

HIF-1 α induces cell cycle arrest by functionally counteracting Myc

Minori Koshiji¹, Yukio Kageyama¹,
Erin A Pete¹, Izumi Horikawa²,
J Carl Barrett² and L Eric Huang^{1,*}

¹Laboratory of Human Carcinogenesis, NCI, National Institutes of Health, Bethesda, MD, USA and ²Laboratory of Biosystems and Cancer, NCI, National Institutes of Health, Bethesda, MD, USA

Hypoxia induces angiogenesis and glycolysis for cell growth and survival, and also leads to growth arrest and apoptosis. HIF-1 α , a basic helix–loop–helix PAS transcription factor, acts as a master regulator of oxygen homeostasis by upregulating various genes under low oxygen tension. Although genetic studies have indicated the requirement of HIF-1 α for hypoxia-induced growth arrest and activation of *p21^{cip1}*, a key cyclin-dependent kinase inhibitor controlling cell cycle checkpoint, the mechanism underlying *p21^{cip1}* activation has been elusive. Here we demonstrate that HIF-1 α , even in the absence of hypoxic signal, induces cell cycle arrest by functionally counteracting Myc, thereby derepressing *p21^{cip1}*. The HIF-1 α antagonism is mediated by displacing Myc binding from *p21^{cip1}* promoter. Neither HIF-1 α transcriptional activity nor its DNA binding is essential for cell cycle arrest, indicating a divergent role for HIF-1 α . In keeping with its antagonism of Myc, HIF-1 α also downregulates Myc-activated genes such as *hTERT* and *BRCA1*. Hence, we propose that Myc is an integral part of a novel HIF-1 α pathway, which regulates a distinct group of Myc target genes in response to hypoxia.

The EMBO Journal (2004) 23, 1949–1956. doi:10.1038/sj.emboj.7600196; Published online 8 April 2004

Subject Categories: chromatin & transcription; cell cycle

Keywords: cell cycle; HIF-1 α ; hypoxia; Myc; *p21^{cip1}*

Introduction

Under low oxygen tension (hypoxia), mammals elicit an array of cellular processes, in an attempt to maintain ATP production by improving oxygen delivery and enhancing glycolysis. It appears that HIF-1 α , a basic helix–loop–helix (bHLH) transcription factor of the PAS family (Wang *et al*, 1995), is responsible for upregulating most, if not all, hypoxia-inducible genes (Semenza, 1999; Wenger, 2002). HIF-1 α is constitutively transcribed and translated (Huang and Bunn, 2003), but is rapidly degraded in normoxia by the ubiquitin-proteasomal pathway (Salceda and Caro, 1997; Huang *et al*, 1998; Kallio *et al*, 1999). Polyubiquitination of HIF-1 α is

mediated by the von Hippel-Lindau tumor suppressor (Maxwell *et al*, 1999; Cockman *et al*, 2000; Kamura *et al*, 2000; Ohh *et al*, 2000; Tanimoto *et al*, 2000), a part of an E3 ubiquitin ligase, that binds to the oxygen-dependent degradation domain (ODD) (Huang *et al*, 1998; Huang and Bunn, 2003) of HIF-1 α . Thus, removal of the ODD renders HIF-1 α stable in normoxia, enabling transcriptional activation of its target genes and induction of hypervascularity *in vivo* (Huang *et al*, 1998; Elson *et al*, 2001). The VHL binding is controlled by hydroxylation of two proline residues in HIF-1 α : Pro-402 (Masson *et al*, 2001) and Pro-564 (Ivan *et al*, 2001; Jaakkola *et al*, 2001). These prolines are modified in normoxia by the HIF prolyl-4-hydroxylases that require oxygen, iron, and 2-oxoglutarate as cofactors (Bruick and McKnight, 2001; Epstein *et al*, 2001). Therefore, depletion of oxygen or iron chelation inhibits the enzymatic activity, resulting in stabilization of HIF-1 α , followed by heterodimerization with ARNT and recruitment of the transcription coactivator p300/CBP (Arany *et al*, 1996; Kallio *et al*, 1998; Kung *et al*, 2000) and SRC-1 (Carrero *et al*, 2000). Consequently, HIF-1 heterodimer binds to the hypoxia-responsive element (HRE) in the promoter, upregulating its target genes including vascular endothelial growth factor (VEGF), glucose transporters, and glycolytic enzymes for cell growth and survival (Semenza, 1999).

On the other hand, hypoxia/HIF-1 α has also been suggested to inhibit cell growth (Carmeliet *et al*, 1998; Gardner *et al*, 2001; Green *et al*, 2001; Goda *et al*, 2003) and to promote apoptosis (Carmeliet *et al*, 1998; Bruick, 2000). Although the results from *Hif1 α* null cells indicate that HIF-1 α is required for hypoxia-induced upregulation of *p21^{cip1}*, a key cyclin-dependent kinase inhibitor that controls G₁ checkpoint, the role of HIF-1 α in cell cycle has been controversial (Carmeliet *et al*, 1998; Gardner *et al*, 2001; Goda *et al*, 2003). Furthermore, it remains puzzling how HIF-1 α activates *p21^{cip1}* because no HIF-1 α -bound HRE has been identified in the promoter. To gain insights into the mechanism underlying hypoxia-induced cell growth arrest, we took advantage of an ODD-deficient HIF-1 α (Δ ODD) that is stable and functional in normoxia (Huang *et al*, 1998), and demonstrated that HIF-1 α , even in the absence of hypoxic signal, induces cell cycle arrest by antagonizing Myc activity, resulting in derepression of *p21^{cip1}* (Herold *et al*, 2002; Seoane *et al*, 2002). Remarkably, neither HIF-1 α transcriptional activity nor its DNA binding is required; instead, HIF-1 α does so via its N-terminal region that interacts with Myc. Thus, our findings signalize a novel HIF-1 α –Myc pathway regulating a new array of hypoxia-responsive genes that are distinct from those activated through HIF-1 α transcriptional activation.

Results

HIF-1 α induces cell cycle arrest by activating *p21^{cip1}*

To demonstrate that it is HIF-1 α , rather than other hypoxia-associated effects, that induces cell cycle arrest, we tested the

*Corresponding author. NIH, National Cancer Institute, Bldg 37, Room 3044B, 37 Convent Dr. MSC4255, Bethesda, MD 20892, USA.
Tel.: +1 301 402 8785; Fax: +1 301 480 1264;
E-mail: huange@mail.nih.gov

Received: 19 November 2003; accepted: 9 March 2004; published online: 8 April 2004

effects of HIF-1 α in normoxia by taking advantage of an ODD-deficient HIF-1 α (Δ ODD). Infection of HCT116 cells with three multiplicities of infection (moi) of adenoviruses expressing the HIF-1 α variant (Ad- Δ ODD) gave rise to pronounced changes in cell cycle (Figure 1A, top panels); there was an \sim 7-fold decrease of cells in the S-phase and a concomitant increase in the G₁-phase. In addition, the G₂-phase was

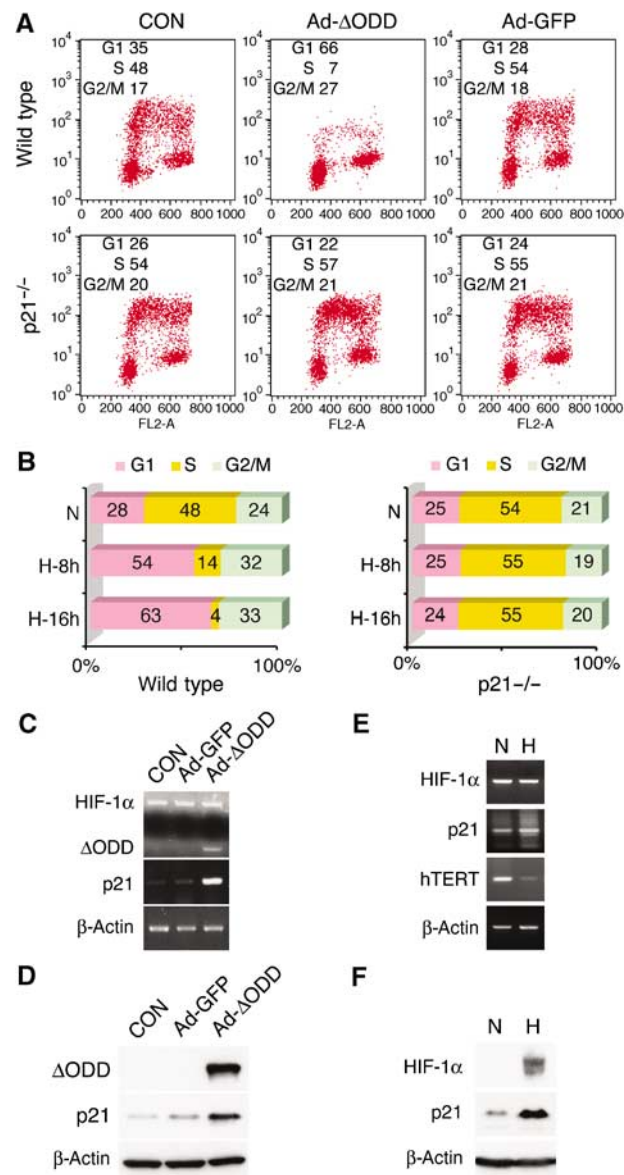


Figure 1 HIF-1 α expression is sufficient to induce cell cycle arrest by activating *p21^{cip1}*. (A) HCT116 wild-type and *p21*^{-/-} variant were analyzed for BrdU incorporation after adenoviral infection, as indicated. The original data are presented, together with the percentage of each phase in the inset. CON, no infection. (B) HCT116 wild-type and *p21*^{-/-} cells were subjected to hypoxia (H) for 8 or 16 h prior to cell cycle analysis. The percentage of each phase is shown in bar graphs. N, normoxia. (C, D) Adenovirus-infected cells were assayed for mRNA (C) and protein (D) levels of specified genes by RT-PCR and Western blot, respectively. Both endogenous HIF-1 α and Δ ODD mRNA levels were determined in the same PCR reactions, and β -actin levels served as loading controls. (E, F) Hypoxic induction of *p21^{cip1}* expression was examined at mRNA (E) and protein levels (F). hTERT mRNA levels were also included as a control (see below).

moderately increased. By contrast, adenoviruses expressing green fluorescent protein (Ad-GFP) produced no obvious effects. The GFP expression was confirmed with fluorescence microscopy and Western analysis (data not shown). As expected, a stable full-length HIF-1 α , resulting from double mutations of Pro-402 and Pro-564, also induced G₁ arrest (Supplementary Figure). In agreement with the effect of adenoviral infections, treatment with 1% oxygen resulted in similar changes in cell cycle (Figure 1B, left panel). Therefore, these results provide evidence that a stable HIF-1 α is sufficient for cell cycle arrest in normoxia.

To test whether HIF-1 α -induced cell cycle arrest requires *p21^{cip1}*, we determined the effect of HIF-1 α in *p21^{cip1}* null HCT116 cells (Bunz *et al*, 1998). As a result of the genetic alteration, these cells exhibited an increased S-phase along with a decreased G₁-phase (Figure 1A, bottom panels). However, neither Ad- Δ ODD infection nor hypoxic treatment (Figure 1B, right panel) altered the cell cycle profile, confirming the requirement of *p21^{cip1}* for HIF-1 α -mediated cell cycle arrest (Carmeliet *et al*, 1998; Goda *et al*, 2003). In keeping with this notion, Ad- Δ ODD infection of wild-type HCT116 cells enhanced *p21^{cip1}* expression at both mRNA and protein levels (Figure 1C and D). Similar results were obtained when cells were treated with hypoxia (Figure 1E and F).

***p21^{cip1}* upregulation is independent of HIF-1 α transcriptional activity and its DNA binding**

To elucidate the mechanism underlying HIF-1 α -mediated *p21^{cip1}* expression, we determined the involvement of HIF-1 α C-terminal activation domain, which is responsible for the transcriptional activity of Δ ODD by binding to p300/CBP. Unlike wild-type HIF-1 α , Δ ODD constitutively activated HIF-1-mediated reporter gene in transient transfection (Figure 2A). We then inactivated Δ ODD transcriptional activity via simultaneous mutations (L795V, C800S, L818S, and L822V) that disrupt p300/CBP binding (Gu *et al*, 2001; Dames *et al*, 2002; Freedman *et al*, 2002). As expected, this Δ ODD LCLL mutant was unable to activate the HIF-1 reporter (Figure 2A). In contrast, substitution of Δ ODD Cys-800 with valine (C800V) further stimulated transcriptional activity in reference to Δ ODD (Gu *et al*, 2001). Likewise, adenoviral infection with the Δ ODD LCLL mutant failed to induce the expression of *VEGF* (Figure 2B) and *Glut-1* (data not shown), another HIF-1 target gene. Therefore, these results confirm that the Δ ODD LCLL mutant is transcriptionally inactive.

Next, we tested the effects of Δ ODD LCLL on cell cycle progression. Surprisingly, cells infected with Ad-LCLL also manifested slowed growth, similar to those infected with Ad- Δ ODD, or treated with desferrioxamine, a hypoxia mimetic (data not shown). Whereas Ad-LCLL infection failed to increase *VEGF* secretion (Figure 2D), cell cycle arrest was observed in the infected HCT116 cells (Figure 2F) and normal human fibroblasts (data not shown). Furthermore, both *p21^{cip1}* mRNA and protein levels were elevated upon Ad-LCLL infection (Figure 2C and E), arguing against the requirement of HIF-1 α transcriptional activity for *p21^{cip1}* activation.

This surprising finding suggested that HIF-1 α activates *p21^{cip1}* in a way that is distinct from the activation of classic hypoxia-inducible genes such as *VEGF* and glycolytic enzyme genes. To test this hypothesis, we investigated whether HIF-1 α DNA binding is required for growth arrest. To this end, the basic region required for HIF-1 α DNA binding was mutated

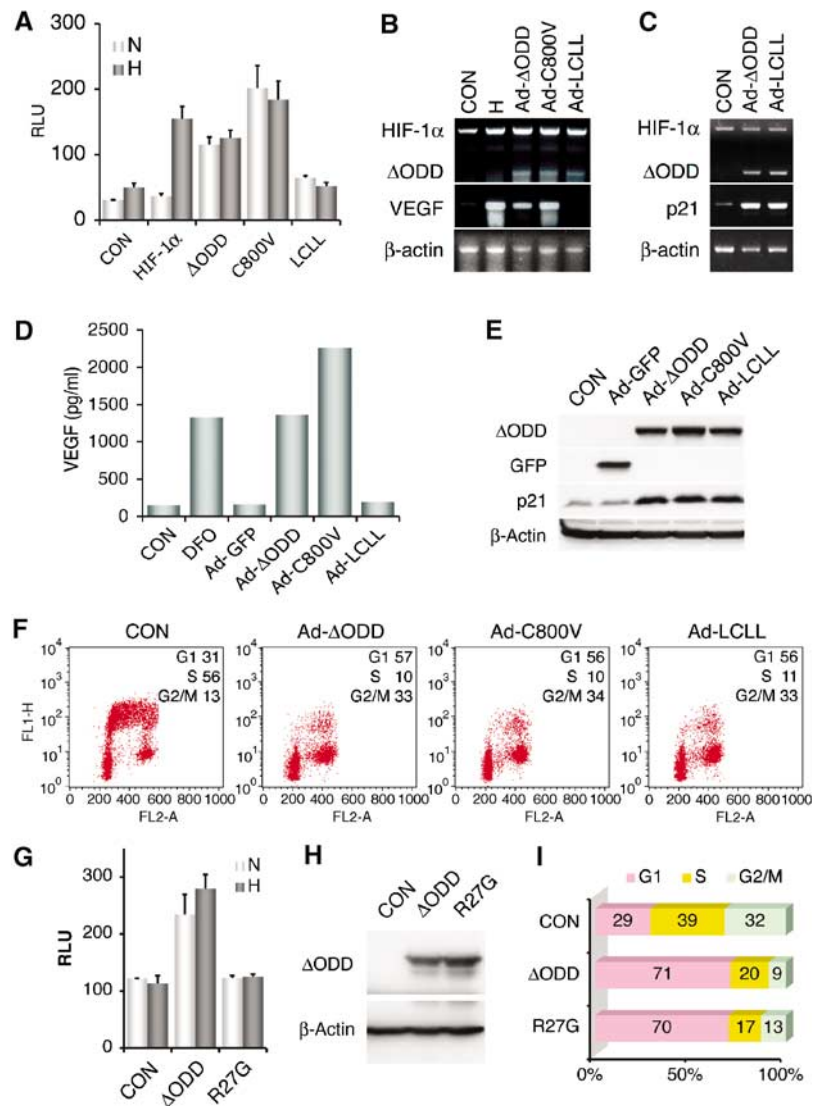


Figure 2 HIF-1 α induction of *p21^{cip1}* and G₁ arrest is independent of its DNA-binding and transcriptional activity. (A) Transcriptional activity of HIF-1 α , Δ ODD, and Δ ODD plus C800V or LCLL mutations was determined in Cos-7 cells in a HIF-1-mediated reporter system (Huang *et al*, 1996). Relative luciferase units (RLU) are presented with means plus standard errors from three independent experiments. (B, D) HCT116 cells were assayed for *VEGF* expression by RT-PCR (B) or *VEGF* secretion by ELISA (D) after adenoviral infection as indicated. DFO, desferrioxamine. (C, E) Δ ODD LCLL mutant was analyzed for its effects on *p21^{cip1}* expression at mRNA (C) and protein (E) levels. (F) Effect of the Δ ODD LCLL mutant on cell cycle profile was determined as above, with the percentage of each phase indicated in the inset. (G–I) Δ ODD and its R27G mutant were assayed in Cos-7 cells for HIF-1-mediated reporter activity (G) and Western analysis of protein levels (H), and in HCT116 cells for induction of cell cycle arrest (I). CON, transfected with pcDNA3.

by replacing Arg-27 with glycine. Despite its equivalent level of expression to that of Δ ODD, the Δ ODD R27G mutant failed to activate the HIF-1 reporter (Figure 2G and H), indicating the loss of DNA-binding ability. However, the mutant was still able to induce G₁ arrest (Figure 2I). Thus, we conclude that neither HIF-1 α transcriptional activity nor its DNA binding is required for *p21^{cip1}* activation.

HIF-1 α functionally antagonizes Myc

As *p21^{cip1}* is transcriptionally repressed by Myc (Herold *et al*, 2002; Seoane *et al*, 2002; Gartel and Shchors, 2003), we hypothesized that HIF-1 α -mediated *p21^{cip1}* activation is a result of HIF-1 α counteraction of Myc repression. To this end, a Myc-activated *hTERT* promoter (Horikawa *et al*, 2002) was tested for the HIF-1 α antagonism. Whereas transient Myc expression resulted in a moderate increase

(Eisenman, 2001; Levens, 2002) in *hTERT* reporter activity, co-expression of Δ ODD significantly inhibited the induction (Figure 3A). Likewise, Ad- Δ ODD infection also markedly downregulated the expression of Myc-activated genes including *hTERT* and *BRCA1* (Figure 3B). Real-time PCR confirmed a seven-fold reduction in *hTERT* expression (data not shown). In addition, infection with the transcriptionally inactive mutant Ad-LCLL also inhibited the Myc-activated genes (Supplementary Figure). It is noteworthy that neither Ad- Δ ODD nor hypoxia (data not shown) altered *c-myc* mRNA and protein levels, supporting the notion that HIF-1 α functionally antagonizes Myc.

To confirm the requirement of Myc for HIF-1 α effects on *p21^{cip1}*, *hTERT*, and *BRCA1*, we employed *c-myc* small interfering RNA (siRNA) that effectively abrogated *c-myc* expression (Figure 3C). Consequently, *hTERT* and *BRCA1* expression

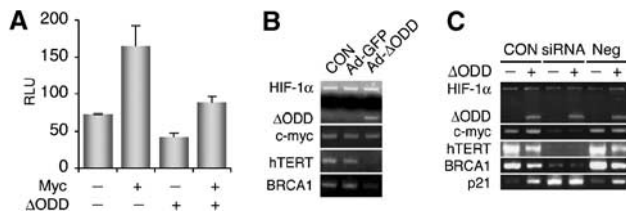


Figure 3 HIF-1 α counteracts Myc activity. (A) Effect of Δ ODD on Myc transcriptional activity was examined with an *hTERT* luciferase reporter in HCT116 cells. The graph represents three independent experiments in duplicate. (B) Myc target genes, as indicated, were analyzed by RT-PCR after adenoviral infection. *c-myc* mRNA levels were also determined. (C) After transfection with *c-myc* siRNA (siRNA), HCT116 cells were infected with Ad- Δ ODD and analyzed with RT-PCR for Myc target gene expression, as indicated. CON, no siRNA. Neg, negative control siRNA.

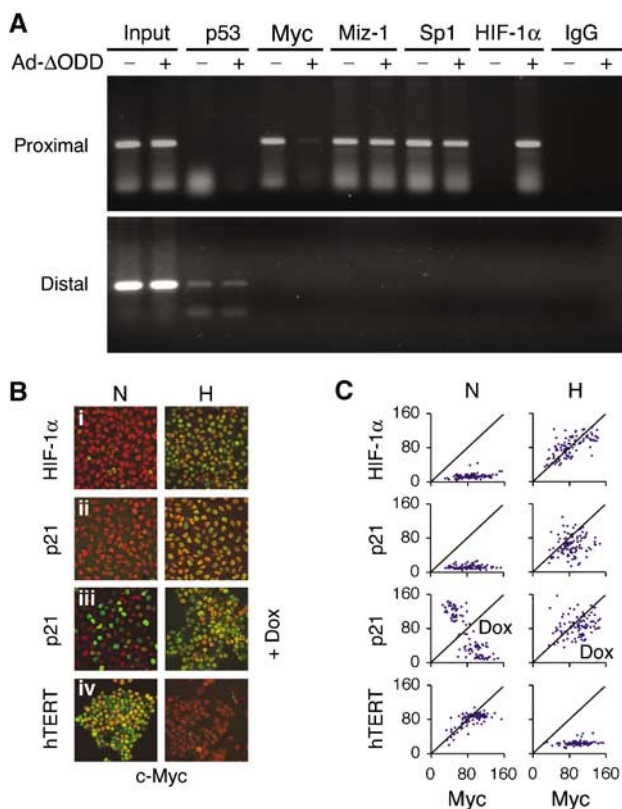


Figure 4 HIF-1 α displaces Myc binding from *p21^{cip1}* promoter and overrides Myc target gene expression. (A) After Ad- Δ ODD infection of HCT116 cells, chromatin immunoprecipitation assays were performed with indicated antibodies at the top. Both the proximal and distal regions of *p21^{cip1}* promoter were analyzed. Input, genomic DNA prior to immunoprecipitations. (B) Normoxic (N) and hypoxic (H) HCT116 cells were stained by immunofluorescence with anti-Myc antibody (in red) and antibodies against HIF-1 α , *p21^{cip1}*, or hTERT (in green). Only merged images are presented. The cells in panel iii were treated with doxorubicin (Dox). (C) Myc expression levels from 100 individual cells in (B) were plotted in dots along the x-axis against those of HIF-1 α , *p21*, or hTERT in the y-axis. Cells expressing both proteins are depicted in dots along the diagonal lines.

was inhibited, whereas *p21^{cip1}* expression was upregulated, confirming an essential role of Myc in regulating these genes. More importantly, *c-myc* siRNA abrogated the HIF-1 α antagonism, as evidenced by the lack of further changes in the

expression of *p21^{cip1}*, *hTERT*, and *BRCA1*. These results confirm an indispensable role of Myc in HIF-1 α counteraction.

Hypoxia-induced HIF-1 α dictates Myc target gene expression

To gain insights into the underlying mechanism of the HIF-1 α antagonism, we determined HIF-1 α and Myc binding to the *p21^{cip1}* promoter. The results from chromatin-immunoprecipitation assays showed that in normoxia Myc, but not HIF-1 α , bound the proximal region of the promoter (Figure 4A, top panel), consistent with a previous report (Seoane *et al*, 2002). In addition, both Miz-1 and Sp1 also interacted with this region (Gartel and Shchors, 2003). Upon Ad- Δ ODD infection or hypoxia (data not shown), HIF-1 α occupied the same region of the *p21^{cip1}* promoter, whereas Myc binding was significantly weakened, suggesting that HIF-1 α has a higher affinity than Myc for the *p21^{cip1}* promoter. However, when the upstream p53-binding region of the promoter was analyzed, only p53 binding was detected (bottom panel), arguing for the specificity of Myc and HIF-1 α binding. Therefore, these results indicate that HIF-1 α activation of *p21^{cip1}* is mediated by the displacement of Myc from the *p21^{cip1}* promoter.

To substantiate the biological relevance of HIF-1 α effects on Myc target gene, we evaluated the hypoxic effects on Myc target gene expression. Immunofluorescence staining showed that vast majority of the cells co-expressed Myc with *p21^{cip1}* and HIF-1 α under hypoxia (Figure 4B and C). Although treatment with DNA-damaging agents such as doxorubicin rendered cells expressing Myc or *p21^{cip1}*, but not both (Seoane *et al*, 2002), additional hypoxic treatment converted the cells to co-expression of the two, indicating an authoritative role of hypoxia in Myc target gene expression. Consistently, hypoxia abolished normoxic co-expression of hTERT and Myc. Similar results were obtained with Ad- Δ ODD infection (data not shown). The immunofluorescence staining of Myc and *p21^{cip1}* was validated by knocking down their expression with siRNAs; both Myc and *p21^{cip1}* siRNAs ablated the staining of the corresponding genes and altered the expression patterns (Supplementary Figure). Thus, these results support that hypoxia-induced HIF-1 α dictates Myc target gene expression.

HIF-1 α interacts with Myc in vivo

To ascertain the biochemical aspect of the HIF-1 α antagonism, we asked whether HIF-1 α displaces Myc through protein-protein interactions. To this end, both Δ ODD and Myc were transiently co-expressed in normoxic cells for co-immunoprecipitation assays. Figure 5A showed that Myc was weakly detected in an anti-hemagglutinin (HA) immunoprecipitation targeting Δ ODD. Conversely, Δ ODD was present in the anti-Myc immunocomplexes (Figure 5B), suggesting *in vivo* complex formation between HIF-1 α and Myc. The complex specificity was confirmed further by antibody omissions and isotype-matched normal immunoglobulins (Figure 5C). To provide the physiological relevance of this finding, we examined endogenous HIF-1 α and Myc interaction in hypoxic cells. Again, Myc was weakly detected in an anti-HIF-1 α co-immunoprecipitation (Figure 5D). Therefore, we conclude that hypoxia-induced HIF-1 α weakly interacts with Myc *in vivo*.

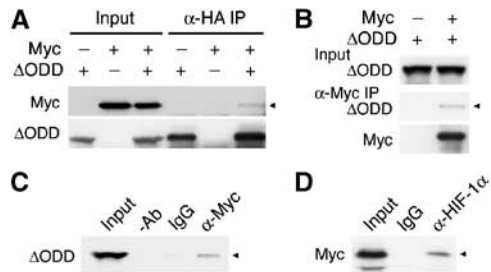


Figure 5 HIF-1 α forms a weak complex with Myc *in vivo*. (A, B) Δ ODD and Myc, as indicated, were co-expressed in 293 cells. Co-immunoprecipitations with antibodies against HA (α -HA IP) or Myc (α -Myc IP) were performed, followed by Western analyses detecting co-immunoprecipitates (marked with arrow heads), as well as immunoprecipitates. *Input*, whole-cell extracts subjected to direct Western analysis. (C) Omission of antibody ($-Ab$) and use of normal immunoglobulin (*IgG*) were included as controls for an anti-Myc co-immunoprecipitation. (D) The interaction of endogenous HIF-1 α and Myc in HCT116 cells was shown in an anti-HIF-1 α co-immunoprecipitation, followed by anti-Myc Western blotting.

The N-terminal HIF-1 α is sufficient for cell cycle arrest

In search of the minimum requirement for HIF-1 α to form a complex with Myc, we tested a set of HIF-1 α mutants with C-terminal deletions (Figure 6A). Whereas HIF-1 α mutants (amino acids 1–400 and 1–329) remained interactive with Myc (Figure 6B), further removal of the PAS-B domain from the C terminus abolished the interaction (Figure 6C), suggesting that Myc interaction requires the N-terminal HIF-1 α containing bHLH and PAS domains (Semenza, 1999). To validate the functional relevance of the N-terminal HIF-1 α interaction with Myc, we tested the effects of the N-terminal HIF-1 α fragments on cell cycle checkpoint. In agreement with the above results, HIF-1 α 1–400 not only stimulated *p21^{cip1}* reporter expression (Figure 6D) but also induced G₁ arrest (Figure 6E). In contrast, HIF-1 α 1–167 showed no effect. To confirm the dispensable role of HIF-1 α DNA binding in cell cycle arrest, we again mutated the basic region with R27G mutation. As expected, a similar G₁ arrest was detected with this mutant (Supplementary Figure). Therefore, we conclude that the N-terminal HIF-1 α is sufficient for functionally counteracting Myc, resulting in derepression of *p21^{cip1}*.

Discussion

In this study, not only did we provide evidence that HIF-1 α , even in the absence of hypoxic signal, is sufficient to induce cell cycle arrest by upregulating *p21^{cip1}* expression, but more significantly we unravelled the mechanism by which HIF-1 α activates *p21^{cip1}*. In particular, we demonstrated that HIF-1 α counteracts Myc activity by displacing Myc binding from the *p21^{cip1}* promoter. Remarkably, it is the N-terminal HIF-1 α comprising bHLH and PAS domains that functionally antagonizes Myc, a novel mechanism distinct from HIF-1 α C-terminal transactivation of the classic hypoxia-inducible genes (e.g. *VEGF*, *Glut-1*). Therefore, HIF-1 α employs at least two mechanisms for regulating gene expression: in addition to its C-terminal transactivation of genes possessing HREs, HIF-1 α , via its N-terminal counteraction of Myc activity, independently overrides the expression of Myc target genes lacking the canonical HRE. Interestingly, HIF-1 α splice variants devoid of the C-terminal transactivation domains

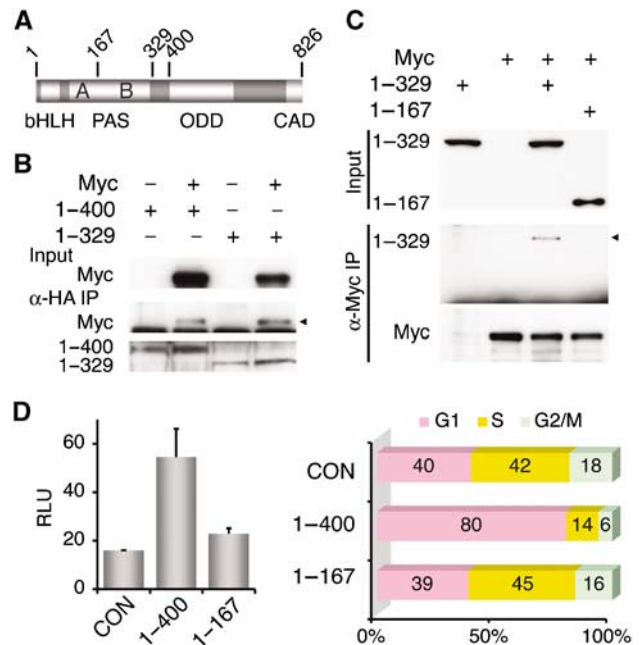


Figure 6 The N-terminal HIF-1 α interacts with Myc and induces cell cycle arrest. (A) A schematic representation of HIF-1 α . Structural domains are indicated at the bottom (Huang and Bunn, 2003), and the relevant codon numbers at the top. (B, C) HIF-1 α mutants (amino acids 1–400, 1–329, and 1–167) were transfected into 293 cells, and analyzed by co-immunoprecipitation assays to determine the minimum requirement for Myc binding. (D, E) The N-terminal HIF-1 α (1–400 and 1–167) was examined for its ability to activate *p21^{cip1}* promoter in Cos-7 cells (D), and to alter cell cycle in HCT116 cells (E). CON, transfected with pcDNA3.

have been reported (Chun *et al*, 2001, 2002), although their role in counteracting Myc activity remains to be determined. The HIF-1 α antagonism of Myc is not limited to *p21^{cip1}* activation, because HIF-1 α also downregulates the expression of *hTERT* and *BRCA1*, known targets of Myc (Eisenman, 2001; Levens, 2002). In addition, HIF-1 α has also been implicated in hypoxic induction of cyclin-dependent kinase inhibitor *p27^{Kip1}* (Goda *et al*, 2003), another Myc target (Obaya *et al*, 2002) that might be regulated by the same mechanism. It should be stressed that none of these genes possesses a canonical HRE in the promoter. Thus, HIF-1 α counteraction of Myc may serve as a general mechanism underlying up- or downregulation of Myc target genes in response to hypoxia (Figure 7).

Our finding that neither HIF-1 α DNA binding nor its transcriptional activity is required for *p21^{cip1}* activation and cell cycle arrest does not support the possibility that HIF-1 α directly binds to the promoter of *p21^{cip1}*, which contains ACGTG sequence (Salnikow *et al*, 2000), *hTERT*, and *BRCA1*, thereby transcriptionally activating or inactivating gene expression. Furthermore, this finding also argues against the hypothesis that the HIF-1 α antagonism of Myc results directly from HIF-1 α transcriptional activation of target genes that in turn repress Myc activity, because it is highly improbable that an HIF-1 α mutant lacking DNA-binding and transcriptional activity is able to transactivate Myc repressor genes. Although direct HIF-1 α DNA binding to the promoter cannot be completely ruled out, the overall evidence favors the HIF-1 α –Myc interaction as a primary mode

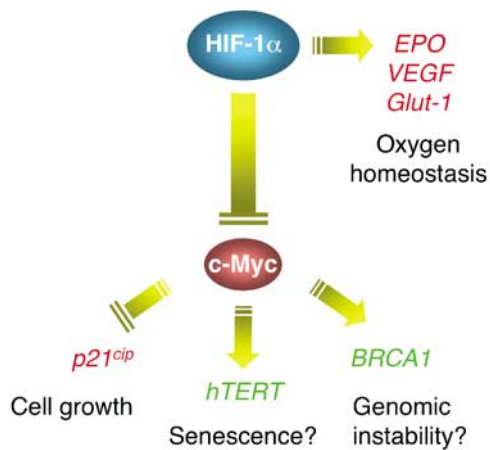


Figure 7 An HIF-1 α -Myc pathway regulating hypoxia-responsive genes lacking the canonical HRE. Apart from activating the classic hypoxia-inducible genes such as erythropoietin (*EPO*), *VEGF*, and *Glut-1*, HIF-1 α functionally counteracts Myc, thereby overriding Myc target gene expression. The upregulated genes are in red, and the downregulated in green.

of action in altering the Myc target gene expression under hypoxia.

We showed that the HIF-1 α antagonism is mediated by Myc displacement, resulting in derepression of *p21^{cip1}* promoter. In fact, not only hypoxia, but a stable HIF-1 α in normoxia as well, is able to displace Myc binding, suggesting an authoritative role of HIF-1 α in Myc target gene expression. This notion is consistent with and supported by another line of evidence that hypoxia overrides Myc target gene expression, as shown in Figure 4B and C. HIF-1 α displacement of Myc is also in good agreement with the observation of weak HIF-1 α -Myc interaction *in vivo*, presumably resulting from the transient protein-protein interactions during competition, or the residual Myc binding after competition. The biological significance of this weak interaction, however, is underscored by the finding that the N-terminal HIF-1 α not only binds Myc, but also activates *p21^{cip1}* promoter and thereby induces cell cycle arrest (Figure 6), arguing for the functional relevance of the interaction.

It is noteworthy that, despite its constitutive binding to the distal region of *p21^{cip1}* promoter (Figure 4A), p53 is not absolutely required for hypoxia-induced *p21^{cip1}* activation (unpublished data), in agreement with a previous report (Goda *et al*, 2003). This finding is supported by the observation that hypoxia-induced p53 is transcriptionally incompetent for activating downstream target genes (Koumenis *et al*, 2001). Therefore, it is likely that p53 has a limited, yet nonessential role in facilitating hypoxic activation of *p21^{cip1}* promoter.

Myc is known to play a pivotal role in a transcription factor network that regulates cellular proliferation, growth, differentiation, and apoptosis (Dang, 1999). It has been reported recently that Myc is essential for vasculogenesis and angiogenesis during development and tumor progression (Baudino *et al*, 2002), apparently in disagreement with the theory of HIF-1 α counteraction of Myc. Furthermore, Myc has also been shown to activate glucose transporter 1 and glycolytic genes (Osthus *et al*, 2000), transcriptional targets of HIF-1 α as well. These seemingly contradictory observations can be reconciled, however, by the interpretation that Myc is only

responsible for normoxic induction of these genes, whereas in hypoxia HIF-1 α seizes control of gene expression as a result of its counteraction of Myc. Therefore, both HIF-1 α and Myc may function coherently to enhance glycolysis in tumor tissues. A crucial experiment to test this hypothesis would be to examine the requirement of Myc for increased glycolysis in hypoxia. Another possible explanation is that Myc downregulates the expression of HIF-1 α E3 ubiquitin ligase, the von Hippel-Lindau protein (O'Connell *et al*, 2003), thereby giving rise to a more stable HIF-1 α that accounts for glycolysis. Given the complexity of tumor biology, how HIF-1 α and Myc contribute to glycolysis and angiogenesis needs in-depth investigation.

Although HIF-1 α -induced cell cycle arrest may seem paradoxical to its documented role in tumor growth, recent reports from well-controlled studies support our hypothesis that HIF-1 α inhibits cell proliferation. HIF-1 α ^{-/-} tumors unexpectedly grew faster (Carmeliet *et al*, 1998) and became more invasive than its HIF-1 α ^{+/+} counterpart, when oxygen supply was adequate (Blouw *et al*, 2003). Furthermore, HIF- α stabilization failed to promote, but rather decreased, tumor growth (Mack *et al*, 2003). Interestingly, a stable HIF-2 α (Kondo *et al*, 2002), but not HIF-1 α (Maranchie *et al*, 2002), overrides the tumor-suppressing activity of the von Hippel-Lindau protein, suggesting differential roles for HIF-1 α and HIF-2 α in tumor growth. Undoubtedly, further investigation is warranted for a better understanding of the divergent roles of HIF-1 α and HIF-2 α in tumorigenesis.

Materials and methods

Cell culture and hypoxic treatment

HCT116^{p53+/+} and HCT116^{p21-/-} (gifts from B Vogelstein) were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum. Normal human fibroblasts (MRC5, GM07532), 293, and Cos-7 cells were grown in DMEM medium supplemented with 10% bovine calf serum. Hypoxic treatment was carried out in an incubator (NAPCO) with 1% O₂, 5% CO₂, and 94% N₂, or in the presence of 200 μ M desferrioxamine as indicated.

Adenoviruses

Recombinant adenoviruses expressing ODD-deleted HIF-1 α , or its variant C800V and LCLL were created with the AdEasy adenoviral vector system (Stratagene). Adenoviral infection was carried out in 5% fetal bovine serum media for 1 h at 37°C. In general, 3 moi was used for tumor cell lines and 10 moi for nontransformed cells. Cells were harvested after 8 h infection.

Cell cycle analysis

Cells were labeled with 10 μ M bromodeoxyuridine (BrdU) for 30 min, and fixed in 70% ethanol for 1 h. Subsequently, cells were treated with 2 N hydrochloric acid plus 0.5% Triton X-100. After wash, cells were stained with anti-BrdU antibody (BD Biosciences) and propidium iodide, and analyzed by FlowJo (TreeStar). Figures are representatives of at least three independent and reproducible experiments.

Cell cycle profile was also determined from cells transfected with 1 μ g of indicated p(HA)HIF1 α mutant constructs plus 0.1 μ g pEGFP-F (Clontech). The GFP-positive population was analyzed with propidium iodide staining (Nagashima *et al*, 2001).

Transfection

Reporter assays were performed essentially as described previously with 0.25 μ g reporter and 0.1 μ g pEYFP-Nuc for normalization (Huang *et al*, 2002). hTERT promoter activity was assayed with 0.1 μ g pMyc (Horikawa *et al*, 2002) and 0.2 μ g p(HA)HIF1 α (401 Δ 603) (Huang *et al*, 1998). HIF-1 reporter assays were performed with pEpoE-luc containing a 3' enhancer of erythropoietin (Huang *et al*, 1996) co-transfected with 0.1 μ g pARNT and

p(HA)HIF1 α , p(HA)HIF1 α (401 Δ 603), or its variants. *p21^{cip1}* reporter (pWWP-luc, a gift from B Vogelstein) was assayed with 0.1 μ g p(HA)HIF1 α (1-400) and 0.1 μ g pMyc.

The interaction between Myc and HIF-1 α was determined by transfecting cells in six-well plates with 0.6 μ g Myc and 1 μ g p(HA)HIF1 α (401 Δ 603), p(HA)HIF1 α (1-400), p(HA)HIF1 α (1-329), or p(HA)HIF1 α (1-167) (Huang *et al*, 1998), followed by co-immunoprecipitations.

Western blot and co-immunoprecipitation

Western blot was conducted as described previously (Huang *et al*, 1998). Co-immunoprecipitation was carried out in NETN buffer (Gu *et al*, 2001), followed by Western blot to identify precipitates and co-precipitates. One-fifth of the whole-cell extracts were subject to direct Western blot for quantification of the input amounts. Monoclonal antibodies against HIF-1 α and p21^{cip1} were purchased from BD Biosciences, and anti-HA antibody from Roche. Polyclonal anti-Myc antibody was from Upstate.

For detection of endogenous HIF-1 α -Myc complex, HCT116 cells grown in two 10 cm plates were treated with 12.5 μ M Cbz-LLL in hypoxia for 4 h. Subsequently, cells were lysed in a lysis buffer containing 25 mM Tris, 300 mM NaCl, 1% Triton X-100, and protease inhibitor Complete (Roche). The lysates were pre-cleared by incubation with protein A agarose beads for 1 h, and divided into two: one for immunoprecipitation with polyclonal anti-HIF-1 α antibody (Santa Cruz) and the other with normal rabbit IgG. A monoclonal anti-Myc antibody (Oncogene) was used for Western blot.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instructions. One-step RT-PCR was carried out using AccessQuick RT-PCR kit (Promega) with 0.5 μ g total RNA. Primers for HIF-1 α flank the ODD so that both endogenous and ODD-deleted HIF-1 α can be detected simultaneously.

Chromatin immunoprecipitation assays

Following 16-h treatment with hypoxia or adenoviral infection, HCT116 cells were crosslinked with formaldehyde as described previously (Boyd *et al*, 1998). Chromatin immunoprecipitations were performed by using the Chromatin Immunoprecipitation Assay Kit (Upstate) following the manufacturer's instructions. Antibodies used include rabbit polyclonal anti-Myc (Upstate), mouse monoclonal anti-p53 (BD Biosciences), goat polyclonal anti-Miz-1, mouse monoclonal anti-Sp1, rabbit polyclonal anti-HIF-1 α , and normal rabbit IgG (Santa Cruz Biotechnology). The PCR primers spanning the distal region of the *p21^{cip1}* promoter (-2312 to -2131) are 5'-CAGGCTGTGGCTCTGATTGG-3' (forward) and 5'-TTCAGAGTAACAGGCTAAGG-3' (reverse), and those covering the

proximal region (-194 to +88), 5'-ACCGGCTGGCCTGCTGGAAC-3' (forward) and 5'-TCTGCCCGCTCTCTCACCT (reverse).

Enzyme-linked immunoabsorbant assay (ELISA)

VEGF levels in the cell culture medium were determined with a human VEGF ELISA kit (American Research Products). The VEGF level was quantified by comparing its optical density to the standard curve in accordance with the manufacturer's protocol.

Immunofluorescence staining and confocal microscopy

HCT116 cells were maintained in normoxia or incubated in hypoxia for 8 h. Some were pretreated with 0.34 μ M doxorubicin. Cells were then fixed in 4% paraformaldehyde in PBS for 15 min, and permeabilized with 0.1% Triton X-100 in PBS for another 15 min. Subsequently, cells were incubated with 5% chicken serum. Primary antibodies used at 1:100 dilutions include rabbit anti-Myc (Upstate), mouse anti-Myc (Oncogene), mouse anti-p21 (BD Biosciences), rabbit anti-HIF-1 α (Santa Cruz), and goat anti-hTERT (Santa Cruz). Secondary antibodies against mouse, rabbit or goat were conjugated with FITC, Texas red (Molecular Probes) or AMCA (Jackson Immuno Research). Confocal laser scanning was carried out using a Zeiss LSM 510 microscope.

The area and mean pixel intensities of Myc, HIF-1 α , p21^{cip1}, and hTERT were quantified, respectively, from 100 individual cells and analyzed with Quantitative analysis software (Zeiss). Myc expression levels in each cell were plotted in *x*-axis in dots, and the HIF-1 α , p21^{cip1}, or hTERT expression levels in the corresponding cells were in *y*-axis to demonstrate the co-expression of these proteins in single cells. Cells co-expressing both proteins are distributed along the diagonal line.

RNA interference

Silencer *c-myc* siRNA, negative control siRNA, and Silencer siRNA transfection kit were purchased from Ambion, and used according to the manufacturer's instructions. At 36 h after transfection with siRNA, cells were infected with Ad- Δ ODD.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Xin W Wang for the helpful suggestions on the cell cycle study, Bert Vogelstein for reagents, and Curt C Harris for critical reading of the manuscript. We are grateful to the Gene Expression Laboratory of the SAIC Frederick Inc. for adenovirus productions, and Susan Garfield and Remy Pedeux for their advice on immunofluorescence staining and confocal microscopy.

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