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# Ubiquitination is absolutely required for the degradation of hypoxia-inducible factor - 1 alpha protein in hypoxic conditions

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ABSTRACT

The hypoxia-inducible factor (HIF) is recognized as the master regulator of hypoxia response. HIF-α subunits expression are tightly regulated. In this study, our data show that ts20 cells still expressed detectable E1 protein even at 39.5° C for 12 h, and complete depletion of E1 protein expression at 39.5° C by siRNA enhanced HIF-1α and P53 protein expression. Further inhibition of E1 at 39.5 °C by siRNA, or E1 inhibitor Ube1-41 completely blocked HIF-1α degradation. Moreover, immunoprecipitations of co-transfection of HA-ubiquitin and FLAG—HIF—1α plasmids directly confirmed the involvement of ubiquitin in the hypoxic degradation of HIF-1α. Additionally, hypoxic HIF-1 α degradation is independent of HAF, RACK1, sumoylation or nuclear/cytoplasmic localization. Taken together, our data suggest that constitutive HIF-1α protein degradation in hypoxia is absolutely ubiquitination-dependent, and unidentified E3 ligase may exist for this degradation pathway.



## 1. Introduction

Low oxygen levels (hypoxia) is a common physiological and pathological process, which is widely implicated in inflammation, ischemic lesions and cancer [1]. The cellular adaption to hypoxia includes the induction of angiogenesis, a switch from aerobic metabolism to anaerobic glycolysis, and the expression of a variety of stress proteins regulating cell death and/or survival. The hypoxia-inducible factor (HIF) is recognized as the master regulator of hypoxia response, activating the transcription of a large number of genes critical for the adaptation to hypoxia [1,2]. HIF also contributes to tumor growth and its increased expression has been correlated with poor patient prognosis [2]. Recent advances in cancer biology highlighted the HIF pathway as a crucial survival pathway for which novel strategies of cancer therapy could be developed [3].

The HIF transcription factor is a heterodimer: the oxygen-regulated HIF-α subunit and the constitutively expressed HIF-1β subunit (also known as the aryl hydrocarbon receptor nuclear translocator, ARNT) [4]. The heterodimers translocate into the nucleus, where they interact with specific DNA sequences called HIF-responsive elements (HREs). By binding to the HRE, HIF may either activate or repress gene expression. At least three different genes have been identified that encode a subunit of HIF, namely HIF1α, HIF2α and HIF3α [5]. Under normoxic conditions, HIF-α subunits are tightly regulated by a set of enzymes called HIF prolyl hydroxylase (PHDs). PHDs hydroxylate HIF-α subunits at specific prolyl residues (Pro402/Pro564 in HIF-1α and Pro405/Pro531 in HIF-2α). The hydroxylated HIF-α subunits are recognized by the von-Hippel Lindau (VHL) tumor suppressor E3 ligase, targeted for degradation by proteasome pathway. During hypoxia, HIF-1α accumulates, dimerizes with HIF-1β, and forms the transcriptionally active HIF-1 complex. Similar increases in HIF-1 complexes are found in normoxic VHL-deficient cells because of their inability to degrade HIF-α proteins [6]. Although primarily regulated by changes in cellular oxygen tension, it has now become clear that HIF-α may be also activated by a wide range of growth-promoting stimuli and oncogenic pathways such as insulin, insulin-like growth factor-1, epidermal growth factor (EGF) and mutant Ras, Src and/or PI-3K pathways [7–10].

Compared to the VHL pathway, the regulation of these oxygen-

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independent pathways is less affected by oxygen availability. VHL- and oxygen-independent degradation of HIF-1α has been observed on overexpression of GSK3 or FOXO4; and following treatment with histone deactylase (HDAC) inhibitors or heat shock protein 90 (Hsp90) inhibitors [11–15]. Also, hypoxia-Associated Factor (HAF) is an important new mediator of VHL-independent HIF-1α degradation [16]. Liu et al. indicated that RACK1 competes with HSP90 for binding to the PAS-A domain of HIF-1α, promoting the $O_2$/PHD/VHL-independent and proteasome-dependent degradation of HIF-1α [17]. Recently, constitutive ubiquitin-independent degradation of HIF-1α and HIF-2α via the proteasomal pathway has also been proposed [18]. In this study, we present data showing that constitutive hypoxic HIF-1a degradation is absolutely ubiquitin-dependent, and HAF and RACK1 are not involved in this degradation pathway.

## 2. Materials and methods

### 2.1. Cell culture

Cell lines Hep3B, HepG2, HeLa, T-24 were from ATCC.

ts20 cells were originally from Dr. H. L. Ozer (Rutgers). Cells, with the exception of ts20, were cultured at 37 °C, 5% $CO_2$ in recommended medium supplemented with 10% fetal bovine serum. ts20 cells were cultured at 35 or 39.5 °C according to the experimental requirements. For hypoxia treatment, cell dishes were placed in an oxygen station (In VIVO2, Ruskin Tech), flushed with gas containing 1% $O_2$, 5% $CO_2$, and 94% $N_2$ and incubated for the indicated times.

### 2.2. Chemicals and reagents

Common chemicals, solvents, and general reagents were from Sigma. Cycloheximide and desferrioxamine were from Sigma; MG132 was from Bio Mol (Plymouth Meeting, PA); E1 inhibitor was from Biogenova(Potomac, Maryland).

### 2.3. Immunoblots and Immunoprecipitations

Antibodies were purchased as follows.

monoclonal anti-human HIF-1α and P53 from BD Biosciences (San Diego, CA); monoclonal anti-mouse HIF-1α from R&D Systems; monoclonal anti-FLAG M2 from Sigma; monoclonal anti-mouse E1 ubiquitin activating enzyme antibody and polyclonal anti-mouse Uba6 antibody from Abcam(Cambridge, MA); monoclonal anti-Actin from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-HA from Cell signaling(Danvers, MA).

Immunoblots and Immunoprecipitations were performed as described [14]. Cells were lysed in lysis buffer (1% Triton, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride) in the presence of a 1 × protease inhibitor mix. The primary antibody incubation was performed for 4 h at 4 °C followed by precipitation with protein A-Sepharose (Pierce). Precipitates were extensively washed in lysis buffer for subsequent immunoblots.

### 2.4. Plasmids and transfections

FLAG-tagged full-length HIF-1α, HIF-1α P402A, P564G were kindly provided by Dr. Qingcheng Yang (Shanghai, China). HIF-1αP2MΔ400–600, HIF-1αP2MΔ296–400, HIF-1αP2MΔ81–200, HIF-1αP2MΔ616–658 and HIF-1αP2MK391/477 were generated using the QuikChange Site-directed mutagenesis kit (Stratagene). All mutations and deletions were performed in the HIF-1αP402A/P564G. HA-tagged ubiquitin was cloned into PCDNA3 vector (Invitrogen) by PCR-based strategies. All plasmids were validated by DNA sequence analysis. Transfections were performed using Lipofectamine 2000 (Invitrogen) based on manufacturer's instructions.

### 2.5. Small interfering RNAs (siRNAs) and transfections

The siRNA specifically targeting E1 ubiquitin activating enzyme, Uba6, RACK1, Ubc9 and negative control siRNA (siControl) were purchased from Life technologies (Waltham, MA). Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) by following the manufacturer's protocol.

## 3. Results

### 3.1. HIF-1α protein is constitutively degraded in hypoxic conditions

Recent work by Kong et al. [18] showed that HIF-α proteins are constitutively degraded in hypoxic conditions in HT1080, ts20 cells. To test this phenomenon in other cell lines, we treated Hep3B, HepG2, HeLa and T24 cells in hypoxia plus desferrioxamine (Dfx) and exposed to cycloheximide. As shown in Fig. 1A, HIF-1α is steadily decayed in these cell lines with time compared to untreated cells. Similar results were obtained in Hep3B cells, which were transiently transfected with FLAG-tagged full-length HIF-1α plasmid containing the mutations of putative ODD hydroxylation sites (P402A, P564G) both in hypoxia and normoxia (Fig. 1B). These results confirm the observation that HIF-1α protein is constitutively degraded in hypoxic condition in a VHL independent manner.

### 3.2. Requirement of ubiquitination for HIF-1α degradation in hypoxia

Previous work suggested that the degradation of HIF-α proteins during hypoxia appeared to be independent of ubiquitination [18]. This conclusion was primarily based on the use of a cell line (ts20) that expresses a temperature sensitive E1-enzyme. However, this conclusion needs reevaluation as recent evidence suggested that these cells, even at the non-permissive temperature, still have significant E1 activity [19]. When E1 was completely suppressed by siRNA or E1-inhibitors, we found that HIF-1α degradation under hypoxia was markedly reduced (Fig. 2A-C). In particular, Fig. 2A shows that even at 39.5° C for 12 h, ts20 cells still expressed detectable E1 protein, while complete depletion of E1 protein expression at 39.5° C by siRNA increased HIF-1α (upper panel) and P53 protein expression, this suggests that even at 39.5° C HIF-1α and P53 are still degraded by ubiquitination-mediated proteasome system. Further, proteasome inhibitor-MG132 or E1 inhibitor (Ube1-41) [20] reversed the degradation of HIF-1α following treatment with cycloheximide at 39.5° C (non-permissive temperature) and hypoxia plus desferrioxamine in ts20 cells (Fig. 2B and C). Together, these data suggest that HIF-1α degradation in hypoxia is absolutely dependent on ubiquitination.

Direct confirmation that HIF-1α was ubiquitinated in hypoxic cells was obtained by immunoprecipitation assays. FLAG-tagged plasmids containing cDNAs encoding either a wild type HIF-1α, HIF-1α with mutations of both prolyl-residues (HIF-1αP2M) or a complete deletion of the ODD (HIF-1αΔODD) were co-transfected with HA-ubiquitin (H-Ub) into Hep3B cells. Then the cells were exposed to MG132 (5uM) for 6 h following 48 h transfection and immunoprecipitated (IP) with anti-FLAG antibodies. As shown in Fig. 3, immunoblotting with anti-HA antibodies showed that all HIF-1α proteins were ubiquitinated, while proteasome inhibitor MG132 treatment enhanced ubiquitination. However, ubiquitination does not depend on prolyl-hydroxylation nor requires ODD-

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