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Review

Carrot and stick: HIF-α engages c-Myc in hypoxic adaptation

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The past decade of research on hypoxic responses has provided a considerable understanding of how cells respond to hypoxic stress at the molecular level, thanks to the identification and molecular cloning of the hypoxia-inducible transcription factor, HIF-1α. Numerous target genes have since been identified to account for various aspects of the hypoxic response, including angiogenesis and glycolysis. Yet, fundamental questions remain regarding the mechanisms by which hypoxia controls cell proliferation, genetic instability, mitochondrial biogenesis, and oxidative respiration in cancer cells. Although the proto-oncoprotein c-Myc appears to be the diametrical opposite of HIF-1α in most of these processes, recent studies indicate that c-Myc is an integral part of the HIF-α–c-Myc molecular pathway in the hypoxic response. It has been shown that HIF-α engages with Myc by various mechanisms to achieve oxygen homeostasis for cell survival. This article focuses on the intricate roles of c-Myc in the hypoxic response, discusses various mechanisms controlling c-Myc activity by HIF-α for the regulation of hypoxia-responsive genes, and emphasizing the outcome of gene expression apparently dependent upon hypoxic conditions, cellular context, and gene promoter.
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Solid tumors tend to develop hypoxia (oxygen deprivation), which arises out of the rapid cell proliferation that outstrips the supply of oxygen from the blood vessels. Consequently, tumor hypoxia activates hypoxia-inducible factor (HIF)-1α and HIF-2α,[1,2] two of the well-characterized hypoxia-inducible transcription factors critical for tumor angiogenesis, glycolysis, and metastasis.[3–5] Under physiological conditions, hypoxia activates the ubiquitous HIF-1α, whereas HIF-2α expression is more tissue specific. Yet in human cancers of diverse origins, both HIF-1α and HIF-2α, referred to collectively hereinafter as HIF-α, are frequently overexpressed and activated.

Hypoxia-inducible factor-α is the regulatory subunit of the HIF heterodimeric complex paired with the aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIF-1β).[6] They belong to the Per-ARNT-Sim (PAS) superfamily[7] of transcription factors containing basic helix–loop–helix domains (Figure 1a). Whereas PAS domains confer target gene specificity through protein–protein interactions,[8] the oxygen-dependent degradation domain, unique to HIF-α, mediates oxygen-dependent proteolysis.[9] As such, despite constitutive transcription and translation of the *HIF1A* and *EPAS1* genes (encoding HIF-1α and HIF-2α, respectively), HIF-α protein levels remain low in oxygenated conditions because of proteolysis via the ubiquitin–proteasome pathway.[9–12] HIF-α degradation requires the pVHL-containing E3 ubiquitin ligase,[13–15] which recognizes two hydroxylated proline residues of HIF-α (P402 and P564 in HIF-1α; Figure 1a).[16–18] HIF-α is hydroxylated by three prolyl hydroxylases, EglN1, EglN2, and EglN3 (better known as PHD2, PHD1, and PHD3, respectively), which sense oxygen tension and transduce oxygen signals through hydroxylation.[19–21] Interestingly, HIF-1α is also subjected to asparaginyl hydroxylation at N803 (Figure 1a), which is located in the C-terminal transactivation domain that recruits the transcription co-activator p300/CBP.[22] Thus, the asparaginyl hydroxylation hinders p300/CBP binding, thereby inhibiting HIF-α transactivation.[23–25] By contrast, hypoxia inhibits hydroxylation by limiting oxygen availability, resulting in HIF-α stabilization, which triggers the HIF-α activation cascade involving dimerization with ARNT, recruitment of p300/CBP, and binding to the hypoxia-responsive element (HRE) in the promoter of hypoxia-responsive genes for transcriptional activation (Figure 1b).[4]

These remarkable advances have smoothed the way for the identification of an ever-expanding body of hypoxia-responsive genes that account for many aspects of tumor hypoxia, including angiogenesis, glycolysis, low pH, and cell survival. Yet, unanswered questions remain regarding how hypoxia alters cell proliferation, induces genetic instability, and reprograms cell metabolism and mitochondrial biogenesis,

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Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix–loop–helix; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; INR, initiator element; ODD, oxygen-dependent degradation; PAS, Per-ARNT-Sim
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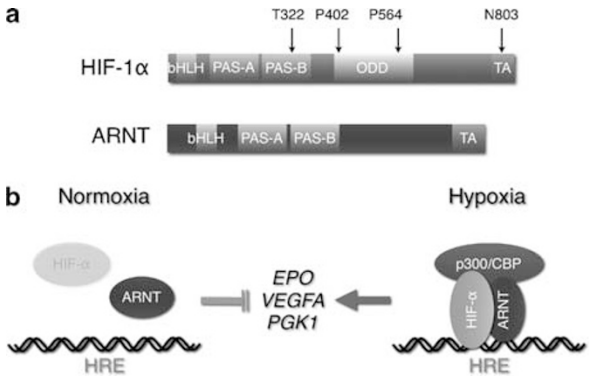

Figure 1 Schematic representation of the HIF-1 molecule and HIF-mediated transcriptional activation of hypoxia-responsive genes. (**a**) HIF-1 is a heterodimer composed of HIF-1α and ARNT. Both subunits contain bHLH (basic helix–loop–helix), PAS (Per-ARNT-Sim), and TA (transactivation) domains. The PAS domain comprises PAS-A and PAS-B subdomains. In addition, HIF-1α harbors an ODD (oxygen-dependent degradation) domain. Four indicated HIF-1α amino-acid residues are subject to post-translational modification. (**b**) In normoxia, HIF-α is unstable and therefore incapable of transactivation. Under hypoxia, however, HIF-α accumulates, leading to dimerization with ARNT, recruitment of p300/CBP, and binding to the HRE (hypoxia-responsive element) in the promoter of hypoxia-responsive genes, as indicated, for transcriptional activation

which are critical for tumor survival and progression. Recent studies, however, have begun to shed new light on these questions by unraveling the intricate role of the proto-oncoprotein c-Myc in relation to HIF-α in adaptation to the tumor microenvironment.[26–29]

c-Myc plays a central role in a transcription factor network that regulates cellular growth, differentiation, apoptosis, and metabolism.[30,31] It is frequently overexpressed in human cancers because of genetic rearrangements such as gene amplifications and chromosomal translocation. Deregulated c-Myc expression has been shown to drive vasculogenesis, reduce cell adhesion, and promote metastasis.[32] c-Myc can activate and repress transcription of its target genes. When in a binary complex with its partner Max, it activates transcription by binding to an E-box consensus sequence. However, Max also pairs with the c-Myc antagonist Mad1 or Max interactor 1 (Mxi1) to occupy the same E-box element for gene repression. In addition, the c-Myc–Max can be recruited by Miz1 for gene repression to core promoter sequences that lack the E-box sequence. Other transcription factors including Sp1 can also tether c-Myc to core promoter sequences.[32] Although HIF-α and c-Myc share common target genes, such as those involved in glycolysis and angiogenesis, they apparently have opposing effects on cell proliferation, mitochondrial biogenesis, and DNA repair. How both HIF-1α and HIF-2α employ diverse mechanisms by either collaborating with or counteracting c-Myc to mediate adaptive responses to hypoxia, why HIF-1α differs functionally from HIF-2α in engaging with c-Myc, and how HIF-1α and HIF-2α sometimes have opposite effects on c-Myc are discussed below. These interesting yet complex findings suggest that target gene promoter, cellular context, and hypoxic environment determine the way by which HIF-α engages with c-Myc and consequently the outcome of target gene expression.

HIF-1α Regulates Cell Cycle and DNA Repair Genes by Counteracting c-Myc Activities Through c-Myc Displacement

The c-Myc ternary repressive complex containing Miz1 is known to repress the cyclin-dependent kinase inhibitor gene *CDKN1A* (encoding $p21^{cip1}$) for cell-cycle progression.[33,34] By contrast, hypoxia upregulates *CDKN1A* gene in an HIF-1α-dependent way.[35,36] Furthermore, HIF-1α expression is sufficient to stimulate *CDKN1A* expression in normoxia, leading to cell-cycle arrest.[26,37] Of particular interest is that such gene upregulation, in contrast with the HIF-α activation cascade (Figure 1b), is independent of HIF-1α DNA-binding and transactivation domains.[26] In fact, the N-terminal portion of HIF-1α that harbors PAS domains (Figure 1a) is sufficient to induce *CDKN1A* expression and cell-cycle arrest, suggesting alternative mechanisms for HIF-1α activation of the *CDKN1A* gene. Indeed, HIF-1α activates *CDKN1A* gene expression by displacing the inhibitory c-Myc from binding to the *CDKN1A* proximal promoter, resulting in gene derepression (Figure 2a). Likewise, another cyclin-dependent kinase inhibitor gene *CDKN1B* (encoding $p27^{kip1}$), also suppressed by c-Myc, can be upregulated by hypoxia by a similar mechanism.[28,37] Thus, HIF-1α antagonizes repressive c-Myc activity for gene activation. It should be noted that unlike c-Myc displacement from the *MSH2* promoter (see below), the biochemistry of c-Myc displacement by HIF-1α in the *CDKN1A* and *CDKN1B* gene promoters has not been well characterized.

In the absence of Miz1, c-Myc forms an activating complex with Max for gene activation.[38] Would HIF-1α counteract an activating c-Myc, thereby resulting in gene repression? Very few genes have been reported to be downregulated by hypoxia, and the underlying mechanisms hitherto have been elusive. Evidently, hypoxia-induced gene repression can hardly be explained by the direct role of HIF-1α in transcriptional activation through binding to the HRE (Figure 1b). However, several studies have shown that c-Myc displacement accounts for hypoxic repression of c-Myc–activated

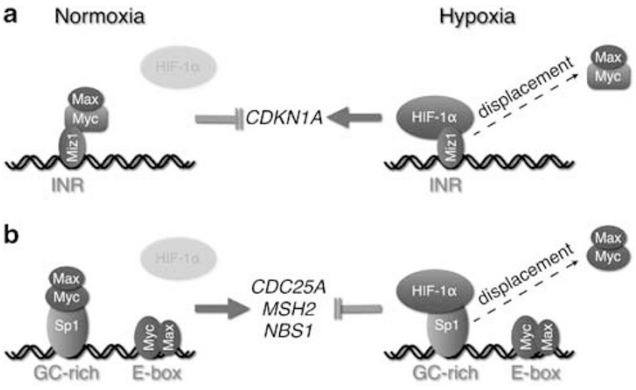

Figure 2 HIF-1α regulates hypoxia-responsive gene expression through c-Myc displacement. (**a**) c-Myc acts as a repressor by binding to Miz1 at the initiator element (INR) of the *CDKN1A* gene in normoxia. Hypoxia-induced HIF-1α displaces c-Myc from the promoter, resulting in gene de-repression. (**b**) Activating c-Myc drives gene transcription (as indicated) through interaction with the transcription factor Sp1 that is bound directly to the GC-rich region of the promoter and direct binding to an E-box element in the intron. Hypoxic stabilization of HIF-1α induces selective c-Myc displacement and, in turn, gene repression. It is noteworthy that c-Myc binding to the E-box remains intact under hypoxia in the *CDC25A* and *NBS1* genes

genes including the DNA repair genes *MSH2*, *MSH6*,[27] and *NBS1*[39] and the cell-cycle gene *CDC25A*;[40] that is, these c-Myc–activated genes are inhibited by hypoxia resulting from c-Myc displacement (Figure 2b). Although all of these genes share a common mechanism for gene repression, noticeable differences have been observed that distinguish one from another. For example, hypoxic downregulation of mismatch repair genes *MSH2* and *MSH6* has the distinction of p53 dependence. Consistently, c-Myc displacement in the *MSH2* and *MSH6* promoters requires wild-type p53. By contrast, hypoxic downregulation of *NBS1* and *CDC25A* genes is p53 independent. Unlike the regulation of DNA repair genes, HIF-1α expression alone neither inhibits *CDC25A* expression nor induces c-Myc displacement, despite the requirement of HIF-1α for mediating hypoxia-induced gene repression and c-Myc displacement. Biochemical and promoter analyses are needed to identify the binding partners responsible for all these subtle distinctions.

A more intriguing question, however, is how HIF-1α displaces an activating c-Myc from the target gene promoter. As for the *CDKN1A* upregulation, HIF-1α uses its N-terminal portion, the PAS-B subdomain (Figure 1a) in particular, for gene repression.[27,39] HIF-1α competes with c-Myc for binding to the transcription factor Sp1 that directly interacts with the *MSH2* promoter. Such competition is consistent with a tenuous interaction between HIF-1α and c-Myc in the cytosol.[26] Accordingly, c-Myc interacts with Sp1 for gene activation in normoxia where HIF-1α levels are low. Under hypoxia, HIF-1α occupies Sp1 via c-Myc displacement, resulting in gene inactivation (Figure 2b).

The requirement of Sp1 for c-Myc displacement raises the question as to whether c-Myc displacement occurs when c-Myc is bound directly to an E-box element. Interestingly, although both *NBS1* and *CDC25A* genes harbor an E-box element in their introns, no significant changes in c-Myc binding to the E-box have been detected when gene expression is suppressed.[39,40] Therefore, it appears that HIF-1α selectively targets c-Myc bound indirectly to DNA via another transcription factor for displacement (Figure 2b). Such a selective mechanism may explain the relatively weak suppression (approximately two- to threefold) of these genes

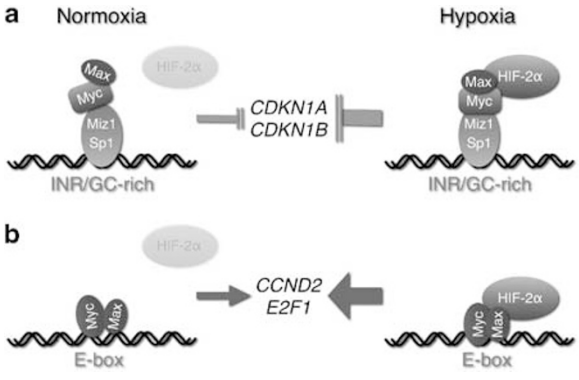

Figure 3 HIF-2α, in contrast with HIF-1α, enhances c-Myc activity. (**a** and **b**) Hypoxia-induced HIF-2α increases c-Myc binding to its partners (Miz1, Sp1, and Max), resulting in further repression of *CDKN1A* and *CDKN1B* when c-Myc is repressive (**a**) and enhanced transcription of *CCND2* and *E2F1* when c-Myc is activating (**b**)

by hypoxia, because of the remnant c-Myc-activating activity bound to the E-box. Furthermore, it stands to reason that HIF-1α might displace c-Myc from Sp1 more readily than from an E-box element, assuming a stronger binding affinity to the DNA sequence. However, more quantitative analyses of c-Myc binding to the E-box element in c-Myc-activated genes such as *ODC*, *CCND2*, and *E2F1* (encoding ornithine decarboxylase 1, cyclin D2, and E2F transcription factor 1, respectively) have shown decreased c-Myc promoter occupancy under hypoxia,[28] suggesting the occurrence of c-Myc displacement from the E-box element, albeit to a lesser extent. Furthermore, HIF-1α also induces a shift in the heterocomplex formation from activating c-Myc–Max to repressive Mad1–Max or Mxi1–Max, thereby further contributing to the inactivation of c-Myc target genes.[28,41]

HIF-2α, in Contrast with HIF-1α, Promotes Cell Proliferation by Enhancing c-Myc Activities Through Stimulation of Max Binding

Although both HIF-1α and HIF-2α follow the same HIF-α activation cascade (Figure 1b) for gene activation, studies have shown that HIF-2α possesses divergent (patho)physiological functions despite striking similarities to HIF-1α in amino-acid sequence and protein structure.[42–47] At the molecular level, several HIF-2α preferentially regulated genes have been identified, including *CCND1*, *EPO*, *POU5F1*, *TGFA*, and *VEGFA* (encoding cyclin D1, erythropoietin, octamer-binding transcription factor 4, transforming growth factor α, vascular endothelial growth factor A, respectively).[46,48,49] However, how HIF-2α specifically targets these genes remains unclear, although the involvement of ETS transcription factors in HIF-2α target gene selection has been suggested.[50]

Thus, given the high percentage of identity in amino-acid sequence particularly to the HIF-1α PAS-B domain, does HIF-2α also engage in c-Myc displacement? Interestingly, HIF-2α is not involved in the c-Myc counteraction because of its inability to compete with c-Myc for Sp1 binding.[39] Accordingly, HIF-2α fails to repress any of the foregoing DNA repair genes.[27,39] What distinguishes HIF-2α from HIF-1α in c-Myc displacement is a specific phosphorylation of HIF-2α at codon T324 (equivalent to HIF-1α T322; Figure 1a) by protein kinase D1, which prevents HIF-2α from competing for Sp1 binding. Conversely, HIF-1α, which is not subjected to phosphorylation at T322, is capable of c-Myc displacement.

Interestingly, what is remarkable is not what HIF-2α cannot do, but what it can. It has been uncovered that HIF-2α's 'hidden talent' is to enhance c-Myc activities by stimulating the interaction with Max.[28] As a result, HIF-2α promotes, rather than inhibits, cell-cycle progression in hypoxic cells by further inhibiting the expression of cyclin-dependent kinase inhibitor genes *CDKN1A* and *CDKN1B* (Figure 3a) and by augmenting the expression of cell-cycle genes *CCND2* and *E2F1* (Figure 3b). Moreover, the effects of HIF-2α on gene expression correlate with increased c-Myc target-gene promoter occupancy in hypoxic cells, and HIF-2α facilitates formation of c-Myc–Max complex through binding to Max. Evidently, further molecular and biochemical characterization

of HIF-2α interactions with Max should provide a mechanistic understanding of the functional difference between HIF-1α and HIF-2α in cell-cycle regulation. It will also be interesting to determine whether these opposing effects on cell cycle by HIF-1α and HIF-2α occur in the same tumor cell or if one dominates the other depending on their expression levels.

HIF-α Controls Mitochondrial Biogenesis by Inhibiting c-Myc-Mediated Transcription Through the Induction of c-Myc Degradation and Mxi1 Expression

In contrast with the opposing effects on c-Myc by HIF-1α and HIF-2α, the two HIF-α isoforms have been reported to work in the same direction to inhibit mitochondrial biogenesis.[29] Peroxisome proliferators-activated receptor γ coactivator 1β is a transcription factor that is regulated by c-Myc and implicated in mitochondrial biogenesis. Study has shown that HIF-α overexpression in a pVHL-deficient renal carcinoma cell line inhibits PGC-1β expression, resulting in decreased mitochondrial DNA content, mitochondrial mass, and oxygen consumption.[29] Both HIF-1α and HIF-2α are involved in the transcriptional repression of *PPARGC1B* (encoding PGC-1β). In contrast to the direct role of HIF-1α in c-Myc target gene repression (Figure 2b), the HIF-α isoforms inhibit *PPARGC1B* expression in two indirect ways (Figure 4a). On the one hand, HIF-α transcriptionally activates the *MXI1* gene (encoding Mxi1 that competes for Max binding).[29,41] On the other hand, HIF-α contributes to c-Myc proteolysis under severe or prolonged hypoxia via the ubiquitin–proteasome pathway without affecting *MYC* mRNA levels. Such a double-punch mechanism of c-Myc inactivation has also been shown to be responsible for transcriptional repression of other c-Myc-activated genes such as *CAD* (encoding carbamoyl-phosphate synthetase 2/aspartate *trans*-carbamylase/dihydroorotase) and *ODC1*. Therefore, both HIF-α isoforms induce c-Myc degradation and *MXI1* gene expression to inhibit c-Myc activity.

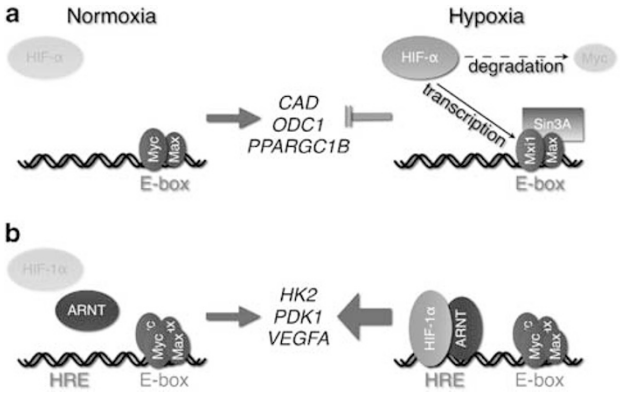

Figure 4 HIF-α inhibits the expression of c-Myc target genes in two independent ways, while deregulated c-Myc cooperates with HIF-1α for the activation of hypoxia-responsive genes. (**a**) Chronic hypoxia induces HIF-α-dependent c-Myc degradation and transcriptional upregulation of the c-Myc antagonist gene encoding Mxi1, which forms a complex with Max and recruits Sin3A for the repression of c-Myc target genes as indicated. (**b**) Deregulated c-Myc cooperates with HIF-1α under hypoxia to augment transcription of genes, as indicated, through binding to E-box and HRE, respectively

Although the contribution of HIF-α to c-Myc degradation is not well understood, the above studies support the notion that c-Myc can be regulated at different levels under different hypoxic conditions and perhaps in different cell types. Treatment with mild hypoxic conditions at 0.5–1% oxygen levels for less than 24 h leads to no significant change in c-Myc protein levels;[26–28] however, severe hypoxic conditions such as 0.1% oxygen or prolonged treatment result in c-Myc degradation without affecting *MYC* mRNA levels.[29,41] Yet, transcriptional downregulation of *MYC* gene by hypoxia has also been suggested to be a possible mechanism controlling the expression of *AFP* gene (encoding α-fetoprotein),[51] which is activated by c-Myc. Furthermore, given *MYC* gene being a transcriptional target of the β-catenin/T-cell factor-4 complex, such mechanism apparently accounts for the derepression of *CDKN1A* gene in hypoxic colon cancer cells, where HIF-1α disrupts the complex formation by competing for β-catenin binding, thereby reducing c-Myc levels.[52] Therefore, c-Myc activity can be affected by various hypoxic conditions.

In c-Myc-Deregulated Cells, HIF-1α Cooperates with c-Myc to Enhance Common Target Gene Expression

In human cancers, c-Myc deregulation is mostly a result of genetic rearrangements that disable homeostatic mechanisms controlling c-Myc protein levels. Thus, c-Myc may no longer be subjected to hypoxic regulation as discussed above. It has been shown that in a Burkitt lymphoma cell line where c-Myc levels are controlled by tetracycline, HIF-1α cooperates with, rather than antagonizes, c-Myc activities to enhance the expression of shared target genes including *HK2* (encoding hexokinase 2), *PDK1* (encoding pyruvate dehydrogenase kinase isozyme 1), and *VEGFA* (Figure 4b).[53] HK2 catalyzes ATP-dependent phosphorylation of glucose to form glucose-6-phosphate in the first step of glycolysis, whereas PDK1 inhibits mitochondrial respiration by inactivating pyruvate dehydrogenase, which converts pyruvate to acetyl-CoA. It is interesting to note that although coexpression of HIF-1α and c-Myc enhances glucose metabolism, *HK2* is the only glycolytic gene to be upregulated in this system. The expression of *LDHA*, another glycolytic gene best known to be responsive to HIF-1α or c-Myc, is not further enhanced by the two. Consistent with previous reports that the *PDK1* gene is a target of HIF-1α,[54,55] c-Myc augments PKD1 expression in the presence of HIF-1α for lactate production. In addition, HIF-1α and c-Myc also cooperate to activate the *VEGFA* gene. Such collaboration is associated with discrete and/or overlapping binding of HIF-1α and c-Myc to the target gene promoters. It remains unclear, however, what determines the interaction and/or cooperation between HIF-1α and c-Myc in the promoters of these common target genes. Whether such collaboration occurs for enhanced gene expression in other c-Myc deregulated tumor cells is yet to be determined. Furthermore, biochemical characterization of additional interacting proteins may help reconcile the differences between cooperative and antagonistic effects (discussed above) of HIF-1α on c-Myc.

Concluding Remarks

The revelation of the intricate roles of c-Myc in hypoxic responses has shed light on some of the fundamental aspects of tumor growth and progression. Although c-Myc is regarded as a central transcriptional hub in the control of growth and proliferation through binding to several thousand genomic loci,[56] its activities are subjugated under hypoxic conditions by HIF-α to serve the interests of tumor cells for adaptation and survival. Various mechanisms are employed to control c-Myc activities in an attempt to adjust to hypoxic conditions by balancing the needs for cell proliferation and metabolism, angiogenesis, DNA repair, and mitochondrial biogenesis.[57–59] Regardless of c-Myc cooperation with or counteraction by HIF-α, the identification of such HIF-α–c-Myc molecular pathway might have just opened the door to delve further into the cellular responses to the hypoxic microenvironment.

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