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ELL is an HIF-1 α partner that regulates and responds to hypoxia response in PC3 cells

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Abstract

Background—ELL plays an important role in tumorigenesis and animal development. HIF-1 is a transcriptional factor that functions as a master regulator of O₂ homeostasis. Our previous studies showed that a binding partner of ELL, U19/Eaf2, can modulate HIF-1 α activity and hypoxia response, suggesting that ELL may also influence HIF-1 α pathway and hypoxia response.

Methods—Co-localization and co-immunoprecipitation were performed to test the interaction between ELL and HIF-1 α . PC3 cells with stable ELL knockdown and PC3 cells with stable ELL overexpression, along with their controls, were established using lentiviral expression system. Western blot and Real-time PCR were performed to test the effect of ELL on HIF-1 α protein and its down-stream gene transcription. To elucidate potential effect of hypoxia on ELL, cell growth and colony formation assay were performed using PC3 subline with stable ELL overexpression.

Results—ELL is associated with HIF-1 α in transfected cells. In PC3 prostate cancer cells, ELL inhibited HIF-1 α protein level and down-stream gene expression. As expected, ELL inhibited cell growth and colony formation under normoxia. Interestingly, the inhibition was alleviated under hypoxia.

Conclusions—Our findings suggest that ELL and HIF-1 α are binding partners and can modulate the functions of each other in hypoxia.

Keywords

ELL; Hypoxia; HIF-1 α ; prostate cancer

Introduction

Hypoxia has emerged as a primary physiological regulator of tumor progresses from a non-angiogenic to angiogenic phenotype (1,2). The transcriptional response to hypoxia is mediated through hypoxia-inducible factor-1(HIF-1) which belongs to a family of basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) transcription factors. HIF-1 is comprised of an α and a β subunit (3-6). HIF-1 α is highly inducible by hypoxia and acts as the master regulator of oxygen homeostasis in many cell lines (7-9). Under normoxic conditions, prolyl-4-hydroxylase domain (PHD) enzymes hydroxylate key proline residues located within the oxygen-dependent degradation domain (ODD) of HIF-1 α . Hydroxylated HIF-1 α can be bound by pVHL and undergo ubiquitination and subsequently degradation by the 26S proteasome. Under hypoxic conditions, HIF-1 α is stable and translocates to the nucleus where it heterodimerizes with HIF-1 β and binds to hypoxia response elements (HREs) within regulatory regions of target genes (10,11). Overexpression of HIF-1 α has been observed in many cancers, including prostate cancer (12,13). Elucidating mechanisms that regulate HIF-1 α activity will provide new insights into the mechanism of carcinogenesis.

The *ELL* (eleven-nineteen lysine-rich leukemia) gene on chromosome 19p13.1 was originally identified as a gene that undergoes frequent translocations with the *MLL* gene on chromosome 11q23 in acute myeloid leukemia (14,15). *ELL* can inhibit cell growth and induce apoptosis (16,17). Previous functional studies showed that *ELL* is an RNA polymerase II elongation factor that can both enhance the overall rate of elongation and inhibit promoter-specific transcription initiation (18,19). Moreover, *ELL* can modulate gene expression via acting as a selective co-regulator for steroid receptors (20). *ELL* is an essential gene in embryonic development because *ELL* gene knockout causes embryonic lethality in mice (21). However, the mechanisms by which *ELL* regulates animal development and tumorigenesis remain largely unclear.

ELL has been reported to bind to other proteins, including *ELL*-associated factors 1 and 2 (EAF1 and EAF2) and p53 in acute myeloid leukemia(3,22-25). p53 is a well-established tumor suppressor. EAF2 is up-regulated by androgens in the prostate, also was named up-regulated gene 19 (U19) (25). Recent studies including mouse knockout experiment indicate that EAF2/U19 is a novel tumor suppressor. The binding of *ELL* to p53 and/or EAF family proteins is likely to have significant impact on cellular activities (24,25) and also argues a potential role for *ELL* in tumorigenesis. Our previous studies showed that U19/Eaf2 is a binding partner of pVHL and can modulate HIF-1 α activity and hypoxia response (26). As a binding partner of EAF2, *ELL* may also influence HIF-1 α pathway and hypoxia response.

In this study, we investigated the interactions between *ELL* and HIF-1 α mainly in PC3 prostate cancer cell model. We choose PC3 as a model because prostate cancer is the most frequently diagnosed noncutaneous malignant neoplasia and the second most common cause of cancer-related deaths in males in the United States (27). We have generated data suggesting that *ELL* and HIF-1 α are binding partners and can modulate each other's functions in hypoxia response of PC3 prostate cancer cells.

Materials and methods

1. Plasmid construction

Full-length human HIF-1 α cDNA was cloned into pEGFP-C1 and pCMV-HA (Clontech, Mountain View, CA). Full-length human *ELL* cDNA, a gift from Dr. A. Shilatifard, was cloned into pEGFP-C1, pDSRed2-C1, and pCMV-Myc (Clontech). A human *ELL*-expressing lentiviral vector was generated by cloning full-length human *ELL* cDNA into the

pCDH1-MCS1-EF1-copGFP vector (System Biosciences, Mountain View, CA). All vectors were sequenced verified.

2. Cell culture, transfection and hypoxia

COS-7 and PC3 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). COS-7 and PC3 cells were grown under standard conditions in DMEM and RPMI1640, respectively; both supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA). Cells were transiently transfected using OPTI-MEM medium (Invitrogen) and Lipofectamine 2000 (Invitrogen). For hypoxic stimulation, cells were cultured in a multi-gas incubator, MCO-17A (SANYO, Japan) set to 1% O₂ and 5% CO₂.

3. Immunofluorescent microscopy

COS-7 or PC3 cells in 12-well plates were transiently transfected and fluorescence detected 24h later using a Nikon TE2000-U microscope (Melville, NY). Images were taken using PHOTOMETRICS® Cool SNAP fx digital camera (Roper Scientific, Inc. Trenton, NJ) and analyzed with MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Nuclei were stained with 1 µg/ml Hoechst 33342 for 2 minutes at room temperature.

4. Co-immunoprecipitation and Western blot

COS-7 cells on 10 cm culture dishes were transfected with 10 µg of plasmid DNA, and 24h later, lysed in a modified RIPA buffer. Equal amounts of cell extract were incubated with HA-probe agarose conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The immunoprecipitates were then washed, eluted, separated on a 10% SDS-PAGE, and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Membranes were probed with 1/1000× Anti-HA, Anti-GFP, Anti-GAPDH (Santa Cruz Biotechnology), or Anti-HIF-1α primary antibodies (Novus Biologicals, Littleton, CO) at 4°C overnight, followed by incubation with 1/5000× horseradish peroxidase (HRP)-linked secondary antibodies (Santa Cruz Biotechnology) for 1h at room temperature. Western blots were developed by the ECL detection system (Amersham Pharmacia, Piscataway, NJ).

5. Virus production and transduction of PC3 cells

We prepared lentivirus by co-transfecting packaging vectors with either the ELL/pCDH1-MCS1-EF1-copGFP or empty pCDH1-MCS1-EF1-copGFP vectors into 293TN cells. The medium was replaced 16h after transfection with DMEM containing 10% FBS. We then collected viral particles from the medium 48h after transfection using 0.45-µm filters (Nalgene, Rochester, NY) and low-speed centrifugation. PC3 cells were incubated with lentivirus and 8µg/ml Polybrene (Sigma), and three days later, GFP-expressing cells were FACS sorted. For shRNA studies, the University of Pittsburgh Cancer Institute Vector Core Facility generated pLKO.1-puro-TurboGFP (shControl) and pLKO.1-puro-shELLlentivirus. PC3 cells were infected with equal volumes of each virus in the presence of 8 µg/ml polybrene, and media was replaced the following day. After 24h, we began selection using 1µg/ml puromycin. All stably-infected cells were cultured for less than eight passages before being used in the experiments. The expression of endogenous ELL in PC3 sublines was determined by Western blot using a custom made polyclonal anti-human ELL antibody (ProteinTech Group, Inc., Chicago, IL)

6. Real-time quantitative RT-PCR

Total RNA was isolated from cells by the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA was synthesized by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA). Real-time quantitative PCR was performed using the SYBR

Green PCR Master Mix (Invitrogen). PCR primers included: ELL: 5'-ACTGCATCCAGCAGTATGTCTCCA-3' and 5'-TCCGAAACTGAACCTTCTTGCCCA-3'; VEGF: 5'-CCTTGCTGCTCTACCTCCAC-3' and 5'-ATGATTCTGCCCTCCTCCTT-3'; Glut-1: 5'-TCCACGAGCATCTTCGAGAAG-3' and 5'-TACT GGAAGCACATGCCAC-3'; and 18S: 5'-CATTCGTATTGCGCCGCT-3' and 5'-CGACGGTATCTGATCGTC-3'. mRNA abundance was calculated by using the $2^{-\Delta\Delta C_t}$ formula.

7. Cell growth and colony formation assay

Lentivirus-infected cells were cultured at a density of 1×10^4 cells/well in 6-well plates. After 24h, the medium was replaced, and the cells subjected to normoxia or hypoxia. Cells were then trypsinized and counted using a hemocytometer. For colony formation assays, 1,000 lentivirus-infected PC3-vector and PC3-ELL cells were seeded separately onto 10 cm tissue culture dishes. After 24h, cells were placed under normoxia or hypoxia for an additional 7 days, and then stained with 0.5% crystal violet. Stained colonies larger than 1 mm in diameter were counted. Each colony formation assay was carried out in triplicate and repeated at least two times.

8. Statistical analysis

Student's *t*-test was utilized to compare the difference between two groups. *P* value < 0.05 was considered to be statistically significant.

Results

1. ELL and HIF-1 α co-localize in the nuclei of transfected cells

Our previous studies showed that U19/Eaf2, a binding partner of ELL, can modulate HIF-1 α activity and response to hypoxic conditions(26). Since ELL binds U19/Eaf2, ELL may also influence HIF-1 α pathway and cellular response to hypoxia. First, we tested if ELL and HIF-1 α can co-localize in the cell. Transient transfection of green fluorescent protein (GFP)-tagged human ELL expression vector alone showed that GFP-ELL localized to the nuclei of the transfected PC3 and COS-7 cells (Fig. 1A). Co-transfection of HA-tagged HIF-1 α induced GFP-ELL to form a speckled nuclear pattern, in contrast to the diffuse nuclear localization of GFP-ELL alone. This suggests that HIF-1 α can interact with ELL (Fig. 1A). When we co-transfected GFP-tagged HIF-1 α with red fluorescent protein (RFP)-tagged ELL into PC3 and COS-7 cells, GFP-HIF-1 α and RFP-ELL co-localized in the same nuclear speckles (Fig. 1B). We also tested whether GFP-HIF-1 α and RFP-ELL co-localize under hypoxic conditions. GFP-HIF-1 α and RFP-ELL co-localized and formed nuclear speckles in both PC3 and COS-7 cells under hypoxia (Data not shown). These observations suggest that ELL and HIF-1 α are associated with each other under both normoxic and hypoxic conditions.

2. ELL and HIF-1 α can be co-immunoprecipitated

To verify that ELL and HIF-1 α can be present in the same complexes in living cells, COS-7 cells were cotransfected with expression vectors encoding HA-tagged human HIF-1 α and GFP-tagged human ELL and then cultured under both normoxic (20% O₂) and hypoxic (1% O₂) conditions. Whole cell lysates (WCL) were immunoprecipitated with HA-probe agarose conjugate and subsequently immunoblotted with an anti-GFP antibody. GFP-ELL co-precipitated by anti-HA antibody in the presence of HA-HIF-1 α (Fig. 2). Interaction of ELL with HIF-1 α under these conditions was not mediated through GFP, since no GFP was detected in the immunoprecipitates of cells transfected with the empty vector expressing only GFP. We also performed co-immunoprecipitation experiment using Flag-tagged ELL

and Myc-tagged HIF-1 α and detected co-precipitation of Flag-ELL and Myc-HIF-1 α in transiently transfected COS-7 cells (Data not shown). These results demonstrate that ELL can interact with HIF-1 α under both normoxic (20% O₂) and hypoxic (1% O₂) conditions.

3. ELL knockdown increases the levels of HIF-1 α protein and its down-stream gene expression in PC3 cells

To determine the effect of ELL on HIF-1 α , we established PC3 cells stably expressing shRNA specific for ELL to inhibit the expression of ELL using a lentiviral shRNA expression system. As shown in Fig. 3A and 3B, ELL protein and mRNA levels were knocked down approximately 50% in shELL-b cells, compared to the control PC3 cells.

To examine the effect of ELL knockdown on HIF-1 α , we analyzed its protein levels. As shown in Fig. 3E, under normoxic condition, the expression of HIF-1 α was very low. However, when cultured in hypoxic conditions, there was a significantly increase in HIF-1 α protein level (Fig. 3E). The knockdown of ELL efficiently increased HIF-1 α protein levels under hypoxic condition (Fig. 3E). To determine if ELL affected HIF-1 α transcriptional activity, we assayed for mRNA levels of HIF-1 α -targeted genes VEGF and Glut-1. PC3 cells with shRNA control (shControl) or stable ELL knockdown (shELL-b) were cultured in normoxic (20% O₂) or hypoxic (1% O₂) conditions for 6 hours, and then relative VEGF and Glut-1 mRNA levels were measured by real-time quantitative RT-PCR. As depicted in Fig. 3C and 3D, hypoxic conditions significantly increased the transcription of VEGF and Glut-1 in both shControl and shELL-b cells. shELL-b cells, which have decreased ELL expression, displayed enhanced mRNA levels of VEGF and Glut-1 under both normoxic and hypoxic conditions as compared to the shControl cells ($p < 0.05$). As a control, we tested the effect of hypoxia on GAPDH expression. Real-time RT-PCR detected little or no effect of hypoxia on GAPDH mRNA level (Data not shown), which is in agreement with previous findings that hypoxia does not affect GAPDH expression in PC3 cells (28,29). The enhancement of HIF-1 α down-stream gene expression in shELL-b cells may be attributed to the upregulation of HIF-1 α protein levels.

4. ELL overexpression inhibits HIF-1 α protein levels and HIF-1 α down-stream gene expression in PC3 cells

To further study the involvement of ELL on HIF-1 α pathway, we established PC3-ELL, PC3 cells with stable overexpression of ELL by lentiviral expression system, along with PC3-vector, PC3 cells infected with empty lentiviral vector. As shown in Fig. 4A and 4B, ELL protein and mRNA levels were dramatically increased in PC3-ELL cells as compared to the PC3-vector control cells.

To investigate the effect of ELL overexpression on HIF-1 α , PC3-ELL cells were first analyzed for HIF-1 α protein levels. Under hypoxic conditions, HIF-1 α protein accumulated in both PC3-vector and PC3-ELL cells, however, overexpression of ELL inhibited the HIF-1 α accumulation in PC3-ELL cells (Fig. 4E). We also explored whether overexpression of ELL impinges on mRNA expression of HIF-1 α -target genes VEGF and Glut-1. PC3-vector and PC3-ELL cells were cultured in normoxic (20% O₂) or hypoxic (1% O₂) conditions for 6 hours. Total RNA was isolated and then analyzed by real-time quantitative RT-PCR to determine the levels of VEGF and Glut-1 mRNA. As expected, in Fig. 4C and 4D, 1% O₂ significantly increased the transcription of VEGF and Glut-1 in both PC3-vector and PC3-ELL cells. Overexpression of ELL inhibited mRNA level of VEGF and Glut-1 under both normoxic and hypoxic conditions, with the extent of inhibition more dramatic under hypoxic condition. These results suggest that overexpression of ELL decreases HIF-1 α protein levels and HIF-1 α down-stream gene expression.

5. Hypoxia alleviates ELL inhibition of PC3 cell growth

The interactions between HIF-1 α with ELL proteins suggest that ELL function could be influenced by hypoxic conditions. A previous study showed that overexpression of ELL inhibits cell proliferation and induces apoptosis (17). To determine whether hypoxia can influence ELL inhibition of cell growth, lentivirus-infected PC3 stable cells were plated at equal density and cultured under normoxia or hypoxia for 96 h and then cell numbers were quantified using a hemocytometer. As a control, Fig. 5A showed that hypoxia had little or no effect on ELL protein levels. As expected, the growth of PC3-ELL was significantly inhibited as compared to the PC3-vector under normoxic condition ($p < 0.05$) (Fig. 5B). However, under hypoxic conditions, PC3-ELL proliferated at similar rate as PC3-vector cells. We also carried out colony formation assay for PC3-vector and PC3-ELL under normoxic and hypoxic conditions (Fig. 5C&D). Hypoxia slightly reduced the number of colonies formed by PC3-vector ($p > 0.05$). However, hypoxia increased the number PC3-ELL colonies and the result was statistically significant ($p < 0.05$). These results demonstrate that ELL inhibited cell growth under normoxic condition and the ELL inhibition of cell growth was alleviated under hypoxic conditions. As HIF-1 α is the master regulator of hypoxic response, the crosstalk between ELL and HIF-1 α may be involved in the hypoxia regulation of ELL activity.

Discussion

The studies herein provide evidence for crosstalk between ELL and HIF-1 α , particularly during hypoxia response in PC3 prostate cancer cells. Our studies showed that ELL and HIF-1 α can be co-immunoprecipitated and are co-localized to the nuclei of transfected cells. The interactions between ELL and HIF-1 α appear to be functionally important. ELL is capable of inhibiting hypoxia-responsive gene expression, and reciprocally, hypoxia alleviates the inhibition of cell growth by ELL. Our results suggest for a physical and functional link between ELL and HIF-1 α signaling pathways.

Our studies showed that under hypoxia condition ELL overexpression inhibited the mRNA levels of VEGF and GLUT-1 more than two-fold, which is significantly more dramatic than their inhibition by ELL under normoxia condition. The inhibition of VEGF and GLUT-1 expression under hypoxia condition is correlated with the reduced expression of HIF-1 α (Fig. 4). The ELL knockdown by shRNA and ELL overexpression caused opposite effects on HIF-1 α protein and down-stream genes. Down-regulation of ELL is associated with elevated levels of HIF-1 α protein and VEGF and GLUT-1 mRNA. Our findings suggest that the regulation of VEGF and GLUT-1 mRNA level by ELL is in part mediated through HIF-1 α . We recognize that ELL repression of endogenous HIF-1 α protein level was only observed in stably infected PC3 cells, but not for the exogenous HA-tagged HIF-1 α protein in transiently transfected COS-7 cells (Fig. 2). The differences in the effect of ELL on endogenous HIF-1 α (Fig. 4) and on transiently transfected HA-HIF-1 α (Fig. 2) may reflect the differences in cell lines used, HA-tagging, and/or transient vs. stable transfection. Regardless, the observation that knockdown of ELL by shRNA resulted in elevated HIF-1 α protein level strongly argues for a suppressive role for ELL in HIF-1 α protein levels *in vivo* in PC3 cells. It is likely that ELL modulation of hypoxia-responsive gene expression is mediated in part through regulating HIF-1 α protein levels. However, we recognize that ELL regulation of VEGF and GLUT-1 expression may involve other signaling pathways, in addition to HIF-1 α .

The expression of VEGF and GLUT-1 under normoxia conditions was also modulated by either ELL knockdown or ELL overexpression (Figs. 3 & 4). This raises a possibility that ELL may modulate the expression of VEGF and GLUT-1 using a mechanism independent of HIF-1 α , especially under normoxic condition. ELL is known to enhance transcriptional elongation. However, its transcriptional elongation function is unlikely responsible for its

ability to repress the mRNA levels of VEGF and GLUT-1 under normoxia condition because the transcriptional elongation activity of ELL is associated with elevated expression of its targeted genes(18). Further analysis will be required to elucidate the mechanism by which ELL represses the expression of VEGF and GLUT-1 mRNA levels.

The finding of physical and functional interactions between ELL and HIF-1 α provides another mechanism for crosstalk between EAF-ELL axis and the HIF-1 α signaling pathway. We previously reported that EAF2/U19 binds to and stabilizes pVHL and subsequently modulates HIF-1 α signaling. ELL is a well-established binding partner for EAF2/U19 (23,25) and HIF-1 α is recognized by pVHL (30-32). Thus, ELL, EAF, pVHL and HIF-1 α could associate with each other and be present in the same protein complex. This also raises the possibility that ELL binding to HIF-1 α is mediated through and/or facilitated by the EAF and pVHL proteins. Thus, ELL and HIF-1 α could interact both directly and indirectly.

We also showed that hypoxia alleviated the ELL inhibition of PC3 cell growth. Since hypoxia had little or no effect on the ELL protein level (Fig. 5B), the inability of ELL overexpression to suppress PC3 growth under hypoxia is unlikely associated with the ELL protein abundance. One possibility is that hypoxia may interfere with the signaling pathway by which ELL inhibits PC3 cell growth. Since the mechanism of ELL repression of cell growth remains virtually unknown, it is difficult to predict the possible mechanisms of hypoxia influence on ELL repression of cell growth. Regardless, our findings provide an example and a model system to explore the mechanism by which hypoxia modulates the function of growth suppressive factors. This suggests that hypoxia may also help prostate cancer cells to escape growth suppression by other growth-inhibitory genes and facilitate tumor progression.

The crosstalk between ELL and HIF-1 α provides a potential link between angiogenesis and hematopoiesis. Few studies reported the potential interactions between angiogenesis-promoting proteins and hematopoiesis-related transcription factors(33). ELL is implicated in hematopoiesis and MLL-ELL fusion protein plays a critical role in the development of acute myeloid leukemia (AML). The finding of ELL modulation of HIF-1 α activity suggests a potential role for HIF-1 α in hematopoiesis and AML. Our finding also suggests that ELL binding to HIF-1 α represents a novel potential mechanism to regulate angiogenesis.

In summary, our studies showed that ELL and HIF-1 α can be present in the same protein complex and influence each other's activity. ELL inhibits the expression of hypoxia-responsive genes and, reciprocally, hypoxia alleviates the ELL repression of prostate cancer cell growth. These studies argue for a functional crosstalk between ELL and HIF-1 α signaling pathways, providing new insights into the mechanisms of ELL and HIF-1 α action.

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Abbreviations

ELL	Eleven-nineteen Lysine-rich Leukemia
U19/Eaf2	ELL-associated factor 2
HIF-1	Hypoxia-inducible factor-1

Fig. 1. ELL co-localizes with HIF-1 α in the nucleus and form speckles

(A) Effect of HIF-1 α on GFP-ELL intracellular localization. Transient transfection of COS-7 and PC3 cells with GFP-ELL, HA-HIF-1 α or CMV-HA showed co-localization in nuclear speckles. (B) Co-localization analysis of RFP-ELL and GFP-HIF-1 α . GFP-HIF-1 α was transiently transfected into COS-7 and PC3 cells with RFP-ELL and Myc-EAF1. Twenty four hours after transfection, the intracellular localization of RFP-ELL and GFP-HIF-1 α was visualized under fluorescent microscope. Merged images were shown to demonstrate the co-localization. Myc-EAF1 was used here to enhance the red fluorescent signal of RFP-ELL.

Fig. 2. Co-immunoprecipitation of ELL with HIF-1 α

COS-7 cells were transfected with HA-HIF-1 α expression vector together with expression vector encoding GFP or GFP-ELL, as indicated. The transfected cells were untreated or subjected to hypoxia (1% O₂) for 6 h prior to lysis. Aliquots of whole cell lysate (WCL) and anti-HA immunoprecipitate (IP) were analyzed by immunoblot assay with indicated antibodies. NS, non-specific.

Fig. 3. ELL knockdown increases the protein level of HIF-1 α and its down-stream genes
 (A) ELL expression in PC3-shControl and PC3-shELL-b, -c, and -d sublines. Aliquots of whole cell lysate were immunoblotted with ELL and GAPDH antibodies. (B) Real-time RT-PCR analysis of ELL mRNA level in PC3-shControl and PC3-shELL-b cells. Mean and standard deviation based on three independent qPCR reactions are shown. The asterisk indicates statistically significant differences, $p < 0.05$. (C and D) Real-time RT-PCR analysis of VEGF and Glut-1 mRNA expression. PC3-shControl and PC3-shELL-b cells were untreated or exposed to hypoxia (1% O_2) for 6 h. Total RNA was isolated and analyzed by qPCR specific for the VEGF and Glut-1 mRNA. All experiments were performed three times independently using triplicate each time, and data represent mean $\pm$ SEM. of all the experiments. (E) Analysis of endogenous HIF-1 α protein level. PC3-shControl and shELL-b cells were untreated or exposed to hypoxia (1% O_2) for 6 h. Aliquots of whole cell lysate were analyzed by Western blotting using antibodies against HIF-1 α and β -actin separately. β -actin served as loading controls. * $p < 0.05$; NS represents non-specific.

Fig. 4. ELL overexpression causes a reduction in endogenous HIF-1 α protein level and its downstream gene expression

(A) Western blot analysis of ELL expression in PC3-vector and PC3-ELL cells. Aliquots of whole cell lysate were immunoblotted with ELL and GAPDH antibodies. (B) Real-time RT-PCR analysis of ELL mRNA level in PC3-vector and PC3-ELL cells. Mean and standard deviation based on three independent qPCR reactions were shown. (C and D) Real-time RT-PCR analysis of VEGF and Glut-1 mRNA expression in PC3-vector and PC3-ELL cells. PC3-vector and PC3-ELL cells were untreated or exposed to hypoxia (1% O₂) for 6 h. Total RNA was isolated and analyzed by qPCR specific for the VEGF and Glut-1 mRNA separately. All experiments were repeated three times using triplicate each time, and data represent mean $\pm$ SEM. of all the experiments. (E) Western blot analysis of HIF-1 α protein level. PC3-vector and PC3-ELL cells were untreated or exposed to hypoxia (1% O₂) for 6 h. Aliquots of whole cell lysate were investigated by Western blotting using antibodies against HIF-1 α and β -actin. β -actin served as loading controls. * $p < 0.05$; NS represents non-specific.

Fig. 5. Alleviation of ELL inhibition of cell growth by hypoxia

(A) Western blot analysis of ELL protein level in PC3-vector and PC3-ELL cells in normoxia or hypoxia for 96 h. Antibodies against ELL and β -actin were used in Western blot analysis of whole cell lysates. β -actin served as loading controls. (B) Lentivirus-infected PC3-vector and PC3-ELL cells were seeded at density of 1×10^4 cells/well in 6-well plates and the cells were then placed under normoxic or hypoxic conditions. Cell numbers were determined 96 hours after the exposure to hypoxia. The experiment was repeated 3 times with triplicate at each time. (C) Lentivirus-infected PC3-vector and PC3-ELL cells were seeded at density of 1×10^3 cells/dish in 10cm dishes and the cells were then placed under normoxic or hypoxic conditions for additional 7 days. Representative images from two separate experiments carried out in triplicate are shown. The images in the squares were also shown at enlarged format. (D) Quantification of colony numbers. Colonies bigger than 1mm in diameter were counted. Each data point represents mean $\pm$ SEM of two independent experiments with triplicate in each.