
Article

PBRM1 cooperates with YTHDF2 to control HIF-1 α protein translation

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Abstract: PBRM1, a component of the chromatin remodeller SWI/SNF, is often deleted or mutated in human cancers, most prominently in renal cancers. Core components of the SWI/SNF complex have been shown to be important for the cellular response to hypoxia. Here we investigated how PBRM1 controls HIF-1 α activity. We find that PBRM1 is required for HIF-1 α transcriptional activity and protein levels. Mechanistically, PBRM1 is important for HIF-1 α mRNA translation, as absence of PBRM1 results in reduced actively translating HIF-1 α mRNA. Interestingly, we find that PBRM1, but not BRG1, interacts with the m6A reader protein YTHDF2. HIF-1 α mRNA is m6A modified, bound by PBRM1 and YTHDF2. PBRM1 is necessary for YTHDF2 binding to HIF-1 α mRNA and reduction of YTHDF2 results in reduced HIF-1 α protein expression in cells. Our results identify a SWI/SNF independent function for PBRM1, interacting with HIF-1 α mRNA and the epitranscriptome machinery. Furthermore, our results suggests that the epitranscriptome associated proteins play a role in the control of hypoxia signalling pathways.

Keywords: keyword 1; PBRM1 2; HIF-1 3; SWI/SNF 4; YTHDF2 5; m6A; 6; Hypoxia.

1. Introduction

The cellular response to decreased oxygen tension (hypoxia) is characterised by changes in the gene expression program primarily mediated by the hypoxia-inducible factor (HIF) family of transcription factors. HIF transcription factors are heterodimers composed of an oxygen labile HIF- α subunit and a constitutively expressed HIF-1 β subunit. In well oxygenated cells HIF- α subunits are targeted for proteasomal degradation by sequential proline hydroxylation and polyubiquitination mediated by proline hydroxylases (PHDs) and the Von Hippel Lindau (VHL) E3 ubiquitin ligase, respectively [1]. In hypoxic cells PHD enzymes are inhibited leading to the stabilisation and accumulation of HIF- α isoforms. This results in active transcription of HIF target genes [2].

In order to facilitate the extensive changes in gene expression necessary for the cellular response to hypoxic stress changes to the chromatin structure must take place for transcription factors such as HIF to access the promoter regions of its target genes. Chromatin exists in a spectrum of closed and open conformations determined by the density of nucleosomes, with active transcription requiring an open conformation of chromatin so that protein complexes can bind to the underlying DNA [3]. One mechanism of regulating chromatin structure is through the action of ATP-dependent remodelling

complexes, which can result in either increased or decreased gene transcription [3]. There are 4 families of ATP-dependent remodelling complexes: imitator switch (ISWI), chromodomain helicase DNA-binding (CHD), INO80 and switch/sucrose non-fermentable (SWI/SNF). Each subfamily is specialised to preferentially achieve particular chromatin outcomes: assembly, access or editing, and has been shown to contribute to gene expression changes in response to hypoxic stress [3,4]

The levels and activity of SWI/SNF chromatin remodelling complexes are important for controlling gene expression in response to hypoxic stress [5]. SWI/SNF complexes are a family of large multi-subunit complexes containing at least 8-12 subunits including an ATPase module, either BRG1 or BRM1, which exert sliding force to displace nucleosomes to allow the binding of the transcriptional machinery to the promoters of target genes [6]. The function of SWI/SNF complexes is defined by the composition of subunits within each subcomplex with PBAF containing PBRM1, ARID2 and PFH10; while BAF complexes contain ARID1A/B protein [6].

We have previously shown that the SWI/SNF components BAF155, BAF57, and BRG1 are required for HIF-1 α mRNA expression, and their depletion impairs the HIF-dependent hypoxic response [7]. Indeed, not only is chromatin remodelling necessary for HIF-1 α mRNA expression, but both BRG1 and BRM are required for full activation of HIF-1 and HIF-2 target gene expression in Hep3B, RCC4T, and SW13 cells [8]. More recent work has shown overexpression of the SWI/SNF subunit, protein polybromo-1 (PBRM1), increases the levels of both HIF-1 α and HIF-2 α target genes (PHD2, Glut1, BNIP3) [9], suggesting PBRM1 could regulate HIF-1 α in a similar way to other SWI/SNF complex members. Interestingly, PBRM1 along with the HIF-1 α and HIF-2 α E3 ligase, VHL, is the most frequently mutated gene in clear cell Renal Cell Carcinoma (ccRCC) [10]

PBRM1 is a large (193 kDa) multidomain protein containing six bromodomains, which bind to acetylated histones, BAH domains which are protein binding domains [11], and an HMG domain which has been shown to bind to the DNA minor groove [12]. Although, PBRM1 is a core component of the PBAF complex, numerous studies have demonstrated nucleosome remodelling-independent functions of PBRM1 including: localising with kinetochores during mitosis [12], sister chromatid cohesion [13], DNA double strand break repair [14], and interferon signalling [15,16]. Therefore, how PBRM1 levels and activity can alter the HIF-dependent hypoxic response is not yet clear.

Here we investigated the involvement of PBRM1 in regulating HIF activity. We find that PBRM1 is required for full HIF activity and HIF-1 α protein levels. However, this regulation occurs through promoting efficient translation of HIF-1 α mRNA, rather than direct effects on HIF-1 α gene transcription. This activity is supported by PBRM1's ability to bind selectively to HIF-1 α mRNA, as well as the RNA-binding protein YTHDF2, which is also required for normal levels of HIF-1 α protein in specific cellular backgrounds. This reveals a function for PBRM1 distinct from chromatin regulation and the rest of the SWI/SNF complex and suggests that the epitranscriptome and its machinery are important for the cellular response to hypoxia.

2. Materials and Methods

Cell culture and treatment

Cell lines were obtained from the European Collection of Cell Cultures. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 2mM L-glutamine, 10% (v/v) Foetal Bovine Serum (FBS) (Gibco), 50units/mL penicillin (Lonza) and 50µg/mL streptomycin (Lonza). Reporter HRE-luciferase cell lines were maintained in these conditions supplemented with 0.5 µg/ml Puromycin. All cell lines were routinely tested for mycoplasma contamination using MycoAlert kit from Lonza. Hypoxia treatments were performed in an InVivo 300 or InVivo2 hypoxia workstation (Ruskin) at 1% O₂, 5% CO₂, and 37°C. Cells were treated with MG132 (Calbiochem) by supplementing the growth media with a final concentration of 20 µM (dissolved in DMSO) 3 hrs prior to cell lysis. Cells were treated with cycloheximide at a final concentration of 0.1 mg/ml. Cells were treated with Actinomycin D at a final concentration of 5µg/ml.

siRNA and DNA transfection

Transfections in HeLa, U2OS, H1299, and A549 were performed using Interferin (Polyplus) following manufacturer's instructions. HEK293 cells were transfected with 0.12M CaCl₂ and HEPES buffered saline (0.156M NaCl, 0.375M Na₂HPO₄, 10mM HEPES) were mixed in water at a final volume of 400µL and added to cells. Cell culture media was changed 24h following transfection and cells were harvested 48h following transfection. GFP-PBRM1 was a kind Gift of Prof. Jessica Downs and was described in [14]. For DNA transfections 1µg of plasmid DNA was transfected per well of a 6-well plate. siRNA oligonucleotides (Eurofins) were transfected at 27nM. The siRNA oligonucleotide sequences were: Scrambled AACAGUCGCGUUUGCGACUGG, PBRM1_A GAAGAAAGCAUUAAGGUAU, PBRM1_B TCAGGACGTCTCATTAGCGAA, YTHDF2 AAGGACGTTCCCAATAGCCAA.

Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% (v/v) NP40, 0.25% (w/v) Na-deoxycholate, 0.1% (w/v) SDS, 10 mM NaF, 2 mM Na₃VO₄ and 1 tablet/10 mL Complete, Mini, EDTA-free protease inhibitors (Roche)), centrifuged for 10 mins at 13,000rpm at 4°C, collecting the supernatant for analysis. SDS-PAGE and immunoblotting were performed using standard protocols. The following primary antibodies were used for immunoblotting: HIF-1α (610958, BD Biosciences), HIF-2α (sc-13596, Santa Cruz), HIF-1β (3718, Cell Signalling), β-Actin (3700, Cell Signaling)/ (60009-1-Ig, Proteintech), PBRM1 (ABE70, Millipore), YTHDF2 (24744-1-AP, Proteintech/80014, Cell Signaling). GFP (2956, Cell Signaling), BRG1 (Santa Cruz, sc-17796). Following incubation with a horseradish peroxidase-conjugated secondary antibody (Cell Signalling Technology), chemiluminescence (Thermo Scientific) was detected.

Luciferase assay

Cells lines stably expressing HRE-luciferase reporter were transfected and/or treated as indicated before lysis in Passive Lysis Buffer (PLB). Luciferase assays were performed according to the manufacturer's instructions (Promega) and activity was measured using a Lumat LB 9507 Luminometer (EG&G). Results were normalised according to protein concentration and reported as a percentage of control.

qPCR

RNA from HeLa cells was extracted using the peqGOLD total RNA kit (Peqlab) and from HEK293 cells using Direct-Zol RNA MiniPrep kit (Zymo Research) according to manufacturer protocols. RNA was reverse transcribed using the Quantitect Reverse Transcription kit (Qiagen). Real-time PCR was performed using Brilliant II Syber green kit (Agilent) on a Mx3005P qPCR machine (Agilent). mRNA levels were calculated based on averaged CT values and normalized to β -actin mRNA levels. The following primers were used for qPCR: HIF-1 α forward CATAAAGTCTGCAACATGGAAGGT, HIF-1 α reverse ATTTGATGGGTGAGGAATGGGTT, HIF-1 β forward CAAGCCCCTTGAGAAGTCAG, HIF-1 β reverse GAGGGGCTAGGCCACTATTC, β -Actin forward CCCAGAGCAAGAGG, β -Actin reverse GTCCAGACGCAGGATG, HK2 forward AGCCCTTCTCCATCTCCTT, HK2 reverse AACCATGACCAAGTGCAGAA, PHD2 forward GAAAGCCATGGTTGCTTGTT, PHD2 reverse TGTCTTCTGGAAAAATTCG, PHD3 forward CTTGGCATCCCAATTCTTGT, PHD3 reverse ATCGACAGGCTGGTCCTCTA, GLUT1 forward TCAAAGGACTTGCCCAAGTTT, GLUT1 reverse GATTGGCTCCTTCTCTGTGG, VEGF forward AGCTGCGCTGATAGACATCC, VEGF reverse CTACCTCCACCATGCCAAGT, BNIP3 forward GCCACCTCGCTCGCAGACAC, BNIP3 reverse CAATCCGATGGCCAGCAAATGAGA.

Polysome profiling

HeLa cells were transfected in 10 cm plates. 1.5 hrs before lysis translation was stimulated by changing the media. 10 mins before lysis 0.1 mg/ml cycloheximide was added. Cells were washed twice with 5ml ice cold PBS containing 0.1 mg/ml cycloheximide. Cells were collected in 300 μ l PBS with cycloheximide, and the PBS was removed by centrifugation. The cells were resuspended in 550 μ l polysome extraction buffer (15 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 300 mM NaCl, 1% Triton X-100, 1 mM DTT, 0.1 mg/ml cycloheximide and RNasein (Sigma)). Cellular debris was pelleted by centrifugation at 17000 g for 1 min at 4°C. 500 μ l of supernatant was added to 10 ml 10-50% sucrose gradients (with extraction buffer without Triton X-100). Gradients were centrifuged using a SW41 rotor at 223000 g for 2 hrs at 4°C. The gradient was collected in 1 ml fractions. RNA was extracted from the solution with a RNAeasy extraction kit (Qiagen) according to the manufacturer's protocol. RNA was converted to cDNA using Quantitect Reverse Transcription kit (Qiagen) on a SureCycler 8800 (Agilent) and a Mx3005P qPCR machine. qPCR was performed using Brilliant II SYBR green (Agilent) on a Mx3005P (Agilent).

Co-immunoprecipitation

Cells were lysed in RIPA buffer and lysate was diluted with an equal volume of buffer (50 mM Tris-HCl (pH 8.0) 0.5 mM DTT, 20% glycerol, 5 mM NaF, 500 mM Na₃VO₄ and 1 EDTA-free protease inhibitor tablet per 10 ml of buffer). 500 μ g of cell lysate per condition was incubated with 2 μ g of each antibody, or 2 μ g of IgG control (Sigma) and rotated at 4 °C overnight. Immune complexes were captured with 20 μ l of protein G Sepharose beads (Generon) by incubating with rotation for 1.5 hrs at 4 °C. Beads were washed 3 times with PBS, then the beads were boiled with 20 μ l of SDS loading buffer.

RNA immunoprecipitation

Either HeLa or H1299 cells were grown in 15 cm plates, washed twice with ice cold PBS, harvested in ice cold PBS and pelleted. Of these cells 10% were used for each condition. Cells were lysed with 100 μ l of RIP lysis buffer (Magna RIP, Millipore) and snap frozen. Cells were thawed and centrifuged at 15000 g. The supernatant was diluted 10 fold with Magna-RIP wash buffer. Magnetic beads were coated with 5 μ g of the appropriate antibody for 30 mins at room temperature. The beads were incubated with the cell extract with rotation overnight at 4°C. The beads were then washed six times with RIP wash buffer. After setting aside 10% of the beads for IP control, the RNA was eluted by treatment with Proteinase K according to the manufacturer instructions, then purified by phenol-chloroform extraction followed by an 80% ethanol wash. RNA was finally dissolved in water, and the RNA was converted to cDNA and quantified with qPCR comparing to 500 ng of the input.

In vitro RNA binding assay

For RNA bait preparation, DNA duplexes containing T7 promoter sequence (5'-TAATACGACTCACTATAG-3') followed by either HIF-1 α UTR (CAGTGCTGCCTCGTCTGA) or luciferase mRNA control sequence (GGAGCCCCTGCTAACGACATTTACAACGAG) were generated by PCR. RNA was generated with transcription by T7 RNA polymerase according to the manufacturer's instructions (Thermo Fisher). Each 20 μ l reaction was spiked with 200 ng DNA template and 0.5 μ M biotin-labelled CTP. The reaction mixture was then treated with 2 Units of DNase and incubated at 37°C. The RNA was purified by RNA purification column (PeqGOLD, Peqlab). Secondary structure was ensured by heating to 90°C followed by 2 mins incubation on ice and adding 50 μ l RNA structure buffer (10 mM Tris-HCl (pH7.4), 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.4 U/ μ l RNase inhibitor). RNA was conjugated to beads by mixing 100 μ l of each RNA with 20 μ l of Streptavidin agarose beads (Sigma, S1638) and incubating with mixing for 30 mins at 4°C. Beads were washed twice with 5 mM Tris-HCl (pH 7.4), 1 M NaCl. A 10 cm plate of PC-3 cells per pulldown were lysed in buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1 mM PMSF, and 0.5 mM DTT, supplemented with an EDTA-free protease inhibitor tablet (Roche)). Cells were homogenised on ice using 10 strokes of a dounce homogeniser, then centrifuged at 16100 g for 15 mins at 4°C. 1 mg of supernatant was incubated with each of the bead-conjugated RNAs for 3 hrs at 4°C then washed 3 times with wash buffer (25 mM Tris-HCl (pH 7.4), 150 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.5% IGEPAL, 1 mM PMSF, 0.4 U/ μ l RNase inhibitor, supplemented with an EDTA-free protease inhibitor tablet (Roche)). Proteins present were analysed by SDS-PAGE and immunoblot.

Recombinant protein expression and in vitro pulldown assay

E. coli BL21 DE3 (Invitrogen) cells were transformed with plasmids containing 6xHis tagged YTHDF2 (1-384, 384-579, or 1-579) and PBRM1 (BAH1&2 967-1287 or HMG 1394-1462 domains). 250 ml secondary cultures were inoculated and grown to OD_{600nm} 0.6, then induced with 0.5 mM IPTG and incubated with shaking overnight at 18°C. Cells were pelleted, resuspended in lysis buffer (0.1 M Tris pH 8, 0.5 M NaCl, 5% Glycerol, 20 mM Imidazole, 1 mM β -ME, protease inhibitor tablet) then sonicated for 60 s in 10s pulses at 80% amplitude on ice. Lysed cells were centrifuged at 40,000 g and clarified lysate was

mixed with 40 μ l of Ni-NTA affinity resin for 4 hrs at 4°C. Beads were washed in lysis buffer for 15 mins, then 1 mg of HeLa cell lysate was added and the mixture was incubated at 4°C overnight with rotation. Beads were then washed 3 times with wash buffer (50 mM Tris pH 7.4, 200 mM NaCl, 5% Glycerol, 0.1% Tween-20, 1 mM DTT). Beads were boiled with SDS loading buffer and analysed by Western blotting.

3. Results

3.1. *PBRM1 is required for HIF transcriptional activity*

As previously discussed, SWI/SNF chromatin remodeller activity has been associated with HIF-dependent and independent responses in hypoxia [7-9]. Furthermore, several SWI/SNF complex members are often mutated in cancer. Of note, PBRM1 is often mutated in renal cancer, where the HIF system plays an important role in the oncogenic process [10]. To specifically examine the effects of PBRM1 in the control of HIF activity, siRNA oligonucleotides were used to deplete PBRM1 in a variety of cell lines containing an HRE-luciferase reporter, prior to exposure to hypoxia. PBRM1 levels were effectively depleted by 2 independent siRNAs as determined by immunoblot analysis (Figure 1A). PBRM1 depletion results in a significant decrease in HIF activity in all cell lines tested (Figure 1A). We then investigated if this was also reflected at the level of endogenous HIF-dependent targets using qPCR. We could detect significant decreases in the levels of HK2, PHD2 and PHD3 mRNA in HeLa cells (Figure 1B) but not GLUT1 mRNA levels (Figure 1B). In HEK293 cells, PBRM1 depletion resulted in decreased mRNA levels of all the HIF targets we investigated (Figure 1C). These results suggest that knockdown of PBRM1 inhibits hypoxia-induced HIF activity in multiple cell lines.

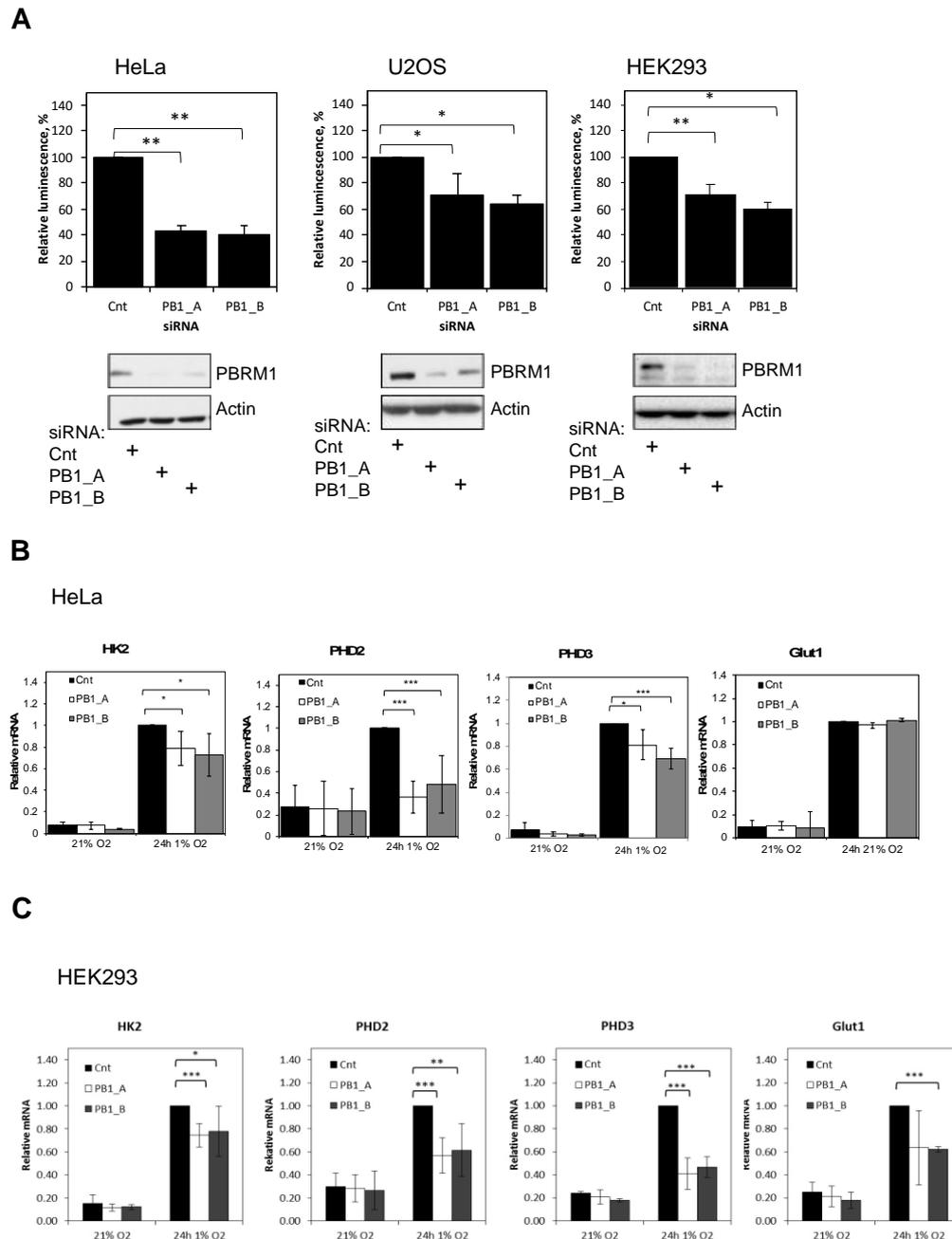


Figure 1. PBRM1 is required HIF transcriptional activity. (A) HeLa, U2OS, and HEK293 HRE-luciferase reporter cell lines were transfected with either control (Cnt) or PBRM1 (PB1) siRNAs for 48 hrs prior to lysis, and exposed to 1% O₂ for 24 hrs prior to lysis. Cell lysates were assayed for luciferase activity and western blot analysis. Graph displays mean and standard deviations (SD) of a minimum of three independent biological replicates. Anova significance indicated (* $p \leq 0.05$, ** $p \leq 0.01$). (B) HeLa cells and (C) HEK293 cells were transfected and treated with hypoxia as before. Total RNA was extracted, and mRNA levels was assessed by qPCR, normalised to Actin, and compared to control siRNA. Graph depicts the means and SD of a minimum of three independent biological replicates with Anova significance indicated (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.2 PBRM1 is required for HIF-1 α protein expression in normoxia and hypoxia

HIF activity is primarily regulated by the availability of the oxygen-sensitive HIF- α subunits. This is dependent on the degradation of HIF- α by the proteasome. To

investigate if PBRM1 has a role in regulating their abundance, levels of HIF-1 α and HIF-2 α were measured by immunoblot analysis in hypoxic cells in which PBRM1 levels were depleted using siRNA. Interestingly, depletion of PBRM1 specifically reduced the levels of hypoxia-induced HIF-1 α but did not alter the levels of the HIF-2 α or HIF-1 β HIF subunits (Figure 2A). Conversely, PBRM1 overexpression increases the levels of hypoxia induced HIF-1 α but again had little effect on other HIF subunits (Figure 2B). We next investigated if inhibiting the proteasome, would alter PBRM1-dependent reduction of HIF-1 α protein levels. Analysis of PBRM1 depletion in HeLa and HEK293 cells showed that HIF-1 α levels could not be restored by treating cells with the proteasome inhibitor MG132 (Figure 2C). This was also the case when we co-depleted a proteasomal component RPN11 with PBRM1 (Sup. Fig. S1A). Consistent with this observation, we could not detect changes in the stability of HIF-1 α protein, when PBRM1 was depleted (Sup. Fig. S1B). We could show that overexpression of PBRM1, lead to increased levels of HIF-1 α in presence or absence of MG132 (Figure 2D). These results were similar to the observed effects following depletion of core SWI/SNF subunits we had previously investigated in U2OS cells [7], suggesting a role in transcriptional regulation via BRG1. To confirm this hypothesis, we analysed the role of PBRM1 in cells that have impaired BRG1, A549 and H1299 lung cancer cells [17]. Surprisingly, PBRM1 depletion in these cells resulted in similar reduction in HIF-1 α levels following MG132 treatment (Figure 2E). These results indicated that PBRM1 regulation of HIF-1 α is occurring through a mechanism independent of BRG1.

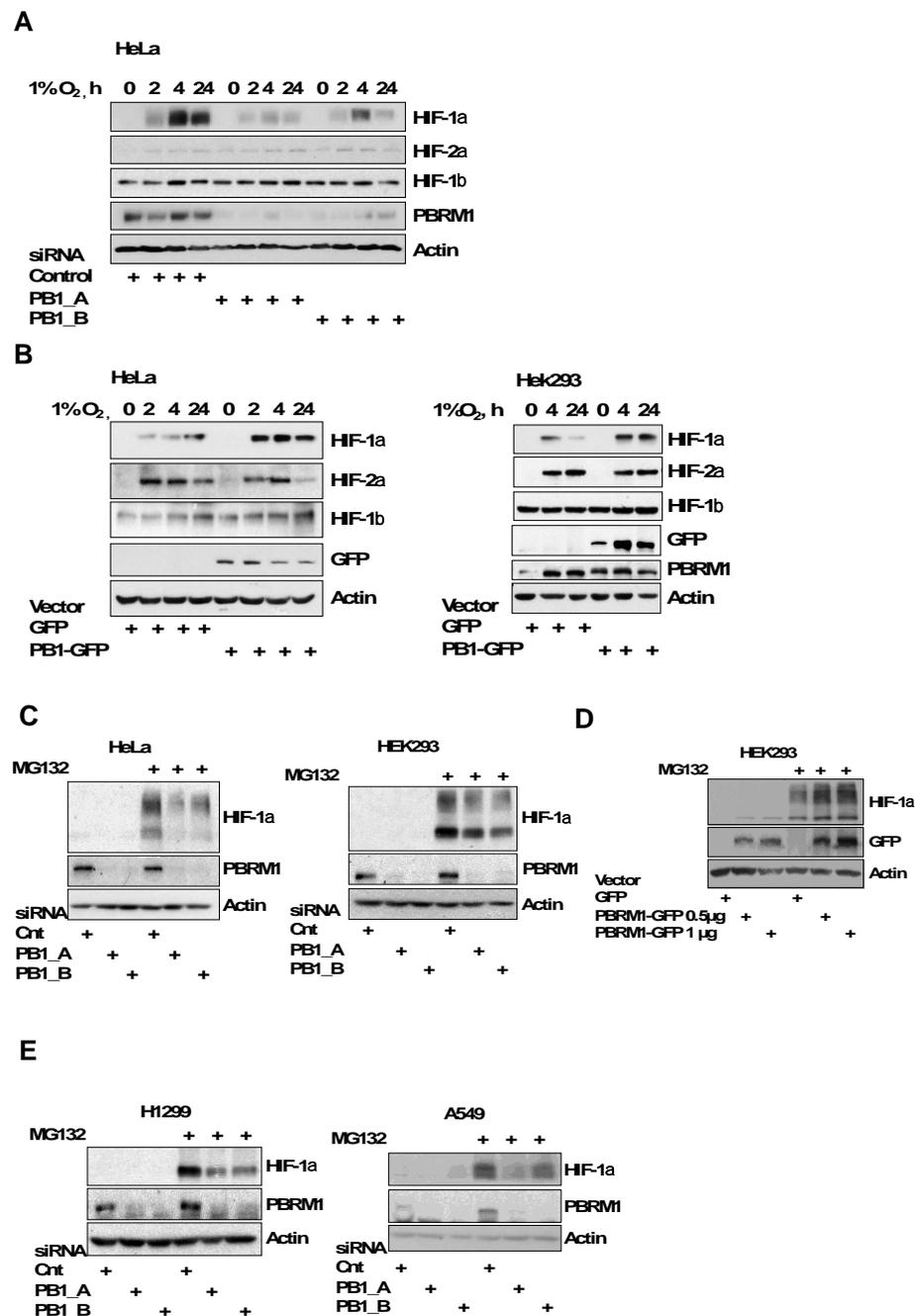


Figure 2. PBRM1 is required HIF-1 α protein levels independently of proteasomal degradation. (A) HeLa cells were transfected for a total of 48 hrs with either control or PBRM1 (PB1) siRNAs before exposure to various durations of treatment with 1% O₂. Whole cell lysates were analysed by western blot to assess HIF isoform protein levels. **(B)** HeLa and HEK293 cells were transfected with 1 μ g of either GFP empty vector or a plasmid containing GFP-PBRM1. Cells were then exposed to 1% O₂ for various periods of treatment. Whole cell lysates were analysed by western blot to assess HIF isoform protein levels. **(C)** HeLa and HEK293 cell lines were transfected with control or PBRM1 siRNAs, then treated with DMSO or MG132 for 3 hrs before lysis. Whole cell lysates were analysed by western blot to assess HIF-1 α protein levels. **(D)** HEK293 cells were transfected with 1 μ g of either GFP empty vector or increasing amounts of a plasmid containing GFP-PBRM1. Cells were then treated with DMSO or MG132 for 3 hrs before lysis. Whole cell lysates were analysed by western blot to assess HIF-1 α protein levels. **(E)** H1299 and A549 cells were transfected with control or PBRM1 siRNAs, then treated

with DMSO or MG132 for 3 hrs before lysis. Whole cell lysates were analysed by western blot to assess HIF-1 α protein levels.

3.3. PBRM1 binds selectively to HIF-1 α mRNA and promote polysome processing

Our analyses of how PBRM1 controls HIF-1 α suggest a mechanism that differs from that of SWI/SNF components BAF155, BAF57, and BRG1, regulation of HIF-1 α transcription [7]. To investigate this, we examined HIF mRNA levels in HeLa cells, after PBRM1 siRNA depletion. HIF-1 α , HIF-2 α , and HIF-1 β transcript levels all increase when PBRM1 is depleted (Figure 3A). Knockdown of BRG1, led to a reduction in HIF-1 α mRNA level as expected (Figure 3B). Furthermore, PBRM1 depletion did not change HIF-1 α mRNA in HEK293 (Figure 3C) and H1299 cells (Figure 3D). We ruled out PBRM1 depletion decreasing HIF-1 α mRNA stability (Figure S2A). Clearly PBRM1 does not regulate HIF activity through transcriptional regulation but has a divergent function to other SWI/SNF complex members.

It is known that PBRM1 can bind to DNA as part of its function on the PBAF complex [12], but we wondered whether it could bind to RNA as a mechanism for regulating the expression of HIF-1 α . Using an RNA immunoprecipitation (RIP) assay we find that PBRM1 can bind to HIF-1 α mRNA (Figure 3E), as well as the mRNAs of other hypoxia-responsive HIF target genes (Sup. Fig. S3A) revealing a novel function of PBRM1. This RNA binding capacity was confirmed with an *in vitro* RNA binding assay demonstrating that PBRM1 can bind to HIF-1 α mRNA via the 5' UTR (Figure 3F). This RNA binding demonstrates specificity, as PBRM1 was unable to bind to a control mRNA sequence.

As PBRM1 depletion could decrease HIF-1 α protein levels but not transcript levels, we next investigated HIF-1 α translation. Global protein synthesis, as well as translation rates of individual mRNAs, can be measured by polysome profiling; a technique in which free ribosomes can be separated from mRNA bound ribosomes (polysomes) on a sucrose gradient. Quantitative PCR analysis of polysome-associated HIF-1 α mRNA revealed that PBRM1 depletion causes a decrease in HIF-1 α mRNA association with actively translating ribosomes consistent with a decrease in HIF-1 α translation efficiency (Figure 3G-H). Importantly, no change was detected in the levels of polysome associated mRNAs for HIF-2 α and HIF-1 β (Sup Fig. S3B). These data suggest that HIF-1 α mRNA translation is sensitive to levels of PBRM1.

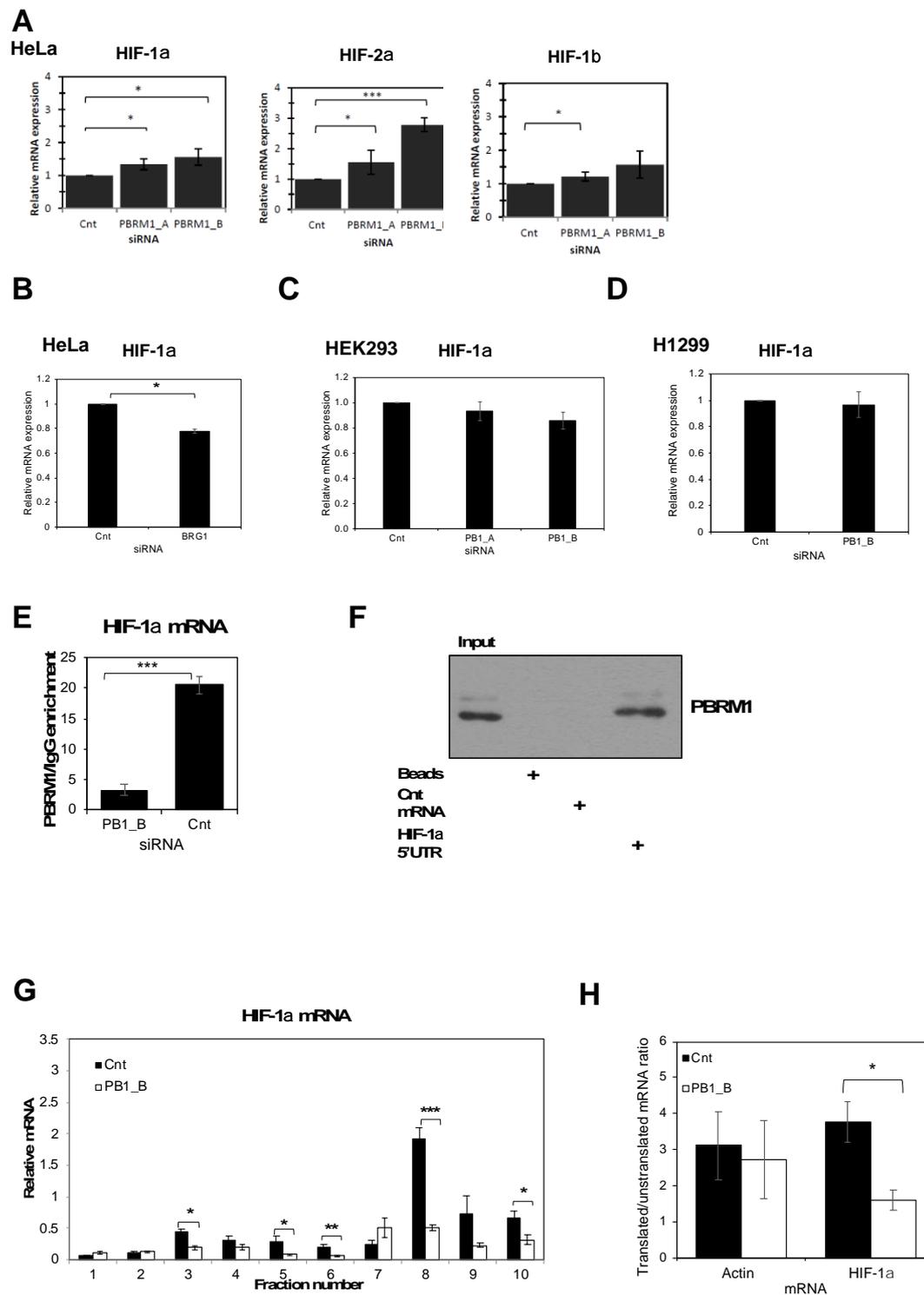


Figure 3. PBRM1 binds HIF-1 α mRNA and regulates HIF-1 α translation through polysome processing. (A) HeLa cells were transfected with control and BRG1 siRNAs for 48 hrs then cells were lysed and the mRNA levels of HIF transcripts were assessed by qPCR compared to actin levels. The means and standard deviations are plotted with Anova significance indicated (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). (B) HeLa, (C) HEK293, and (D) H1299 cells were transfected with control and PBRM1 siRNAs for 48 hrs then cells were lysed and the mRNA levels of HIF transcripts were assessed by qPCR compared to actin levels. The means and standard deviations are plotted with Anova significance indicated (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). (E) HeLa cells were transfected with either control or PBRM1 siRNAs, then 48 hrs later lysed and incubated with PBRM1 antibody coupled to magnetic beads. After washing any RNA present was converted to cDNA and analysed by qPCR using primers for HIF-1 α . The means and standard deviations are plotted

with Anova significance indicated (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). (F) Biotinylated control and HIF-1 α RNA bait were conjugated to beads then incubated with 1 mg of HeLa cell lysate. The beads were washed and PBRM1 protein levels were assessed by Western blot. (G) HeLa cells were transfected with control or PBRM1 siRNA and lysed 48 hours later. Clarified HeLa cell lysate was added to a 10-50% sucrose gradient and centrifuged at 223,000 g then collected in fractions. RNA was extracted and levels of HIF-1 α were determined by qPCR for each fraction and reported relative to Actin levels. The means and standard deviations are plotted with Anova significance indicated (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). (H) The ratio of mRNA between polysomal (7-10) and monosomal (1-6) fractions are shown for Actin and HIF-1 α transcripts with and without mRNA knockdown of PBRM1. The means and standard deviations are plotted with Anova significance indicated (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.4. PBRM1 but not BRG1 can bind to the m6A binding protein YTHDF2

Due to our unexpected observations that PBRM1 controlled HIF-1 α levels through translational control we interrogated the PBRM1 interactome using IP-MS (data not shown) to identify known translational regulators and RNA binding proteins as interactors of PBRM1. Among these we found YTHDF2, which can recognise and bind to m6A methylated RNA [18]. We could validate this observation using western blot, identifying that PBRM1 binds YTHDF2 in normoxia and reduced binding in hypoxia (Figure 4A). Using bacteria expressed recombinant YTHDF2 as bait and total HeLa cell lysate, we could detect a robust interaction with PBRM1 (Figure 4B). However, when YTHDF2 protein was expressed in two parts, this interaction was either abolished (N-terminal part) or significantly reduced (C-terminal part). We could also determine that PBRM1 can interact with YTHDF2 via its HMG domain, while expression of BAH1 and 2 domains was insufficient to bind YTHDF2 (Figure 4C). Furthermore, treatment with RNase A had no effect on the binding between YTHDF2 and the HMG domain of PBRM1 (Figure 4D), indicating that the interaction between YTHDF2 and PBRM1 is not dependent on the presence of RNA, but is more likely mediated by protein-protein interaction. Consistent with our data presented above, immunoprecipitation of the BRG1 complex, which also contains PBRM1, does not bind YTHDF2, suggesting that PBRM1 has functions outside of the canonical PBAF complex (Figure 4E).

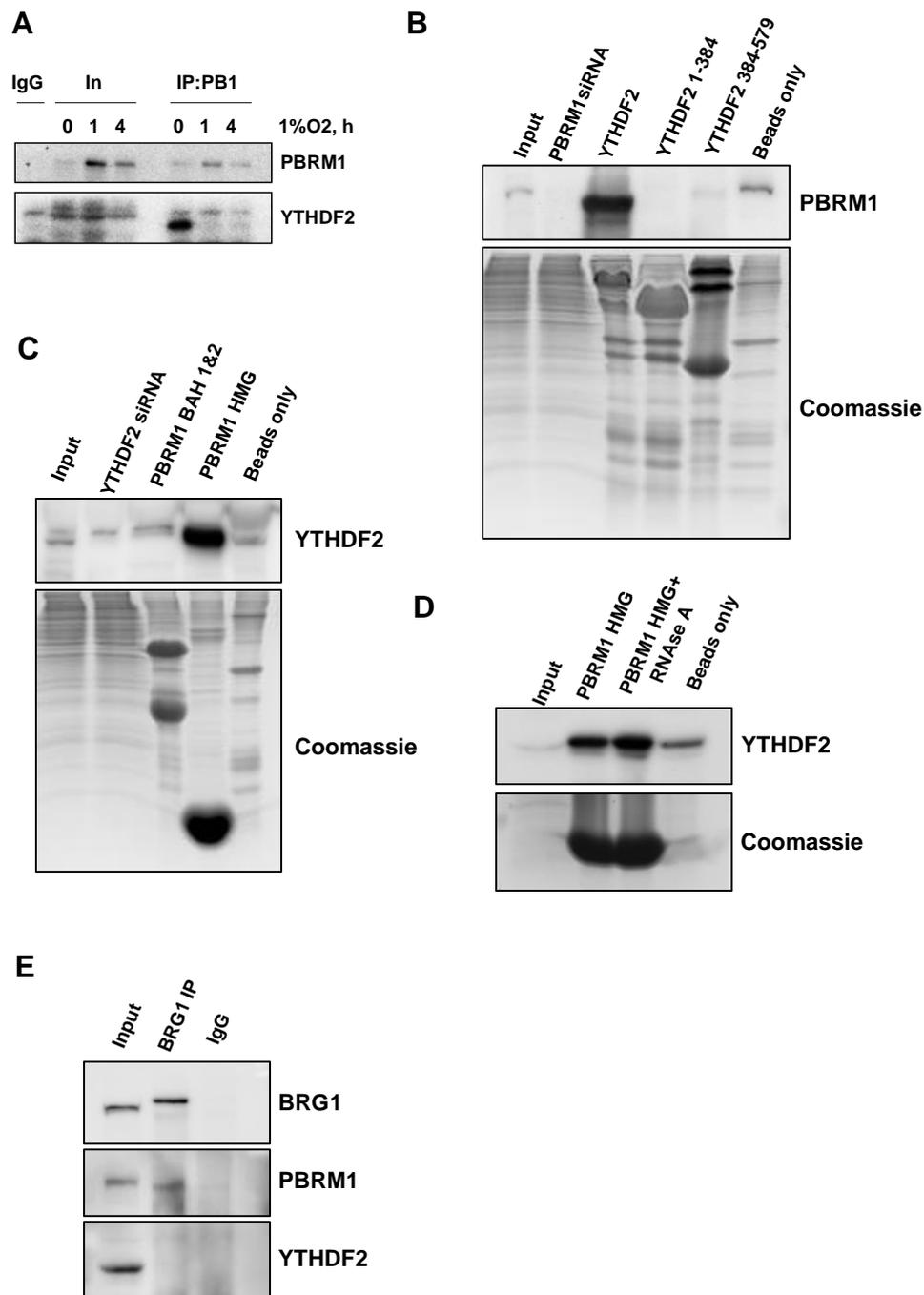


Figure 4. PBRM1 but not BRG1 can bind to the m⁶A binding protein YTHDF2 in cells and in vitro. (A) HeLa cells were exposed to different duration of 1% O₂. 500 µg of cell lysate was immunoprecipitated with either PBRM1 antibody or IgG control. Levels of PBRM1 and YTHDF2 were determined by Western blot. Input represents 10% of protein. (B) Recombinant His-tagged domains of YTHDF2 or (C) PBRM1 bound to Ni-NTA beads were incubated with 1 mg of HeLa total cell lysate overnight at 4°C, then washed and protein levels analysed by western blot. Input represents 10% of protein. (D) Recombinant His-tagged PBRM1 HMG domain (1394-1462) bound to Ni-NTA beads were incubated with 1 mg of HeLa total cell lysate overnight at 4°C, with or without 50 µg of RNase A, then washed and YTHDF2 levels analysed by western blot. Input represents 10% of protein. (E) 500 µg of HeLa cell lysate was immunoprecipitated with either BRG1 antibody or IgG control. Levels of BRG1 and YTHDF2 were determined by Western blot. Input represents 10% of protein.

3.5. PBRM1 is required for YTHDF2 binding to HIF-1 α mRNA and for HIF-1 α protein expression

Our data suggests that HIF-1 α RNA could be N6-Adenosine methylated (m6A). To investigate this further, datasets publicly available were interrogated [19-23]. Our analysis revealed that HIF-1 α has indeed been identified both in human and mouse tissues as being m6A modified, and also bound by YTHDF2 in mouse cells [20] (Sup. Table S1). We next determined if we could validate this observation in our cell system. Indeed, we confirm that HIF-1 α mRNA is m6A modified and can be bound by YTHDF2 (Figure 5A). To determine the interplay between YTHDF2 and PBRM1 in regulating HIF-1 α we depleted PBRM1 by siRNA and assessed HIF-1 α mRNA transcript bound to YTHDF2 (Figure 5B). Our results indicated that YTHDF2 binding to HIF-1 α mRNA requires the presence of PBRM1 (Figure 5B). To investigate the functional significance of PBRM1 binding to YTHDF2, we determined if YTHDF2 was required for HIF-1 α protein expression. Depletion of YTHDF2 decreases HIF-1 α protein following MG132 treatment in both HeLa and H1299 cell lines (Figure 5C-D), demonstrating their requirement for HIF-1 α protein expression.

Taken together our data demonstrate a previously unknown function for PBRM1, outside the SWI/SNF complex and in cooperation with the epitranscriptome, in the control of HIF-1 α mRNA fate.

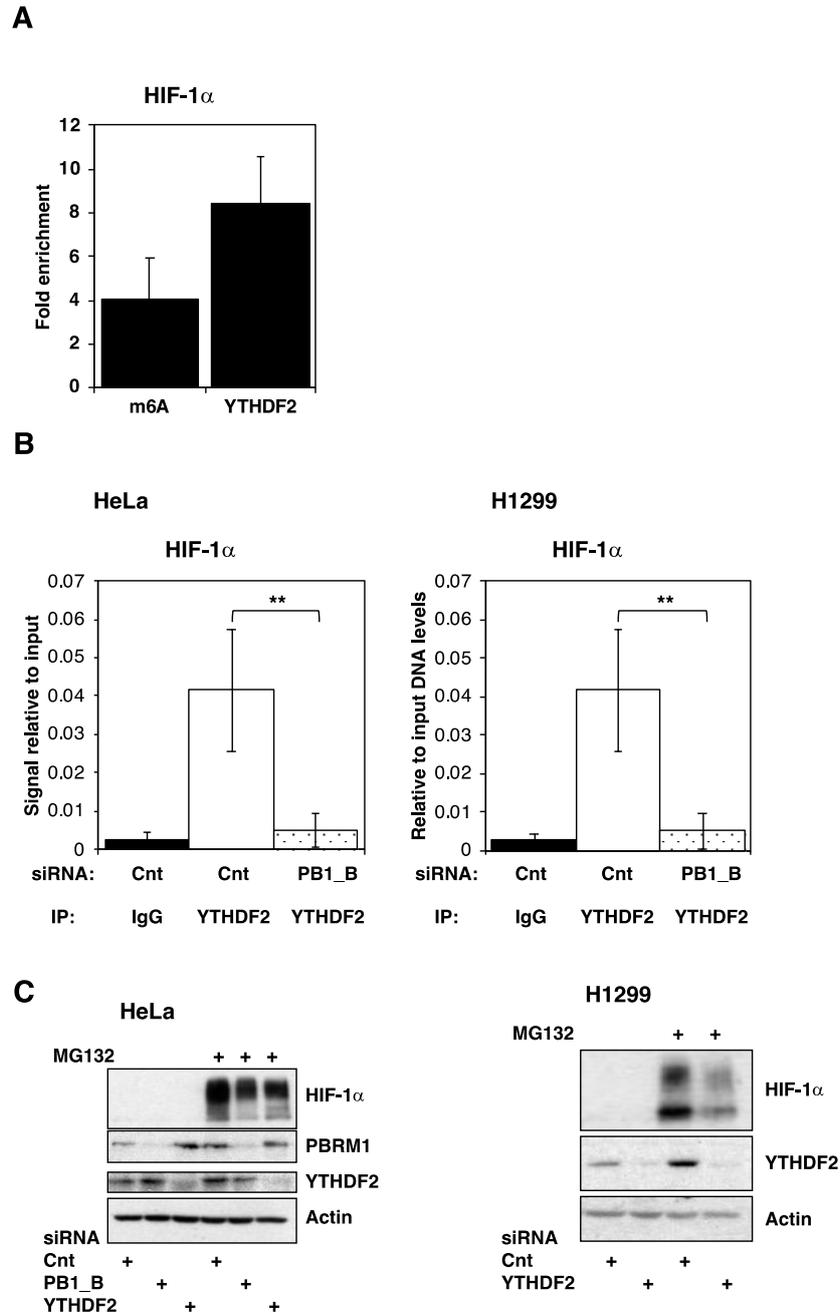


Figure 5. HIF-1 α mRNA is m6A modified and bound by YTHDF2. (A) HeLa cells were lysed and incubated with either m6A or YTHDF2 antibodies, then bound HIF-1 α mRNA was analysed by qPCR. (B) HeLa and H1299 cells were then transfected with either control or PBRM1 siRNAs and incubated with IgG control or YTHDF2 antibodies before analysis of HIF-1 α mRNA levels. The means and standard deviations are plotted with Student's t-test significance indicated (** $p \leq 0.01$). (C) HeLa or H1299 cells were transfected with control, PBRM1, or YTHDF2 siRNAs and either DMSO or MG132 for 3 hrs, then cells were lysed and protein levels determined by western blot.

4. Discussion

Here we investigated the role of PBRM1, a component of the SWI/SNF complex, in the regulation the HIF response. We find that PBRM1 is required for HIF transcriptional activity through promoting HIF-1 α protein synthesis. Unlike, core members of the SWI/SNF complex [7], PBRM1 can promote efficient translation of HIF-1 α mRNA though

polysome processing. This activity is supported by PBRM1's ability to bind selectively to HIF-1 α mRNA, along with the m6A RNA-binding protein YTHDF2, which is also required for normal levels of HIF-1 α protein. Our data reveal a function for PBRM1 that is separate from its involvement in the SWI/SNF complex, as PBRM1 is able to control HIF-1 α protein in the absence of a functional ATPase. Furthermore, we find that HIF-1 α is modified by m6A, suggesting that RNA methylation is required for full efficiency of the hypoxia inducible pathway.

There is a precedent for RNA binding activity within the SWI/SNF complex with the catalytic subunits BRG1 and BRM interacting with lncRNA and mRNA [24,25]. Some RNA processing has been shown to occur co-transcriptionally [26] with the PPB1 subunit of RNA polymerase II acting as a platform for recruiting machinery for 5'-end capping, splicing, polyadenylation, and histone methylation [27,28]. Chromatin structure can influence RNA fate, as H3K4me3 has been shown to recruit RNA capping machinery [29]. SWI/SNF has been shown to affect the elongation rate of RNA Pol II influencing alternative exon inclusion [30]. PBRM1 contains an HMG domain, which is known to bind DNA, and in some cases preferentially bind RNA [31,32]. We find that PBRM1 is able to bind to HIF-1 α mRNA in cells and *in vitro*. This demonstrates a new function for PBRM1, which is independent of its role in a catalytic SWI/SNF complex, as PBRM1 depletion also affects HIF-1 α levels in H1299 cells which are deficient BRG1 [17,33]. It would therefore be of interest to conduct an unbiased analysis using RIP-seq for example to determine how widespread this new PBRM1 function is. An unusual function for PBRM1 was very recently reported [34]. PBRM1 was found to bind to methylated microtubules. This binding was necessary to recruit the SWI/SNF members to the mitotic spindle and ensure genomic stability [34]. Our results similarly show that PBRM1 binds methylated sites, though in RNA, however, this seems to be independent of the other SWI/SNF components.

In hypoxia global translation is reduced, regulated at the stage of initiation [35], posing a paradox with the increases in protein levels of hypoxia-inducible genes. Several mechanisms are proposed to contribute to the selective translation of HIF-1 α mRNA in hypoxia (Reviewed in [36]). Our analysis revealed that HIF-1 α mRNA is m6A modified. This has also been seen in unbiased genome wide analysis for this modification [21,23]. More than 150 different post-transcriptional modification have been identified on cellular RNAs [37], with the most abundant being N6-methyladenosine (m6A) modification within the coding region or UTRs of the RNA, particularly in mRNAs [18]. RNA methylation is "written" by the methyltransferase complex METTL3/14, WTAP, RBM15/15B, and KIAA1429, "erased" by the demethylases FTO and ALKBH5 and recognised or "read" by YTHDF1/2/3 [18]. RNA m6A modification regulates RNA splicing [38], processing [38], decay [39], and translation [20,40]. m6A acts as a binding site for several reader proteins, including YTHDF2. YTHDF2 binding is normally associated with increased of RNA degradation [41]. However, under stress conditions such as heat shock, YTHDF2 translocates to the nucleus and protects 5' UTR m6A which promotes cap-independent translation of target genes [21]. A similar mechanism may operate for HIF-1 α mRNA, as we have found that YTHDF2 becomes more nuclear-located in response to hypoxia (Sup.

Fig. S4). This suggests a role for YTHDF2 in locating to recently transcribed RNA in the nucleus and contributing to hypoxia selective translation in cells.

Although, the analysis of the epitranscriptome has not been conducted in hypoxia, there are existing links between these two aspects. The m6A eraser proteins, FTO and ALKBH5 are 2-oxoglutarate dioxygenases, meaning they use 2-oxoglutarate, iron and oxygen as cofactors. Although, their oxygen affinity might be too high to participate in the hypoxia response [42], ALKBH5 has been shown to be a target of HIF-1 α [43]. This suggests that indeed hypoxia can influence the epitranscriptome in a cell. Furthermore, studies in liver cancer have shown that hypoxia can increase the levels of m6A in mRNA [44]. More recently, METTL3, one of the m6A writers, was shown to promote hypoxia induced hypertension in pulmonary arteries [45]. Our data demonstrating that HIF-1 α is m6A modified adds to the hypothesis that indeed the epitranscriptome is an integral part of the cellular response to hypoxia.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: PBRM1 does not alter HIF-1 α protein stability, Figure S2. PBRM1 does not alter the translation of HIF-1 β or HIF-2 α , Figure S3. Hypoxia induces YTHDF2 nuclear accumulation, Table S1: Analysis of public RIP/PAR-Clip data for HIF-1 α .

Author Contributions: A.S. performed all experiments unless otherwise indicated. M.F. performed the *in vitro* protein binding experiment, the immunofluorescence, and drafted the manuscript. N.K. performed the *in vitro* RNA binding assay and assisted in manuscript preparation. S.R. coordinated the project, oversaw experimental work, and wrote the manuscript.

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