Set7 on HIF-1α mRNA and protein levels. Overexpression of
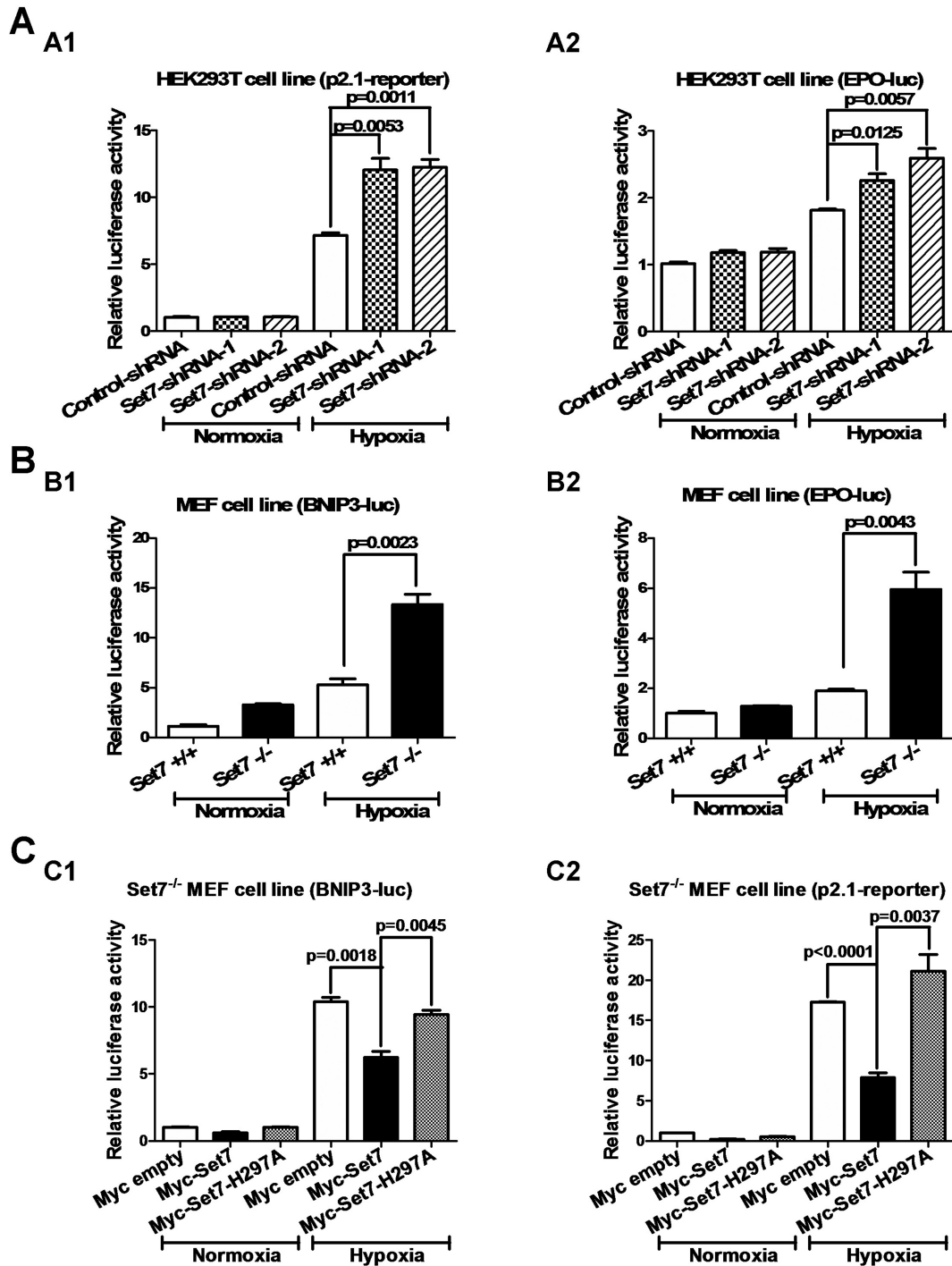
Figure 4. Depletion of Set7 enhances HIF-1α transactivity. (**A**) (A1) The knockdown of Set7 by Set7-shRNA-1 or Set7-shRNA-2 in HEK293T cells significantly enhanced the activity of p2.1 reporter under hypoxic condition ($P = 0.0053$ or $P = 0.0011$, respectively). (A2) The knockdown of Set7 by Set7-shRNA-1 or Set7-shRNA-2 in HEK293T cells significantly enhanced the activity of EPO promoter luciferase reporter under hypoxic conditions ($P = 0.0125$ or $P = 0.0057$, respectively). (**B**) (B1) The activity of BNIP3 promoter luciferase reporter was enhanced in Set7-null MEF cells (Set7$^{-/-}$) compared with that in the wild-type Set7 MEF cells (Set$^{+/+}$) under hypoxic conditions. (B2) The activity of EPO promoter luciferase reporter was enhanced in Set7-null MEF cells (Set7$^{-/-}$) compared with that in the wild-type Set7 MEF cells (Set$^{+/+}$) under hypoxic conditions. (**C**) (C1) The overexpression of the wild-type Set7 but not the mutant Set7 (H297A) in Set7-null MEF cells (Set7$^{-/-}$) inhibited the activity of BNIP3 promoter luciferase reporter. (C2) The overexpression of the wild-type Set7 but not the mutant Set7 (H297A) in Set7-null MEF cells (Set7$^{-/-}$) inhibited the activity of p2.1 reporter. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired t-test).
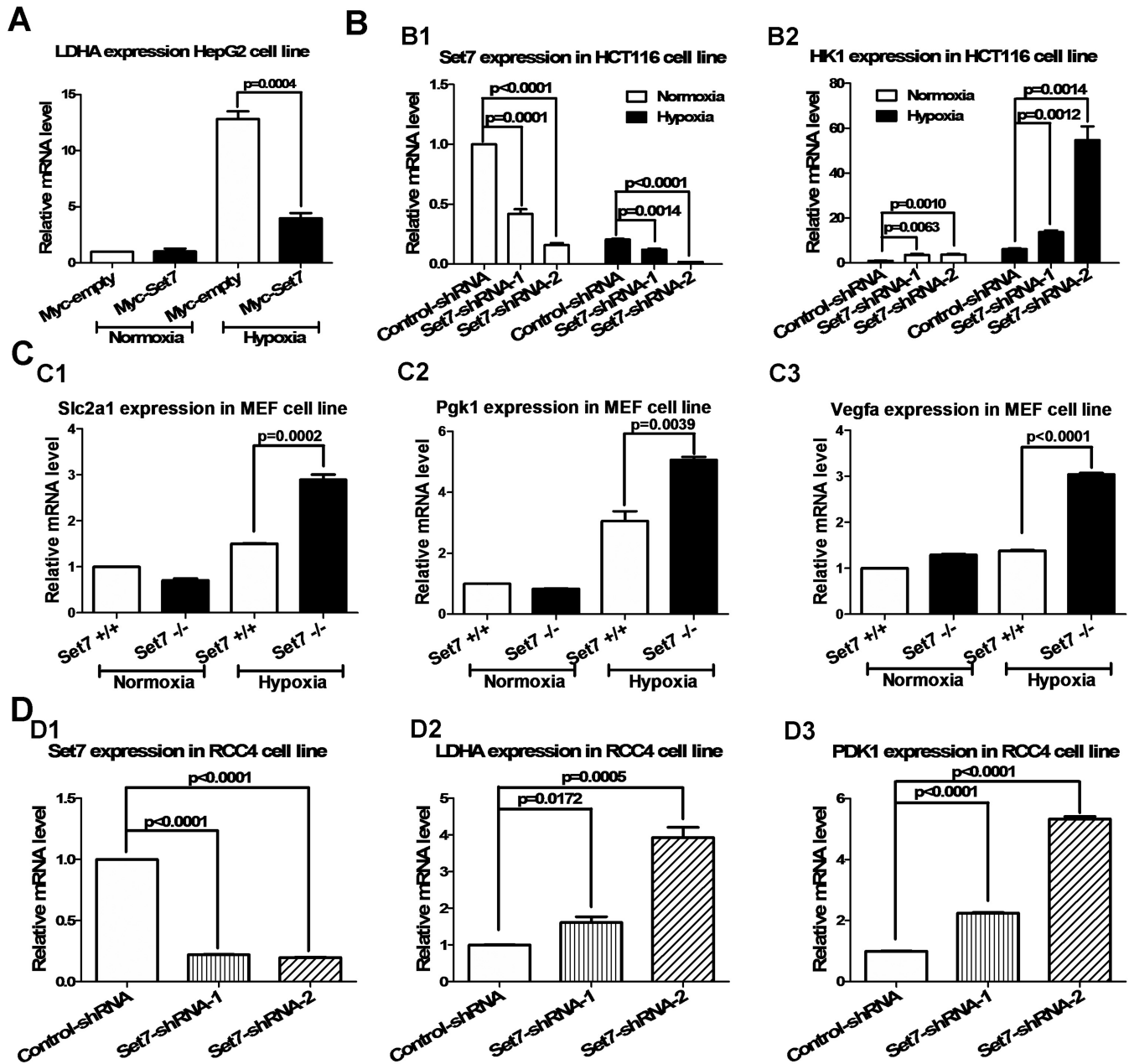
Figure 5. Set7 suppresses HIF-1α target gene expression. (**A**) The overexpression of Set7 reduced *LDHA* expression in HepG2 cells under hypoxia (1% O₂), as revealed by semi-quantitative real-time PCR assays. (**B**) (B1) The efficiency of Set7-shRNA-1 and Set7-shRNA-2-mediated knockdown of Set7 in HCT116 cells was confirmed by semi-quantitative real-time PCR assays. (B2) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in HCT116 cells increased *HK1* expression under normoxic and hypoxic conditions, as revealed by semi-quantitative real-time PCR assays. (**C**) (C1) *Slc2a* expression in Set7-null MEF cells (Set7⁻/⁻) was higher than that in the wild-type MEF cells (Set7⁺/⁺) under hypoxic conditions, as revealed by semi-quantitative real-time PCR assays. (C2) *Pgk1* expression in Set7-null MEF cells (Set7⁻/⁻) was higher than that in the wild-type MEF cells (Set7⁺/⁺) under hypoxic conditions, as revealed by semi-quantitative real-time PCR assays. (C3) *Vegfa* expression in Set7-null MEF cells (Set7⁻/⁻) was higher than that in the wild-type MEF cells (Set7⁺/⁺) under hypoxic conditions, as revealed by semi-quantitative real-time PCR assays. (**D**) (D1) The efficiency of Set7-shRNA-1 and Set7-shRNA-2-mediated knockdown of Set7 in RCC4 cells was confirmed by semi-quantitative real-time PCR assays. (D2) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells increased *LDHA* expression, as revealed by semi-quantitative real-time PCR assays. (D3) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells increased *PDK1* expression, as revealed by semi-quantitative real-time PCR assays. Data were presented as mean ± SEM of three independent experiments performed in triplicate; statistical analysis was performed using GraphPad Prism 5.0 (unpaired *t*-test).
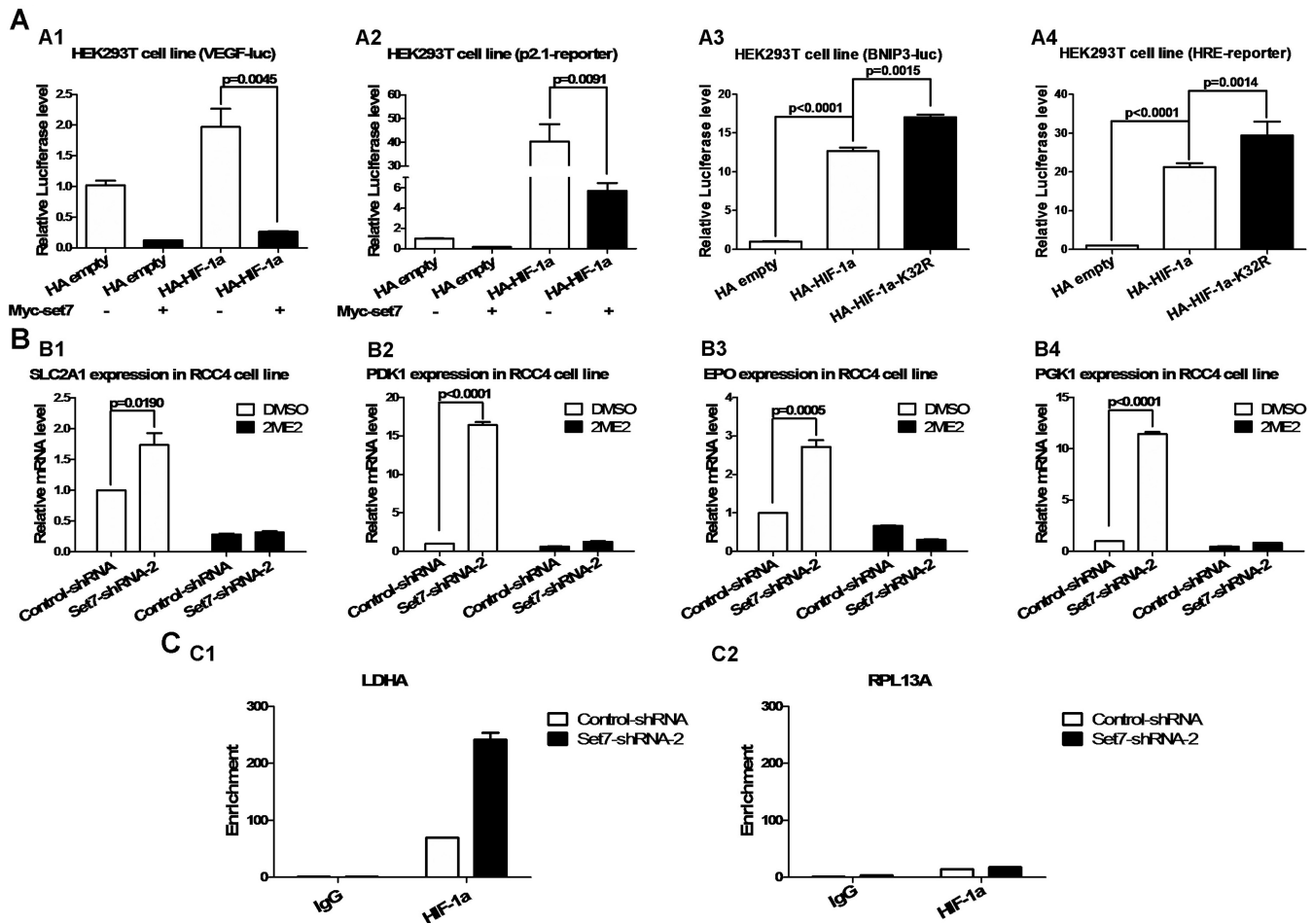
Figure 6. Inhibitory role of Set7 in the hypoxia signaling pathway is mediated by HIF-1α. (**A**) (A1) The overexpression of Set7 inhibited the activity of *VEGF* promoter luciferase reporter activated by the ectopic expression of HIF-1α in HEK293T cells. (A2) The overexpression of Set7 inhibited the activity of p2.1 reporter activated by the ectopic expression of HIF-1α in HEK293T. (A3) The overexpression of the mutant HIF-1α with Set7-methylated site mutated (K32R) significantly enhanced the transactivity of BNIP3 promoter luciferase reporter compared with that of the wild-type HIF-1α in HEK293T cells ($P = 0.0015$). (A4) The overexpression of the mutant HIF-1α with Set7-methylated site mutated (K32R) significantly enhanced the transactivity of the HRE reporter compared with that of the wild-type HIF-1α in HEK293T cells ($P = 0.0014$). (**B**) The knockdown of Set7 by Set7-shRNA-2 in RCC4 cells increased the expression of *SLC2A1*(B1), *PDK1*(B2), *EPO*(B3) or *PGK1*(B4); but inhibition of HIF-1α activity by adding HIF-1α inhibitor, 2ME2 (50 ng/μl, 18 h), abolished this kind of effect under normoxia conditions, as revealed by semi-quantitative real-time PCR assays. (**C**) (C1) ChIP assays show that the knockdown of Set7 in RCC4 cells considerably enhanced the binding ability of HIF-1α to the LDHA promoter. Mouse IgG served as a control for ChIP assays. (C2) The knockdown of Set7 in RCC4 cells does not affect the binding ability of HIF-1α to the RPL13A promoter. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired *t*-test).

Set7 in HEK293T cells did not reduce HIF-1α mRNA level under hypoxic condition (Figure 7A). Consistently, knockdown of Set7 in RCC4 cells also did not enhance HIF-1α mRNA level (Figure 7B). In addition, co-expression of Set7 together with HIF-1α did not alter HIF-1α protein level (Figure 7C). Furthermore, in the presence of cycloheximide (CHX, 50 μg/ml), overexpression of Set7 had no obvious effect on HIF-1α protein stability (Figure 7D). To determine whether the suppression of Set7 on HIF-1α transcriptional activity was due to the effect of Set7 on HIF-1α nuclear translocation (59), we performed subcellular localization assay. After overexpression of HIF-1α together with Set7 or empty vector control, we separated nucleus and cytoplasm fraction and conducted western blot analysis. To ensure the efficiency of fraction separation, we used anti-α-tubulin antibody to monitor cytoplasmic proteins and used anti-Histone H3 antibody to monitor nuclear proteins.

As shown in Figure 7E, overexpression of Set7 did not alter HIF-1α nuclear localization. Taken together, these data suggest that the suppression of Set7 on HIF-1α transcriptional activity was neither due to the effect of Set7 on HIF-1α mRNA and protein levels, nor due to its effect on HIF-1α nuclear localization.

Effect of Set7 on HIF-1α transactivity depends on the activity of Set7 that functions as a methyltransferase

To determine whether the effect of Set7 on HIF-1α activity is dependent on the methyltransferase activity of Set7, we used (R)-PFI-2, a potent and specific inhibitor of Set7 (76). Treatment with (R)-PFI-2 inhibited methylation of HIF-1α by Set7 in HEK293T cells (Figure 8A). The addition of (R)-PFI-2 in RCC4 cells significantly enhanced the expression of HIF-1α target genes, including *SLC2A1*, *LDHA*,
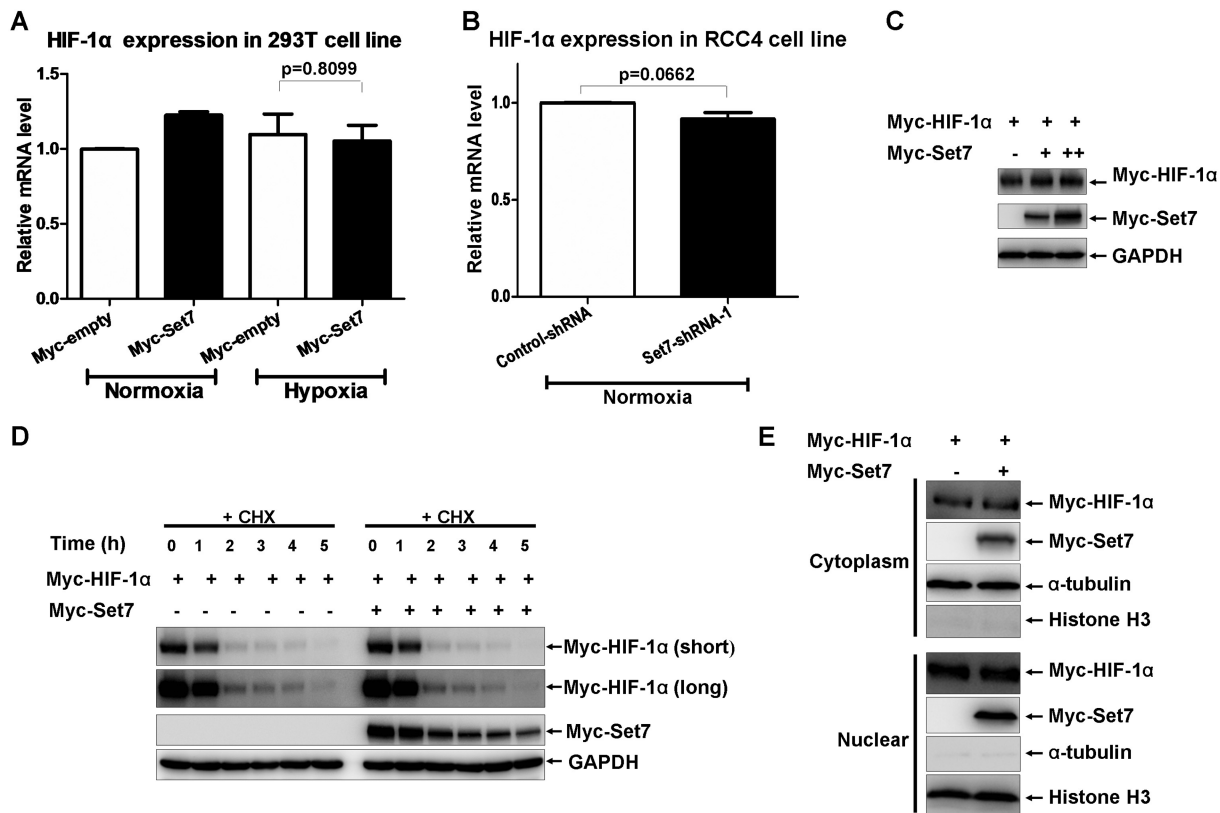
Figure 7. Set7 has no effect on mRNA level, protein level and nuclear translocation of HIF-1α. (**A**) Under hypoxia condition, overexpression of Set7 had no effect on HIF-1α mRNA level in HEK293T cells. (**B**) In RCC4 cells, knockdown of Set7 by Set7-shRNA-1 has no effect on HIF-1α mRNA level. (**C**) Overexpression of Set7 had no obvious effect on HIF-1α protein level in HEK293T cells. (**D**) The protein level of HIF-1α was not affected by overexpression of Set7 in HEK293T cells in the presence of CHX (50 μg/ml). Short, shorter exposure; long, longer exposure. (**E**) Overexpression of Set7 did not affect HIF-1α nuclear translocation. α-tubulin detection was used to monitor cytoplasmic protein and Histone H3 detection was used to monitor nuclear protein.

PDK1, *PGK1*, *EPO* and *VEGF* (Figure 8B). Moreover, the knockdown of Set7 by Set7-shRNA-2 in RCC4 cells eliminated the enhancement effect of (R)-PFI-2 on the expression of HIF-1α target genes, including *SLC2A1*, *PDK1* and *LDHA*; this finding confirms the specific inhibitory role of (R)-PFI-2 on Set7 (Figure 8C1–C3).

These data suggest that methyltransferase activity is essential for Set7 to suppress HIF-1α activity.

Set7 functions as an HIF-1α methyltransferase to regulate glucose homeostasis

The role of HIF-1α in cellular metabolism has been widely investigated (77–81). Under hypoxic conditions, HIF-1α can induce the expression of some genes to promote glucose uptake (44–47). Some genes can also regulate HIF-1α activity to modulate cellular glucose homeostasis (48,62,64,66,82,83). Set7 affects the expression of glycolysis-associated HIF-1α downstream target genes, such as *LDHA*, *HK1*, *PDK1*, *Slc2a1* and *Pgk1* (Figure 5), thus, we postulate that Set7 may affect cellular glucose homeostasis by inhibiting HIF-1α activity. To verify this hypothesis, we examined glucose uptake and ATP levels in cells after Set7 was knocked down or Set7 activity was inhibited. The knockdown of Set7 in RCC4 cells increased glucose uptake and intracellular ATP levels (Figure 9A and

B). The results of 2-NBDG glucose uptake assays showed that Set7$^{-/-}$ MEFs exhibited higher levels of cellular glucose than Set7$^{+/+}$ MEFs under hypoxic conditions (Figure 9C). Furthermore, (R)-PFI-2 treatment upregulated cellular ATP levels and glucose uptake in RCC4 cells (Figure 9D and E). These data suggest that the methyltransferase Set7 regulates glucose homeostasis via HIF-1α.

Set7 is a hypoxia-suppressed gene

To delineate the role of Set7 in the hypoxia signaling pathway, we examined whether hypoxia can regulate Set7 expression. Under hypoxic conditions, Set7 expression was significantly suppressed in the three examined cell lines, as revealed by semi-quantitative RT-PCR assays (Figure 10A1–A3). In HEK293T cells, hypoxia exposure also reduced the protein level of Set7 (Figure 10B). By contrast, the knockdown of HIF-1α by HIF-1α-shRNA-1 and HIF-1α-shRNA-2 increased Set7 protein level (Figure 10C). The inhibition of HIF-α activity by 2ME2 in RCC4 cells induced Set7 expression (Figure 10D). To ensure the reliability of hypoxic conditions in these experiments, we established positive controls by using several well-defined hypoxia-induced genes, including *SLC2A1*, *LDHA*, *PGK1*, *HK1*, *EPO* and *VEGF* (Figure 10A and D). Our findings suggest that Set7 is a hypoxia-suppressive gene.
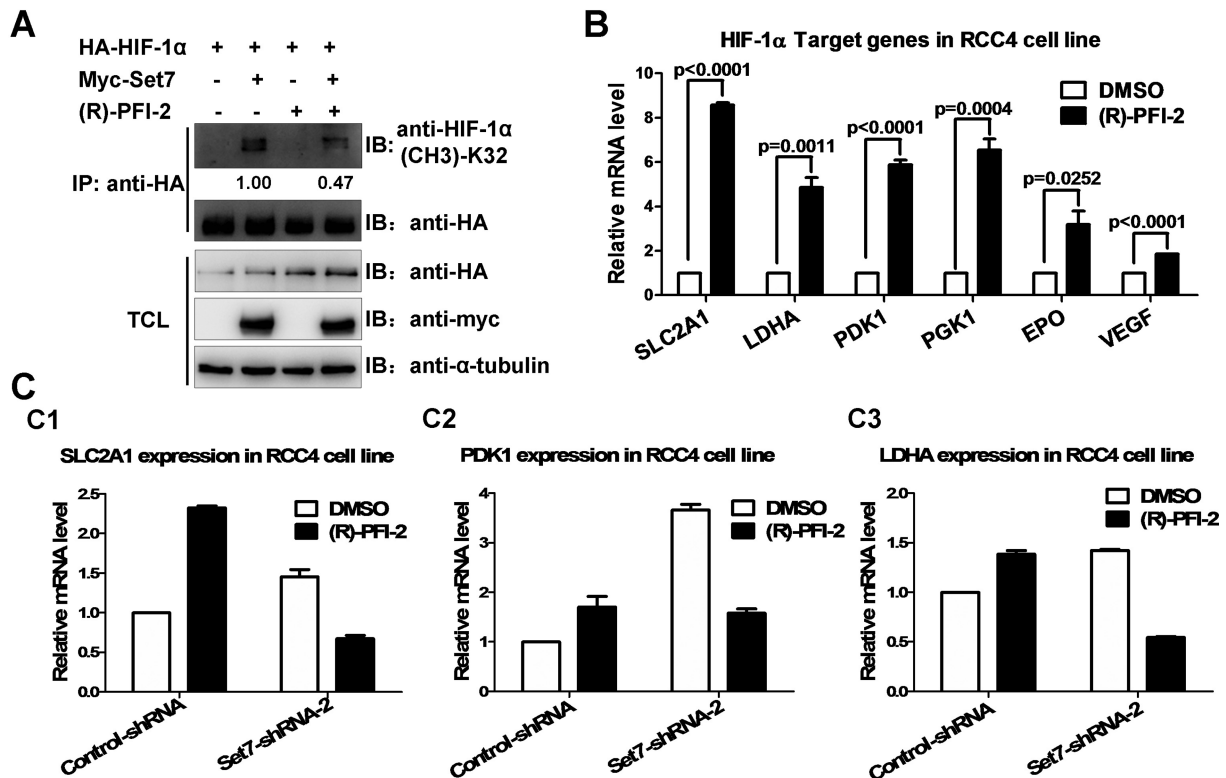
Figure 8. Inhibitory role of Set7 on HIF-1α transactivity is dependent on its methyltransferase activity. (**A**) (R)-PFI-2 (1 μM), a potent and selective inhibitor of Set7, inhibited HIF-1α methylation at K32 catalyzed by Set7, as revealed by immunoprecipitation assays. (**B**) After (R)-PFI-2 (1 μM) treatment was administered for 18 h, the expressions of *SLC2A1*, *LDHA*, *PDK1*, *PGK1*, *EPO* and *VEGF* were increased in RCC4 cells, as revealed by semi-quantitative real-time PCR assay. (**C**) The enhanced expressions of *SLC2A1* (C1), *PDK1* (C2) and *LDHA* (C3) by addition of (R)-PFI-2 (1 μM, 18 h) in RCC4 cells were counteracted by the knockdown of Set7. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired *t*-test).

To determine when HIF-1α is methylated, we performed a time course assay using HEK293T cells. As shown in Figure 10E, HIF-1α was methylated to a higher level after the cells were exposed to hypoxic condition from 1 h to 2 h, then the methylation of HIF-1α decreased after the cells was exposed to hypoxic condition from 4 h even though the total protein level of HIF-1α increased constantly. After the cells were exposed to hypoxic condition for 24 h, the protein level of Set7 decreased dramatically, the methylation of HIF-1α also dropped to low level (Figure 10E). These results suggest that the regulation between Set7 expression and HIF-1α methylation is dynamic. To further figure out its intrinsic relationship will be very interesting.

HIF-2α, as well as HIF-1α, is methylated by Set7

The synthesized peptide based on the HIF-1α protein sequence used to generate antibody is evolutionarily conserved between HIF-1α and HIF-2α (Figure 11A1). The core local recognition sequence of Set7, namely, (K/R) (S/T) K, is also present in HIF-2α, which is evolutionarily conserved among human, macaque, cow, pig, mouse and rat (Figure 11A1 and A2). These findings suggested that HIF-2α may be a substrate of Set7, resembling HIF-1α. To test this hypothesis, we examined the methylation ability of Set7 on HIF-2α by using anti-HIF-1α(CH₃)-K32 antibody after ectopic expression. In HEK293T cells, the ectopic ex-

pression of the wild-type Set7 could methylate the wild-type HIF-2α but not the mutant HIF-2α(K29R); in the latter, the potential methylation site lysine 29 was mutated to arginine (column 2 versus column 5 in the first line of Figure 11B). By contrast, the ectopic expression of the enzymatically dead mutant of Set7, namely, Set7(H297A), did not affect wild-type HIF-2α and mutant HIF-2α(K29R) (column 3 versus column 6 in the first line of Figure 11B). These data suggested that the K29 of HIF-2α is methylated by Set7, and anti-HIF-1α(CH₃)-K32 antibody can detect HIF-2α methylation at lysine 29.

Similar to the inhibitory role of Set7 on HIF-1α, the overexpression of Set7 in HEK293T cells inhibited the reporter activity of the *PAI-1* promoter, a well-defined HIF-2α target gene reporter (Figure 11C) (62,65). In RCC4 cells, the inhibition of Set7 activity by adding (R)-PFI-2 increased the expression of HIF-2α target genes, including *PAI-1*, *CITED2*, *GST* and *POU5F1*, as revealed by semi-quantitative RT-PCR assays (Figure 11D) (62,65). By contrast, the knockdown of Set7 in RCC4 cells by Set7-shRNA-1 and Set7-shRNA-2 promotes the expression of HIF-2α target genes, including *POU5F1* (Figure 11E1), *PAI-1* (Figure 11E2) and *CITED2* (Figure 11E3) (62,65). However, the addition of 2ME2 in RCC4 cells inhibited the activity of HIF-2α; as a result, the upregulation effect of Set7-shRNA-2 on GST expression was completely abrogated (Figure 11F) (62,65).
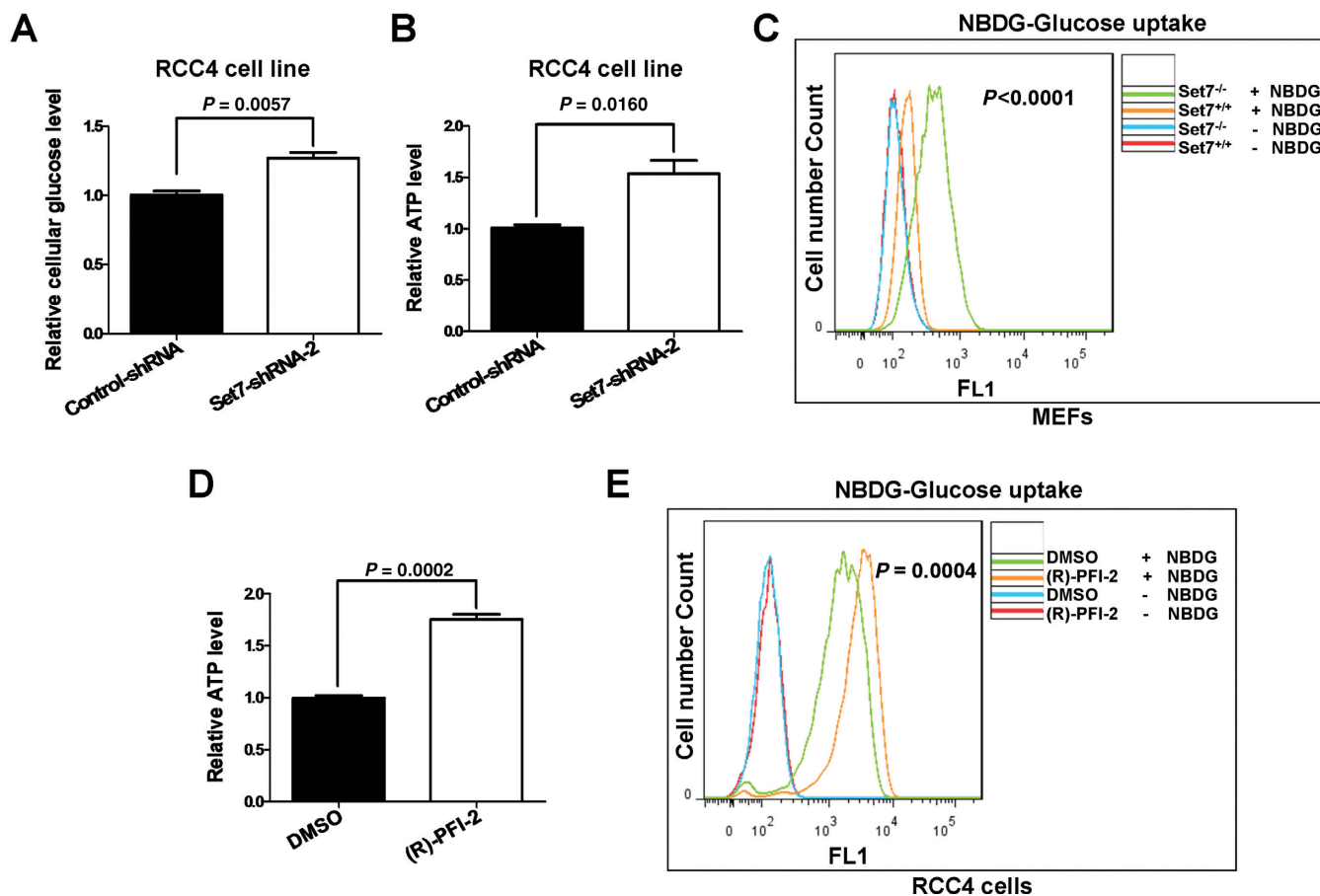
Figure 9. Increased glucose uptake and ATP levels in Set7-deficient cells and cells with Set7 inhibition. (**A**) The knockdown of Set7 by Set7-shRNA-2 via lentivirus infection in RCC4 cells significantly increased cellular glucose levels ($P = 0.0057$), as determined using a glucose uptake assay kit. (**B**) The knockdown of Set7 by Set7-shRNA-2 via lentivirus infection in RCC4 cells significantly increased ATP levels ($P = 0.0160$), as measured using an ATP assay kit. (**C**) The wild-type Set7 (Set7$^{+/+}$) or Set7-null (Set7$^{-/-}$) MEF cells were grown in the presence or the absence of the fluorescent glucose analog NBDG (100 μM) for 1 h; glucose uptake was quantified by flow cytometry (FACS). In the presence of NBDG, Set7$^{-/-}$ MEFs exhibited higher intracellular glucose levels than Set7$^{+/+}$ MEFs (green curve versus orange curve; $P < 0.0001$). Blue and red curves are controls without the fluorescent NBDG glucose analog. (**D**) The inhibited Set7 activity by the addition of (R)-PFI-2 (1 μM) for 24 h in RCC4 cells significantly increased ATP levels ($P = 0.0002$), as determined using an ATP assay kit. (**E**) RCC4 cells were treated with or without (R)-PFI-2 (1 μM) for 24 h, and the cells were grown in the presence or the absence of the fluorescent glucose analog NBDG for 1 h; glucose uptake was then quantified by flow cytometry (FACS). In the presence of NBDG, RCC4 cells with Set7 inhibition (treatment with (R)-PFI-2) exhibited higher intracellular glucose levels than RCC4 cells without Set7 inhibition (treatment with DMSO control) (orange curve versus green curve, $P = 0.0004$). Blue and red curves are controls without the fluorescent NBDG glucose analog. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired *t*-test).

Previous study reported that 786-O cells only harbor wild-type HIF-2α, but not HIF-1α (84). To elucidate the effect of Set7 on HIF-2α, we knocked down Set7 in 786-O cells by Set7-shRNA-1 and Set7-shRNA-2 (Figure 11G1) and examined the expressions of HIF-2α target genes. The knockdown of Set7 in 786-O cells enhanced the expressions of *POU5F1* (Figure 11G2) and *CITED2* (Figure 11G3). Consistently, the overexpression of the wild-type Set7 by lentivirus infection in 786-O cells decreased the protein levels of PAI-1 and SOD-2, which are two well-defined HIF-2α targets. However, the overexpression of the mutant Set7(H297A) elicited no evident effect (Figure 11H). These data suggest that HIF-2α and HIF-1α are novel substrates of Set7, and Set7 inhibits HIF-2α transactivity by monomethylation of HIF-2α at lysine 29.

DISCUSSION

Set7 methylates HIF-1α/2α and negatively regulates HIF-1α/2α transcriptional activity

In this study, we investigate the regulation of the main regulators of the hypoxia signaling pathway by lysine methylation and demonstrate that Set7 methylates HIF-1α at K32 and HIF-2α at K29. HIF-1α and HIF-2α are possible substrates of Set7, as confirmed by the following findings: Set7 methylated HIF-α *in vivo* and *in vitro*; enzymatically dead mutant could not methylate HIF-1α and the specific inhibitor of Set7 blocked HIF-1α methylation.

Thus far, the roles of Set7-mediated methylation on non-histone proteins and corresponding mechanisms have not been elucidated. The ability of Set7 to positively or negatively regulate substrates varies in different substrates (6,8). Several mechanisms have been proposed to explain the role
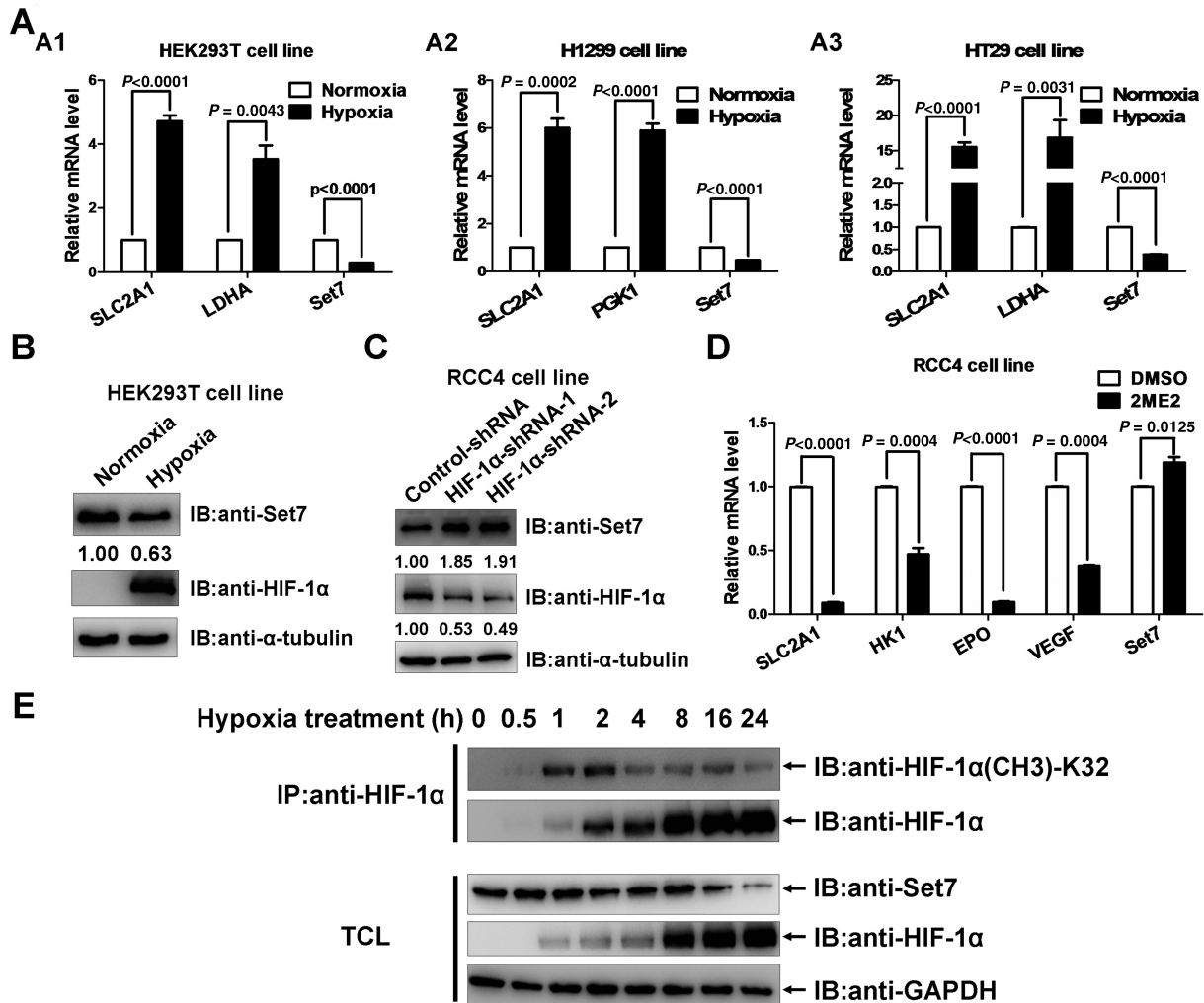
Figure 10. Set7 expression is suppressed under hypoxic conditions. (**A**) Under hypoxic conditions (1% O_2 for 24 h), Set7 expression considerably decreased in HEK293T (A1), H1299 (A2) and HT29 (A3) cells compared with that under normoxic conditions, as revealed by semi-quantitative real-time PCR assays. The expressions of *SLC2A1*, *LDHA* and *PGK1* served as positive controls. (**B**) Under hypoxic conditions, the protein level of Set7 was reduced in HEK293T cells. (**C**) The knockdown of HIF-1α by HIF-1α-shRNA-1 and HIF-1α-shRNA-2 in RCC4 cells increased the protein levels of Set7. (**D**) The inhibition of HIF-1α activity by adding 2ME2 (50 ng/μl, 18 h) significantly increased *Set7* expression ($P = 0.0125$). The expressions of SLC2A1, HK1, EPO and VEGF served as positive controls. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired *t*-test). (**E**) A time course of HIF-1α methylation and Set7 expression under hypoxic condition. In order to detect endogenous HIF-1α methylation, anti-HIF-1α antibody conjugated agarose beads were used for immunoprecipitation.

of lysine methylation by Set7 on non-histone proteins. The enhancement of protein stability and nuclear retention positively regulates the activity of p53 and LIN28A by Set7-mediated monomethylation (11,61); by contrast, the promotion of protein degradation negatively regulates the activity of Sox2 and DNMT1 by Set7-mediated monomethylation (13,85–87). Cytoplasmic retention by Set7-mediated methylation also enhances the Yap function (25). However, all of these mechanisms could not fully explain the effect of Set7 on other substrates, such as AR and FOXO3a (17,18,26,27). Some controversy observations have been reported for the effect of Set7 on FOXO3a (26,27). Methylation of FOXO3a by Set7 decreases FOXO3a protein stability, while moderately increasing FOXO3a transcriptional activity (26). However, it is also reported that methylation of FOXO3a leads to the inhibition of FOXO3a DNA-binding

activity and transactivation without affecting FOXO3a protein stability obviously (17). In this study, we provide evidence to show that lysine methylation of HIF-1α diminished its occupancy on target gene promoters, resulting in the inhibition of gene expression. The conformational change of the HIF-α protein after methylation may reduce the HIF-α protein on the target gene promoter. Of note, the methylation sites (RSK) of HIF-α by Set7 locate in bHLH domain of HIF-α, which may also result in decreasing DNA-binding activity of HIF-α. Alternatively, HIF-1α/2α may mediate the recruitment of Set7 to the HREs of the HIF target genes, resulting in monomethylation of histone H3 at lysine 4; this monomethylation impairs HIF-α binding to HREs and thereby inhibits the transcription of HIF target genes. Notably, a similar mechanism has
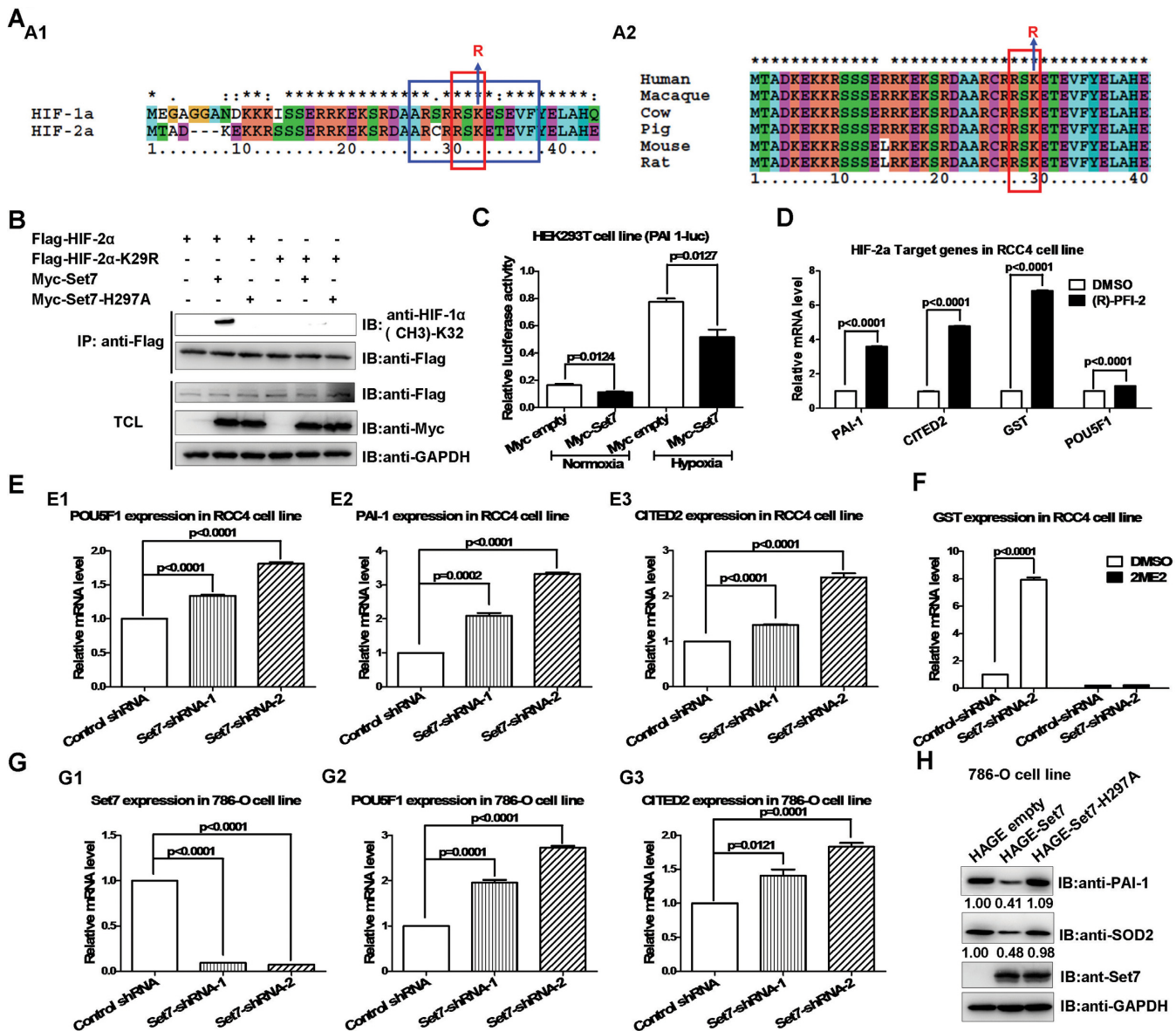
Figure 11. Set7 methylates HIF-2α and inhibits HIF-2α transactivity. (**A**) (A1) Sequence alignment of partial human HIF-1α (amino acids 1–43) and HIF-2α protein (amino acids 1–40). The blue box indicates the peptide sequence (12 amino acids) used for antibody generation [K32 was methylated (Me1) when antibody was generated]; the red box indicates a conserved consensus motif (R/K-S/T-K) methylated by Set7; the blue arrow indicates the conserved lysine mutated to arginine in the methylation assays. (A2) Sequence alignment of the HIF-2α protein (amino acids 1–40) of human, macaque, cow, pig, mouse and rat. The red box indicates a conserved consensus motif (R/K-S/T-K) methylated by Set7; the blue arrow indicates the conserved lysine (K29) mutated to arginine in the methylation assays. (**B**) The wild-type HIF-2α is methylated by wild-type Set7, not by Set7 mutant (H297A); HIF-2α mutant (K29R) could not be methylated by Set7, as detected by anti-HIF-1α (CH3)-K32 antibody after immunoprecipitation assays. (**C**) The overexpression of Set7 suppressed the activity of PAI-1 promoter luciferase reporter in HEK293T cells. (**D**) The inhibition of Set7 activity in RCC4 cells by addition of (R)-PFI-2 (1 μM for 24 h) induced the expression of typical HIF-2α downstream targets, such as *PAI-1*, *CITED2*, *GST* and *POU5F1*, as revealed by semi-quantitative real-time PCR assays. (**E**) (E1) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells considerably increased *POU5F1* expression, as revealed by semi-quantitative real-time PCR assays. (E2) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells considerably increases *PAI-1* expression, as revealed by semi-quantitative real-time PCR assays. (E3) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells considerably increased *CITED2* expression, as revealed by semi-quantitative real-time PCR assays. (**F**) The knockdown of Set7 by Set7-shRNA-2 in RCC4 cells increased *GST* expression, but this effect was not evident when HIF-2α activity was inhibited by addition of 2ME2 (50 ng/μl, 18 h), as revealed by semi-quantitative real-time PCR assays. (**G**) (G1) The efficiency of Set7-shRNA-1 and Set7-shRNA-2 mediated Set7 knockdown in 786-O cells was confirmed by semi-quantitative real-time PCR assays. (G2) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in 786-O cells considerably increased *POU5F1* expression, as revealed by semi-quantitative real-time PCR assays. (G3) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in 786-O cells considerably increased *CITED2* expression, as revealed by semi-quantitative real-time PCR assays. (**H**) The overexpression of Set7 by lentivirus infection in 786-O cells decreased the protein levels of PAI-1 and SOD2, but the overexpression of Set7 mutant (H297A) did not decrease, as revealed by western blot analysis. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired *t*-test).

been proposed to explain the coactivator role of the histone demethylase JMJD2C on HIF-1α (88).

In comparing the target sequences of Set7 bound to histone H3, TAF10 and p53, a structurally conserved binding motif of Set7, namely, K/R-S/T-K (in which K is the target lysine residue) has been recognized (11,12). Several putative substrates of Set7 have been identified on the basis of the consensus target site (68,89). In this study, HIF-1α may be a substrate of Set7 based on the 'RSK' sequence localized in HIF-1α. Subsequent assessments confirmed this hypothesis. The proteins in the protein pool of mammalian cells harboring this consensus motif may be potential substrates of Set7. In the present study, we noticed that the transcriptional activities of the HIF-1α (K32R)/HIF-2α (K29R) mutants were still suppressed by the overexpression of Set7 (data not shown), although the transcriptional activity of HIF-1α (K32R) mutant was higher than that of the wild-type HIF-1α. It appears that Set7 can also methylate other sites, in addition to the K32 in HIF-1α and K29 in HIF-2α. In fact, Set7 binds to amino acids 201–330, amino acids 400–575 and amino acids 576–785 of HIF-1α, in addition to the N-terminus of HIF-1α (amino acids 1–80) that harbors K32. Therefore, other sites in HIF-1α and HIF-2α may be the possible target sites of Set7. However, sites other than K32 or K29 could not be detected in the assays because the antibody used in this study is specific to K32 methylation in HIF-1α and K29 methylation in HIF-2α. But, it is evident that Set7-mediated methylation suppresses HIF-1α/2α transcriptional activity, regardless of the methylated site because (R)-PFI-2, a specific Set7 methyltransferase activity inhibitor, releases the suppressive role of Set7 on HIF-1α/2α activity. Future studies should identify unknown sites methylated by Set7 to fully elucidate the role of Set7 on HIF-α methylation.

Set7 may significantly function in response to hypoxia stress by regulation of HIF-1α/2α

Post-translational modifications of proteins regulate cells in response to different stresses because of rapid response and reversible capability (14,27). As the main regulators of the hypoxia signaling pathway in cells, post-translational modifications of HIF-1α/2α in response to hypoxia have been extensively investigated (48,59,65,66,75,90). Although hypoxia is involved in epigenetic gene regulation by inducing histone demethylase or by activating histone methyltransferase (91–95), whether HIF-1α/2α can directly be methylated or de-methylated and the underlying biological consequences remain poorly described in mechanistic terms; involved factors remain unknown. In this study, evidence indicated that HIF-1α and HIF-2α are methylated by Set7, resulting in the downregulation of HIF target genes; on the other hand, set7 expression is also suppressed by hypoxia; thus, we reveal a novel role of protein methylation in response to hypoxia stress. These data also suggest that Set7 may restrain cells in response to hypoxia to avoid exaggerating reactions. Such reactions cause harmful effects on cells by causing cell apoptosis and autophagy, among others (96–100). Moreover, Set7-HIF-α-Set7 may form a regulatory loop that strictly controls cell responses under hypoxia stress. The destruction of this regulatory loop under some

pathogenic conditions may result in the loss of control of HIF-α activity. As a result, cells may encounter abnormal processes that may lead to pathogenic progression. For this reason, animal models should be established to further explore the role of Set7-mediated HIF-α methylation and to elucidate the physiological relevance of Set7 methyltransferase in the hypoxia signaling pathway.

REFERENCES

1. Pradhan,S., Chin,H.G., Esteve,P.O. and Jacobsen,S.E. (2009) SET7/9 mediated methylation of non-histone proteins in mammalian cells. *Epigenetics*, **4**, 383–387.
2. Egorova,K.S., Olenkina,O.M. and Olenina,L.V. (2010) Lysine methylation of nonhistone proteins is a way to regulate their stability and function. *Biochemistry (Mosc.)*, **75**, 535–548.
3. Morgunkova,A. and Barlev,N.A. (2006) Lysine methylation goes global. *Cell Cycle*, **5**, 1308–1312.
4. Huang,J. and Berger,S.L. (2008) The emerging field of dynamic lysine methylation of non-histone proteins. *Curr. Opin. Genet. Dev.*, **18**, 152–158.
5. Paik,W.K., Paik,D.C. and Kim,S. (2007) Historical review: the field of protein methylation. *Trends Biochem. Sci.*, **32**, 146–152.
6. Biggar,K.K. and Li,S.S. (2015) Non-histone protein methylation as a regulator of cellular signalling and function. *Nat. Rev. Mol. Cell Biol.*, **16**, 5–17.
7. Stark,G.R., Wang,Y. and Lu,T. (2011) Lysine methylation of promoter-bound transcription factors and relevance to cancer. *Cell Res.*, **21**, 375–380.
8. Lanouette,S., Mongeon,V., Figeys,D. and Couture,J.F. (2014) The functional diversity of protein lysine methylation. *Mol. Syst. Biol.*, **10**, 1–25.
9. Nishioka,K., Chuikov,S., Sarma,K., Erdjument-Bromage,H., Allis,C.D., Tempst,P. and Reinberg,D. (2002) Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation. *Genes Dev.*, **16**, 479–489.
10. Wang,H., Cao,R., Xia,L., Erdjument-Bromage,H., Borchers,C., Tempst,P. and Zhang,Y. (2001) Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. *Mol. Cell*, **8**, 1207–1217.
11. Chuikov,S., Kurash,J.K., Wilson,J.R., Xiao,B., Justin,N., Ivanov,G.S., McKinney,K., Tempst,P., Prives,C., Gamblin,S.J. *et al.* (2004) Regulation of p53 activity through lysine methylation. *Nature*, **432**, 353–360.
12. Kouskouti,A., Scheer,E., Staub,A., Tora,L. and Talianidis,I. (2004) Gene-specific modulation of TAF10 function by SET9-mediated methylation. *Mol. Cell*, **14**, 175–182.
13. Esteve,P.O., Chin,H.G., Benner,J., Feehery,G.R., Samaranayake,M., Horwitz,G.A., Jacobsen,S.E. and Pradhan,S. (2009) Regulation of DNMT1 stability through SET7-mediated lysine methylation in mammalian cells. *Proc. Natl Acad. Sci. U.S.A.*, **106**, 5076–5081.

14. Kontaki,H. and Talianidis,I. (2010) Lysine methylation regulates E2F1-induced cell death. *Mol. Cell*, **39**, 152–160.

15. Munro,S., Khaire,N., Inche,A., Carr,S. and La Thangue,N.B. (2010) Lysine methylation regulates the pRb tumour suppressor protein. *Oncogene*, **29**, 2357–2367.

16. Kurash,J.K., Lei,H., Shen,Q., Marston,W.L., Granda,B.W., Fan,H., Wall,D., Li,E. and Gaudet,F. (2008) Methylation of p53 by Set7/9 mediates p53 acetylation and activity in vivo. *Mol. Cell*, **29**, 392–400.

17. Xie,Q., Bai,Y., Wu,J., Sun,Y., Wang,Y., Zhang,Y., Mei,P. and Yuan,Z. (2011) Methylation-mediated regulation of E2F1 in DNA damage-induced cell death. *J. Recept. Signal Transduct. Res.*, **31**, 139–146.

18. Gaughan,L., Stockley,J., Wang,N., McCracken,S.R., Treumann,A., Armstrong,K., Shaheen,F., Watt,K., McEwan,I.J., Wang,C. *et al.* (2011) Regulation of the androgen receptor by SET9-mediated methylation. *Nucleic Acids Res.*, **39**, 1266–1279.

19. Ko,S., Ahn,J., Song,C.S., Kim,S., Knapczyk-Stwora,K. and Chatterjee,B. (2011) Lysine methylation and functional modulation of androgen receptor by Set9 methyltransferase. *Mol. Endocrinol.*, **25**, 433–444.

20. Subramanian,K., Jia,D., Kapoor-Vazirani,P., Powell,D.R., Collins,R.E., Sharma,D., Peng,J., Cheng,X. and Vertino,P.M. (2008) Regulation of estrogen receptor alpha by the SET7 lysine methyltransferase. *Mol. Cell*, **30**, 336–347.

21. Ea,C.K. and Baltimore,D. (2009) Regulation of NF-kappaB activity through lysine monomethylation of p65. *Proc. Natl Acad. Sci. U.S.A.*, **106**, 18972–18977.

22. Yang,X.D., Huang,B., Li,M., Lamb,A., Kelleher,N.L. and Chen,L.F. (2009) Negative regulation of NF-kappaB action by Set9-mediated lysine methylation of the RelA subunit. *EMBO J.*, **28**, 1055–1066.

23. Masatsugu,T. and Yamamoto,K. (2009) Multiple lysine methylation of PCAF by Set9 methyltransferase. *Biochem. Biophys. Res. Commun.*, **381**, 22–26.

24. Yang,J., Huang,J., Dasgupta,M., Sears,N., Miyagi,M., Wang,B., Chance,M.R., Chen,X., Du,Y., Wang,Y. *et al.* (2010) Reversible methylation of promoter-bound STAT3 by histone-modifying enzymes. *Proc. Natl Acad. Sci. U.S.A.*, **107**, 21499–21504.

25. Oudhoff,M.J., Freeman,S.A., Couzens,A.L., Antignano,F., Kuznetsova,E., Min,P.H., Northrop,J.P., Lehnertz,B., Barsyte-Lovejoy,D., Vedadi,M. *et al.* (2013) Control of the hippo pathway by Set7-dependent methylation of Yap. *Dev. Cell*, **26**, 188–194.

26. Calnan,D.R., Webb,A.E., White,J.L., Stowe,T.R., Goswami,T., Shi,X., Espejo,A., Bedford,M.T., Gozani,O., Gygi,S.P. *et al.* (2012) Methylation by Set9 modulates FoxO3 stability and transcriptional activity. *Aging*, **4**, 462–479.

27. Xie,Q., Hao,Y., Tao,L., Peng,S., Rao,C., Chen,H., You,H., Dong,M.Q. and Yuan,Z. (2012) Lysine methylation of FOXO3 regulates oxidative stress-induced neuronal cell death. *EMBO Rep.*, **13**, 371–377.

28. Wang,D., Zhou,J., Liu,X., Lu,D., Shen,C., Du,Y., Wei,F.Z., Song,B., Lu,X., Yu,Y. *et al.* (2013) Methylation of SUV39H1 by SET7/9 results in heterochromatin relaxation and genome instability. *Proc. Natl Acad. Sci. U.S.A.*, **110**, 5516–5521.

29. Lezina,L., Aksenova,V., Ivanova,T., Purmessur,N., Antonov,A.V., Tentler,D., Fedorova,O., Garabadgiu,A.V., Talianidis,I., Melino,G. *et al.* (2014) KMTase Set7/9 is a critical regulator of E2F1 activity upon genotoxic stress. *Cell Death Differ.*, **21**, 1889–1899.

30. Lehnertz,B., Rogalski,J.C., Schulze,F.M., Yi,L., Lin,S., Kast,J. and Rossi,F.M. (2011) p53-dependent transcription and tumor suppression are not affected in Set7/9-deficient mice. *Mol. Cell*, **43**, 673–680.

31. Campaner,S., Spreafico,F., Burgold,T., Doni,M., Rosato,U., Amati,B. and Testa,G. (2011) The methyltransferase Set7/9 (Setd7) is dispensable for the p53-mediated DNA damage response in vivo. *Mol. Cell*, **43**, 681–688.

32. Semenza,G.L. (2014) Hypoxia-inducible factor 1 and cardiovascular disease. *Annu. Rev. Physiol.*, **76**, 39–56.

33. Semenza,G.L. (2014) Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annu. Rev. Pathol.*, **9**, 47–71.

34. Semenza,G.L. (1999) Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annu. Rev. Cell Dev. Biol.*, **15**, 551–578.

35. Walshe,T.E. and D'Amore,P.A. (2008) The role of hypoxia in vascular injury and repair. *Annu. Rev. Pathol.*, **3**, 615–643.

36. Lee,F.S. and Percy,M.J. (2011) The HIF pathway and erythrocytosis. *Annu. Rev. Pathol.*, **6**, 165–192.

37. DeBerardinis,R.J., Lum,J.J., Hatzivassiliou,G. and Thompson,C.B. (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab.*, **7**, 11–20.

38. Wang,G.L., Jiang,B.H., Rue,E.A. and Semenza,G.L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl Acad. Sci. U.S.A.*, **92**, 5510–5514.

39. Semenza,G.L., Jiang,B.H., Leung,S.W., Passantino,R., Concordet,J.P., Maire,P. and Giallongo,A. (1996) Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J. Biol. Chem.*, **271**, 32529–32537.

40. Kaelin,W.G. (2005) Proline hydroxylation and gene expression. *Annu. Rev. Biochem.*, **74**, 115–128.

41. Kaelin,W.G. (2007) Von Hippel-Lindau disease. *Annu. Rev. Pathol.*, **2**, 145–173.

42. Kaelin,W.G. Jr (2008) The von Hippel-Lindau tumour suppressor protein: O2 sensing and cancer. *Nat. Rev. Cancer*, **8**, 865–873.

43. Gossage,L., Eisen,T. and Maher,E.R. (2014) VHL, the story of a tumour suppressor gene. *Nat. Rev. Cancer*, **15**, 55–64.

44. Wheaton,W.W. and Chandel,N.S. (2011) *Hypoxia*. 2. Hypoxia regulates cellular metabolism. *Am. J. Physiol. Cell Physiol.*, **300**, C385–C393.

45. Harris,A.L. (2002) Hypoxia–a key regulatory factor in tumour growth. *Nat. Rev. Cancer*, **2**, 38–47.

46. Melillo,G. (2007) Targeting hypoxia cell signaling for cancer therapy. *Cancer Metastasis Rev.*, **26**, 341–352.

47. Semenza,G.L. (2010) Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene*, **29**, 625–634.

48. Lim,J.H., Lee,Y.M., Chun,Y.S., Chen,J., Kim,J.E. and Park,J.W. (2010) Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha. *Mol. Cell*, **38**, 864–878.

49. Dioum,E.M., Chen,R., Alexander,M.S., Zhang,Q., Hogg,R.T., Gerard,R.D. and Garcia,J.A. (2009) Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. *Science*, **324**, 1289–1293.

50. Bae,S.H., Jeong,J.W., Park,J.A., Kim,S.H., Bae,M.K., Choi,S.J. and Kim,K.W. (2004) Sumoylation increases HIF-1alpha stability and its transcriptional activity. *Biochem. Biophys. Res. Commun.*, **324**, 394–400.

51. Berta,M.A., Mazure,N., Hattab,M., Pouyssegur,J. and Brahimi-Horn,M.C. (2007) SUMOylation of hypoxia-inducible factor-1alpha reduces its transcriptional activity. *Biochem. Biophys. Res. Commun.*, **360**, 646–652.

52. Cheng,J., Kang,X., Zhang,S. and Yeh,E.T. (2007) SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. *Cell*, **131**, 584–595.

53. Carbia-Nagashima,A., Gerez,J., Perez-Castro,C., Paez-Pereda,M., Silberstein,S., Stalla,G.K., Holsboer,F. and Arzt,E. (2007) RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1alpha during hypoxia. *Cell*, **131**, 309–323.

54. Mylonis,I., Chachami,G., Samiotaki,M., Panayotou,G., Paraskeva,E., Kalousi,A., Georgatsou,E., Bonanou,S. and Simos,G. (2006) Identification of MAPK phosphorylation sites and their role in the localization and activity of hypoxia-inducible factor-1alpha. *J. Biol. Chem.*, **281**, 33095–33106.

55. Warfel,N.A., Dolloff,N.G., Dicker,D.T., Malysz,J. and El-Deiry,W.S. (2013) CDK1 stabilizes HIF-1alpha via direct phosphorylation of Ser668 to promote tumor growth. *Cell Cycle*, **12**, 3689–3701.

56. Kalousi,A., Mylonis,I., Politou,A.S., Chachami,G., Paraskeva,E. and Simos,G. (2010) Casein kinase 1 regulates human hypoxia-inducible factor HIF-1. *J. Cell Sci.*, **123**, 2976–2986.

57. Xu,D., Yao,Y., Lu,L., Costa,M. and Dai,W. (2010) Plk3 functions as an essential component of the hypoxia regulatory pathway by direct phosphorylation of HIF-1alpha. *J. Biol. Chem.*, **285**, 38944–38950.

58. Geng,H., Harvey,C.T., Pittsenbarger,J., Liu,Q., Beer,T.M., Xue,C. and Qian,D.Z. (2011) HDAC4 protein regulates HIF1alpha protein lysine acetylation and cancer cell response to hypoxia. *J. Biol. Chem.*, **286**, 38095–38102.

59. Liu,X.H., Kirschenbaum,A., Lu,M., Yao,S., Dosoretz,A., Holland,J.F. and Levine,A.C. (2002) Prostaglandin E2 induces hypoxia-inducible factor-1alpha stabilization and nuclear localization in a human prostate cancer cell line. *J. Biol. Chem.*, **277**, 50081–50086.

60. Jeong,J.W., Bae,M.K., Ahn,M.Y., Kim,S.H., Sohn,T.K., Bae,M.H., Yoo,M.A., Song,E.J., Lee,K.J. and Kim,K.W. (2002) Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. *Cell*, **111**, 709–720.

61. Kassner,I., Andersson,A., Fey,M., Tomas,M., Ferrando-May,E. and Hottiger,M.O. (2013) SET7/9-dependent methylation of ARTD1 at K508 stimulates poly-ADP-ribose formation after oxidative stress. *Open Biol.*, **3**, 120173.

62. Chen,Z., Liu,X., Mei,Z., Wang,Z. and Xiao,W. (2014) EAF2 suppresses hypoxia-induced factor 1alpha transcriptional activity by disrupting its interaction with coactivator CBP/p300. *Mol. Cell. Biol.*, **34**, 1085–1099.

63. Feng,X., Liu,X., Zhang,W. and Xiao,W. (2011) p53 directly suppresses BNIP3 expression to protect against hypoxia-induced cell death. *EMBO J.*, **30**, 3397–3415.

64. Luo,W., Hu,H., Chang,R., Zhong,J., Knabel,M., O'Meally,R., Cole,R.N., Pandey,A. and Semenza,G.L. (2011) Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell*, **145**, 732–744.

65. Pawlus,M.R., Wang,L., Ware,K. and Hu,C.J. (2012) Upstream stimulatory factor 2 and hypoxia-inducible factor 2alpha (HIF2alpha) cooperatively activate HIF2 target genes during hypoxia. *Mol. Cell. Biol.*, **32**, 4595–4610.

66. Zhong,L., D'Urso,A., Toiber,D., Sebastian,C., Henry,R.E., Vadysirisack,D.D., Guimaraes,A., Marinelli,B., Wikstrom,J.D., Nir,T. *et al.* (2010) The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell*, **140**, 280–293.

67. Kim,S.K., Lee,H., Han,K., Kim,S.C., Choi,Y., Park,S.W., Bak,G., Lee,Y., Choi,J.K., Kim,T.K. *et al.* (2014) SET7/9 methylation of the pluripotency factor LIN28A Is a nucleolar localization mechanism that blocks let-7 biogenesis in human ESCs. *Cell Stem Cell*, **15**, 735–749.

68. Couture,J.F., Collazo,E., Hauk,G. and Trievel,R.C. (2006) Structural basis for the methylation site specificity of SET7/9. *Nat. Struct. Mol. Biol.*, **13**, 140–146.

69. Maxwell,P.H., Wiesener,M.S., Chang,G.W., Clifford,S.C., Vaux,E.C., Cockman,M.E., Wykoff,C.C., Pugh,C.W., Maher,E.R. and Ratcliffe,P.J. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*, **399**, 271–275.

70. Wang,G.L. and Semenza,G.L. (1993) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc. Natl Acad. Sci. U.S.A.*, **90**, 4304–4308.

71. Forsythe,J.A., Jiang,B.H., Iyer,N.V., Agani,F., Leung,S.W., Koos,R.D. and Semenza,G.L. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.*, **16**, 4604–4613.

72. Bruick,R.K. (2000) Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc. Natl Acad. Sci. U.S.A.*, **97**, 9082–9087.

73. Mahon,P.C., Hirota,K. and Semenza,G.L. (2001) FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev.*, **15**, 2675–2686.

74. Emerling,B.M., Weinberg,F., Liu,J.L., Mak,T.W. and Chandel,N.S. (2008) PTEN regulates p300-dependent hypoxia-inducible factor 1 transcriptional activity through Forkhead transcription factor 3a (FOXO3a). *Proc. Natl Acad. Sci. U.S.A.*, **105**, 2622–2627.

75. Mabjeesh,N.J., Escuin,D., LaVallee,T.M., Pribluda,V.S., Swartz,G.M., Johnson,M.S., Willard,M.T., Zhong,H., Simons,J.W. and Giannakakou,P. (2003) 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. *Cancer Cell*, **3**, 363–375.

76. Barsyte-Lovejoy,D., Li,F., Oudhoff,M.J., Tatlock,J.H., Dong,A., Zeng,H., Wu,H., Freeman,S.A., Schapira,M., Senisterra,G.A. *et al.* (2014) (R)-PFI-2 is a potent and selective inhibitor of SETD7 methyltransferase activity in cells. *Proc. Natl Acad. Sci. U.S.A.*, **111**, 12853–12858.

77. Greer,S.N., Metcalf,J.L., Wang,Y. and Ohh,M. (2012) The updated biology of hypoxia-inducible factor. *EMBO J.*, **31**, 2448–2460.

78. Majmundar,A.J., Wong,W.J. and Simon,M.C. (2010) Hypoxia-inducible factors and the response to hypoxic stress. *Mol. Cell*, **40**, 294–309.

79. Formenti,F., Constantin-Teodosiu,D., Emmanuel,Y., Cheeseman,J., Dorrington,K.L., Edwards,L.M., Humphreys,S.M., Lappin,T.R., McMullin,M.F., McNamara,C.J. *et al.* (2010) Regulation of human metabolism by hypoxia-inducible factor. *Proc. Natl Acad. Sci. U.S.A.*, **107**, 12722–12727.

80. Semenza,G.L. (2011) Regulation of metabolism by hypoxia-inducible factor 1. *Cold Spring Harb. Symp. Quant. Biol.*, **76**, 347–353.

81. Semenza,G.L. (2009) Regulation of cancer cell metabolism by hypoxia-inducible factor 1. *Semin. Cancer Biol.*, **19**, 12–16.

82. Wang,H.J., Hsieh,Y.J., Cheng,W.C., Lin,C.P., Lin,Y.S., Yang,S.F., Chen,C.C., Izumiya,Y., Yu,J.S., Kung,H.J. *et al.* (2014) JMJD5 regulates PKM2 nuclear translocation and reprograms HIF-1alpha-mediated glucose metabolism. *Proc. Natl Acad. Sci. U.S.A.*, **111**, 279–284.

83. Abu-Remaileh,M. and Aqeilan,R.I. (2014) Tumor suppressor WWOX regulates glucose metabolism via HIF1alpha modulation. *Cell Death Differ.*, **21**, 1805–1814.

84. Khan,M.N., Bhattacharyya,T., Andrikopoulos,P., Esteban,M.A., Barod,R., Connor,T., Ashcroft,M., Maxwell,P.H. and Kiriakidis,S. (2011) Factor inhibiting HIF (FIH-1) promotes renal cancer cell survival by protecting cells from HIF-1alpha-mediated apoptosis. *Br. J. Cancer*, **104**, 1151–1159.

85. Fang,L., Zhang,L., Wei,W., Jin,X., Wang,P., Tong,Y., Li,J., Du,J.X. and Wong,J. (2014) A methylation-phosphorylation switch determines Sox2 stability and function in ESC maintenance or differentiation. *Mol. Cell*, **55**, 537–551.

86. Esteve,P.O., Chang,Y., Samaranayake,M., Upadhyay,A.K., Horton,J.R., Feehery,G.R., Cheng,X. and Pradhan,S. (2011) A methylation and phosphorylation switch between an adjacent lysine and serine determines human DNMT1 stability. *Nat. Struct. Mol. Biol.*, **18**, 42–48.

87. Wang,J., Hevi,S., Kurash,J.K., Lei,H., Gay,F., Bajko,J., Su,H., Sun,W., Chang,H., Xu,G. *et al.* (2009) The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. *Nat. Genet.*, **41**, 125–129.

88. Luo,W., Chang,R., Zhong,J., Pandey,A. and Semenza,G.L. (2012) Histone demethylase JMJD2C is a coactivator for hypoxia-inducible factor 1 that is required for breast cancer progression. *Proc. Natl Acad. Sci. U.S.A.*, **109**, E3367–E3376.

89. Dhayalan,A., Kudithipudi,S., Rathert,P. and Jeltsch,A. (2011) Specificity analysis-based identification of new methylation targets of the SET7/9 protein lysine methyltransferase. *Chem. Biol.*, **18**, 111–120.

90. Liu,X.H., Kirschenbaum,A., Lu,M., Yao,S., Klausner,A., Preston,C., Holland,J.F. and Levine,A.C. (2002) Prostaglandin E(2) stimulates prostatic intraepithelial neoplasia cell growth through activation of the interleukin-6/GP130/STAT-3 signaling pathway. *Biochem. Biophys. Res. Commun.*, **290**, 249–255.

91. Wellmann,S., Bettkober,M., Zelmer,A., Seeger,K., Faigle,M., Eltzschig,H.K. and Buhrer,C. (2008) Hypoxia upregulates the histone demethylase JMJD1A via HIF-1. *Biochem. Biophys. Res. Commun.*, **372**, 892–897.

92. Pollard,P.J., Loenarz,C., Mole,D.R., McDonough,M.A., Gleadle,J.M., Schofield,C.J. and Ratcliffe,P.J. (2008) Regulation of Jumonji-domain-containing histone demethylases by hypoxia-inducible factor (HIF)-1alpha. *Biochem. J.*, **416**, 387–394.

93. Beyer,S., Kristensen,M.M., Jensen,K.S., Johansen,J.V. and Staller,P. (2008) The histone demethylases JMJD1A and JMJD2B are transcriptional targets of hypoxia-inducible factor HIF. *J. Biol. Chem.*, **283**, 36542–36552.

94. Krieg,A.J., Rankin,E.B., Chan,D., Razorenova,O., Fernandez,S. and Giaccia,A.J. (2010) Regulation of the histone demethylase JMJD1A by hypoxia-inducible factor 1 alpha enhances hypoxic gene expression and tumor growth. *Mol. Cell. Biol.*, **30**, 344–353.

95. Lee,J.S., Kim,Y., Bhin,J., Shin,H.J., Nam,H.J., Lee,S.H., Yoon,J.B., Binda,O., Gozani,O., Hwang,D. *et al.* (2011) Hypoxia-induced methylation of a pontin chromatin remodeling factor. *Proc. Natl Acad. Sci. U.S.A.*, **108**, 13510–13515.

96. Zhang,H., Bosch-Marce,M., Shimoda,L.A., Tan,Y.S., Baek,J.H., Wesley,J.B., Gonzalez,F.J. and Semenza,G.L. (2008) Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J. Biol. Chem.*, **283**, 10892–10903.

97. Mazure,N.M. and Pouyssegur,J. (2010) Hypoxia-induced autophagy: cell death or cell survival? *Curr. Opin. Cell Biol.*, **22**, 177–180.

98. Bellot,G., Garcia-Medina,R., Gounon,P., Chiche,J., Roux,D., Pouyssegur,J. and Mazure,N.M. (2009) Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol. Cell. Biol.*, **29**, 2570–2581.

99. Carmeliet,P., Dor,Y., Herbert,J.M., Fukumura,D., Brusselmans,K., Dewerchin,M., Neeman,M., Bono,F., Abramovitch,R., Maxwell,P. *et al.* (1998) Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature*, **394**, 485–490.

100. Greijer,A.E. and van der Wall,E. (2004) The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J. Clin. Pathol.*, **57**, 1009–1014.