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# Forum News & Views

# Targeting the Mitochondria for Cancer Therapy: Regulation of Hypoxia-Inducible Factor by Mitochondria

ERIC L. BELL,[1] TATYANA KLIMOVA, and NAVDEEP S. CHANDEL[1,2]

## ABSTRACT

**As tumors develop, they outgrow the vascular network that supplies cells with oxygen and nutrients needed for survival. In response to decreased oxygen levels, the tumor cells initiate a program of adaptation by inducing the transcription of multiple genes *via* the activation of the transcription factor hypoxia-inducible factor (HIF). Proteins encoded by a subset of genes induced by HIF promote tumorigenesis by acting directly on both the tumor cells and the microenvironment in which the tumor cells reside. The mechanism(s) by which hypoxia activates HIF is a subject of intensive research. Understanding how hypoxia activates HIF will provide targets for the development of therapies that could specifically target growing tumors by not allowing adequate adaptation to hypoxia, which is necessary for cancer progression. Here we outline how mitochondria regulate the activity of HIF during hypoxia.** *Antioxid. Redox Signal.* 10, 635–640.

## INTRODUCTION

As a tumor increases in size, it outgrows the existing vasculature, creating nutrient deprivation that ultimately limits tumor growth. At this point, the tumor will survive but with no net gain in growth because, as the outside of the tumor grows, the center will die of necrosis or apoptosis or both because of nutrient and oxygen deprivation. For a net gain in tumor size to occur, the number of cells that proliferate must outnumber the number of cells that die. Thus, tumors must acquire the ability to obtain nutrients to promote survival and supply cells with the energy needed to proliferate at the abnormal rate observed in tumor cells (5). Tumors achieve this by modifying the surrounding microenvironment to provide an adequate supply pipeline for nutrient delivery at the site of initial growth (angiogenesis), or by disseminating to different areas of the organism where nutrients are more readily available (metastasis) (19). Furthermore, as tumor cells await angiogenesis, they must adapt to the hypoxic environment. The transcription factor hypoxia-inducible factor (HIF) directly transcribes genes involved in the regulation of glycolytic metabolism for hypoxic adaptation as well as genes involved in angiogenesis and metastasis (49).

HIF is a heterodimer of two basic helix loop-helix/Per-ARNT-Sim (PAS) proteins, HIF$\alpha$ and the aryl hydrocarbon nuclear trans-locator (ARNT or HIF-1$\beta$) (55). Both subunits are ubiquitously expressed; however, the $\alpha$ subunit is labile in conditions of normal oxygen (5–21% $O_2$). Under hypoxic (5–0.5% $O_2$) conditions, the $\alpha$ subunit is stabilized, dimerizes with ARNT, and translocates to the nucleus to initiate gene transcription. Three HIF$\alpha$ isoforms have been described, HIF-1$\alpha$, HIF-2$\alpha$, and HIF-3$\alpha$. HIF-1$\alpha$ and HIF-2$\alpha$ are transcriptionally active (41, 55, 56). Their target genes have been described to be both overlapping and distinct.

Deregulation of genes involved in intrinsic cellular processes such as cellular proliferation and apoptosis promote aberrant and unregulated cellular growth, leading to the initiation of cancers (16). Some of the genes induced by HIF control intrinsic cellular processes; therefore, deregulation of HIF can promote the progression of tumorigenesis (Fig. 1). Genes like insulin-like growth factor-2 (IGF-2) and glycolytic enzymes act as intrinsic signals to promote tumorigenesis by modulating cellu-

Department of [1]Medicine and [2]Cell and Molecular Biology, Northwestern University Medical School, Chicago, Illinois.

lar proliferation and survival, thereby providing cells autonomy from extrinsic factors. Vascular endothelial growth factor (VEGF) is a direct target of HIF and promotes the recruitment of endothelial cells to regions of hypoxia to promote the formation of new vascular networks (13). This allows the mass of tumor cells eventually to have a net gain in growth *via* an increased supply of necessary nutrients. Other target genes, such as matrix metalloproteinase-2 (MMP-2), promote invasion of tumor cells, as well as the migration of the cells away from the primary tumor through mesenchymal epithelial transition factor (c-MET) (31, 43). The importance of HIF-mediated transcription in tumorigenesis is highlighted by studies indicating that preventing HIF activation can suppress tumorigenesis (30, 32, 36). Moreover, deletions or mutations of genes involved in suppressing HIF activity, such as the von Hippel–Lindau tumor-suppressor protein (pVHL), promote the onset of various types of cancers (references discussed later). Therefore, defining how a cell senses decreased levels of oxygen to regulate the activity of HIF has broad implications for tumor progression due to hypoxia and may provide targets for therapeutic interventions. This review focuses on how mitochondria function as a key component of oxygen sensing and the possibility of using therapies that target mitochondria in treating cancers.

## OXYGEN REGULATION OF HYPOXIA-INDUCIBLE FACTOR

As previously mentioned, the protein levels of HIF$\alpha$ vary depending on oxygen concentration, whereas HIF$\beta$ protein levels are constitutively stable (26). Oxygen levels regulate the hydroxylation of two proline residues, 402 and 564, within the oxygen-dependent degradation domain (ODDD) of HIF$\alpha$ (37). This hydroxylation reaction is catalyzed by a family of proline hydroxylation enzymes (PHDs) (1, 11). The PHDs require $Fe^{2+}$, oxygen, and 2-oxoglutarate to catalyze the hydroxylation reaction (Fig. 2). Hydroxylated prolines serve as a binding site for pVHL, the substrate-recognition component of the VBC-CUL-2 E3 ubiquitin ligase complex (22–24). Once bound, pVHL tags HIF$\alpha$ with ubiquitin, thereby targeting it for proteasomal degradation (38). The importance of proper regulation of HIF activity is highlighted by the fact that loss of heterozygosity of pVHL is associated with renal cell carcinoma (RCC) (47). The loss of pVHL function results in an increase of HIF levels under normoxia, thereby contributing to the tumorigenicity of RCC *via* aberrant activation of HIF (30, 36).

HIF$\alpha$ activity also is regulated by posttranslational modification of its transactivation domains. The amino-terminal transactivation domain (N-TAD) is located within the ODDD. The carboxy-terminus transactivation domain (C-TAD) contains an asparigine residue that is hydroxylated by FIH (34). This reaction takes place under normoxic conditions (21% $O_2$) and inhibits the transactivation potential of HIF$\alpha$ (33). The hydroxyl group on Asn803 inhibits the interaction of HIF$\alpha$ with the coactivator CBP/p300 (14).

When oxygen levels decrease to $<5\%$ $O_2$, HIF$\alpha$ is not hydroxylated. In the absence of proline hydroxylation, pVHL cannot bind HIF$\alpha$ to initiate ubiquitin-proteasomal degradation. When stabilized, HIF$\alpha$ translocates to the nucleus and dimerizes with HIF$\beta$. Once in the nucleus, the HIF dimer binds to HIF response elements (HREs) located throughout the genome (25). The absence of a hydroxyl group on Asn803 allows HIF to associate with the coactivator CBP/p300 to facilitate the transcription of various target genes. Therefore, HIF activity is tightly controlled by the hydroxylation of various amino acids. Understanding how the hydroxylases that modulate HIF activity are themselves regulated could provide targets for intervention in tumorigenesis as well as other pathologies associated with HIF activity.

## MITOCHONDRIA AS CELLULAR OXYGEN SENSORS

The fact that mitochondria are responsible for the majority of the oxygen consumed within the cell makes them a likely choice for evolutionarily conserved oxygen sensors. Mitochondria contain their own DNA (mtDNA), which encodes 13 genes that are essential for the assembly of a functional electron-transport chain. These genes encode subunits for complexes I, III, IV, and V. The major consumer of oxygen is complex IV. Initial studies indicated that pharmacologic inhibition of complex IV by cyanide did not activate or repress HIF (26). Cells cultured with sublethal levels of ethidium bromide, which inhibits the transcription and replication of mtDNA, resulting in the loss of a functional electron-transport chain, were used genetically to address whether mitochondria are involved in the HIF response (28). These cells devoid of mtDNA, $\rho^0$ cells, are unable to activate HIF$\alpha$ in hypoxic conditions (3, 4). However, these cells are still able to stabilize HIF$\alpha$ in anoxic conditions, indicating that a fundamental difference exists in the mechanism required to stabilize HIF$\alpha$ protein in hypoxic *versus* anoxic conditions (46). These data indicate that the electron-transport proteins are necessary for hypoxic stabilization of HIF$\alpha$ but not anoxic stabilization. The mechanism of HIF$\alpha$ stabilization under anoxic conditions is most likely the direct inhibition of the PHDs due to a lack of oxygen. Because the PHDs require oxygen as a co-substrate, they cannot hydroxylate HIF$\alpha$ in anoxic conditions to initiate degradation, resulting in the stabilization of the HIF$\alpha$ protein.

After initial studies demonstrating that $\rho^0$ cells fail to activate HIF during hypoxia, reports with conflicting data indicated that hypoxic activation of HIF is intact in $\rho^0$ cells (10, 50, 53). However, these data were most likely due to the use of oxygen levels closer to anoxic conditions than hypoxic conditions. Al-

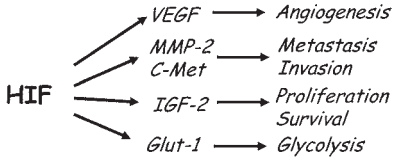


**FIG. 1. HIF targets multiple genes involved in tumorigenesis.**

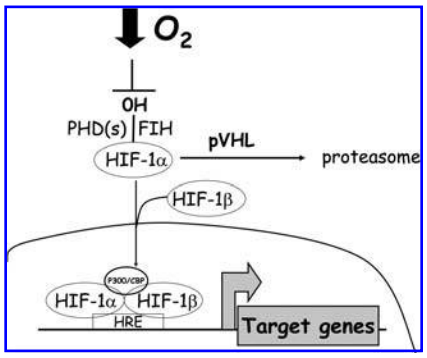


**FIG. 2. HIF-1 is composed of two subunits, oxygen-sensitive HIF-1$\alpha$ and HIF-$\beta$.** HIF-1$\alpha$ is hydroxylated at two different proline residues and an asparagine residue under normoxia. The hydroxylation of proline residues serves as a recognition motif for pVHL. The binding of pVHL targets the HIF-1$\alpha$ protein for ubiquitin-mediated degradation. HIF-1$\alpha$ contains two transactivation domains referred to as TAD-N(531–575) and TAD-C(786–826). The asparagine residue resides in TAD-C. The hydroxylation of asparagine prevents the binding of transcriptional co-activators such as p300/CBP. Under hypoxia, the hydroxylation of proline and asparagine is diminished, which allows the protein to be stabilized and bind to HIF-1$\beta$, as well as p300/CBP, to allow HIF-1–dependent gene transcription.

ternatively, these data could be due to a difference in $\rho^0$ cells generated by ethidium bromide. Recently three independent reports used rigorous genetic methods to manipulate mitochondrial electron transport to determine the requirement of functional mitochondrial electron transport in hypoxic stabilization of HIF$\alpha$ protein. The first study used a genetic approach to knock out cytochrome *c* (35). In the electron-transport chain, cytochrome *c* accepts electrons from complex III and transfers them to complex IV to be used to reduce molecular oxygen to water. Murine embryonic cells lacking cytochrome *c* failed to stabilize HIF$\alpha$ during hypoxic conditions. The other two studies demonstrated that inhibiting complex III function by knocking down the Rieske Fe-S protein inhibits the ability of multiple cell lines to stabilize HIF$\alpha$ in hypoxic conditions (2, 17). However, cells that displayed knockdown of the Rieske Fe-S protein were able to stabilize HIF$\alpha$ in anoxic conditions, further supporting the notion that different mechanisms allow the stabilization of HIF$\alpha$ protein in hypoxic and anoxic conditions. These data provide conclusive evidence that a functional electron-transport chain is required for the hypoxic stabilization of HIF$\alpha$ protein. However, the mechanism by which a functional electron-transport chain activates HIF during hypoxia remains controversial.

## MECHANISMS OF MITOCHONDRIAL OXYGEN SENSING

In the absence of electron transport, cells do not consume oxygen or generate ROS from the mitochondria. These functions of mitochondria have been independently proposed as potential mechanisms by which the mitochondrial electron-transport chain activates HIF during hypoxia (Fig. 3). Molecular oxygen is used as the terminal electron acceptor in the mitochondrial electron-transport chain when cytochrome *c* oxidase (COX) converts oxygen to water (45). This property of mitochondria combined with the requirement of the PHDs for molecular oxygen as a co-substrate is the basis for a model in which mitochondria oxygen consumption is the regulator of HIF activation *via* PHD regulation. This model hypothesizes that during conditions of limited oxygen, mitochondria create an oxygen gradient within the cells as a result of their ability to consume oxygen (7, 18). This gradient would effectively sequester molecular oxygen away from the cytosolic PHDs, thus inhibiting their ability to hydroxylate HIF$\alpha$. However, cells that are respiratory deficient and are not $\rho^0$ cells can still stabilize HIF$\alpha$ protein during hypoxia (2). Further experiments must be performed to resolve whether mitochondrial oxygen consumption is the mechanism by which mitochondria sense hypoxic conditions.

Another model of mitochondrial oxygen sensing is based on ROS generation by mitochondria. It has been demonstrated that cytosolic ROS levels paradoxically increase in hypoxic conditions (3). The increase in ROS during hypoxia is reversible because reoxygenation to normoxia decreases the ROS signal. It is important to note that this is different from the reoxygenation-induced generation of ROS observed in ischemia–reperfusion models in which cells are exposed to anoxia coupled with acidosis during the ischemia phase, and the reoxygenation is restoration of both pH and oxygen levels. The increase in cytosolic ROS during hypoxia is required to stabilize HIF$\alpha$ protein (4). Cells deficient in cytochrome *c* or Rieske iron-sulfur

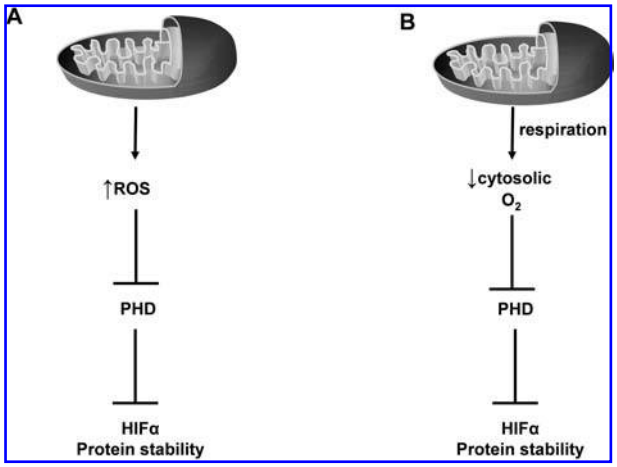


**FIG. 3. Currently two models explain the role of the mitochondrial electron-transport chain in oxygen sensing.** The first model postulates that mitochondria release ROS during hypoxia to inhibit hydroxylation of HIF$\alpha$ protein, thereby causing the protein to escape proteasomal degradation. The second model hypothesizes that mitochondrial respiration limits oxygen availability to the hydroxylases, thereby not allowing the hydroxylation reaction to occur. This results in accumulation of HIF$\alpha$ protein during hypoxia.

protein that are unable to stabilize HIF during hypoxia also do not display an increase in ROS generation during hypoxia. These cells are also deficient in oxygen consumption; therefore, these studies do not differentiate between the ability of mitochondria to generate ROS or consume oxygen. Incubating cells with pharmacologic antioxidants such as ebselen and MitoQ attenuates HIF activation in hypoxic conditions (17, 44). Recently it was reported that MitoQ treatment affects respiration; thus, again, the effect of MitoQ cannot be solely attributed to its antioxidant properties (42). However, studies using protein antioxidants support the involvement of ROS. Expression of protein antioxidants, such as glutathione peroxidase (GPX) and catalase, also attenuates HIF$\alpha$ protein stabilization and activation, but expression of superoxide dismutase (SOD) has no effect (2). SOD converts superoxide to $H_2O_2$, whereas GPX and catalase convert $H_2O_2$ to water. The specificities of these antioxidants for different forms of ROS leads to the conclusion that $H_2O_2$ is the ROS moiety required for stabilization of HIF$\alpha$ protein. HIF$\alpha$ protein is stabilized when cells are pulsed with 25 $\mu M$ *t*-butyl $H_2O_2$, a more stable form of $H_2O_2$, in normal oxygen conditions, indicating that $H_2O_2$ is sufficient to activate HIF-mediated transcription (4). ROS are a normal byproduct of electron transport within the mitochondria, so the ability of cells to increase cytosolic ROS in hypoxic conditions provides a possible link between mitochondrial electron transport and HIF activation. However, where within the electron-transport chain these ROS originate remains unknown. Furthermore, genetic studies must uncouple ROS generation from oxygen consumption to address which function of mitochondrial electron-transport chain is the major regulator of HIF during hypoxia.

A major contention with the ROS model with respect to hypoxic activation of HIF is whether hypoxia increases ROS. Multiple initial studies demonstrated that hypoxia increases mitochondrial free radical production during hypoxia in cell-culture models and in animal models by using the oxidant-sensitive dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (3, 8, 27, 57). However, reports in the literature demonstrate a decrease in ROS levels with other dyes that measure oxidative stress such as dihydrorhodamine 123 or horseradish peroxidase (HRP)-enhanced luminol chemiluminescence (12, 39, 54). All of these dyes have limitations in their measurements of intracellular oxidative stress (51). To resolve whether hypoxia increases or decreases ROS, several groups have taken different approaches to assess ROS. Poyton and colleagues (6) used oxidative protein carbonylation to assess whether mitochondria increase oxidative stress during low oxygen concentrations. They observed that yeast cells increased protein carbonylation, and the mitochondrial respiratory chain was responsible for this carbonylation. Recently, Guzy et al. (17) used a sensitive fluorescence resonance energy transfer (FRET) probe to assess redox status in the cytosol during hypoxia. This probe consists of fusion proteins containing a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) linked by a redox-sensitive hinge that contains cysteine thiols that become cross-linked by oxidant stress. These thiol groups become oxidized during an oxidant stress, causing the CFP and YFP to move apart and the FRET intensity ratio to increase. The redox-sensitive FRET probe, when expressed in cells, responds to hypoxia by producing a dose-dependent increase in the FRET ratio. FRET can be difficult to use; thus the recent development of redox-sensitive GFP probes will help resolve whether hypoxia increases or decreases ROS.

## MITOCHONDRIAL CROSSTALK WITH THE PHDS

The immediate upstream regulator of HIF activity is the PHDs, which hydroxylate HIF$\alpha$ protein. How mitochondrial ROS regulate hydroxylation of HIF$\alpha$ protein remains unknown. Exogenous $H_2O_2$ stabilizes HIF$\alpha$ protein in normal oxygen conditions, implying that ROS inhibit the hydroxylation reaction. Presently, two mechanisms exist by which ROS could prevent hydroxylation of HIF-1$\alpha$ protein. ROS can regulate the redox state of iron through the Fenton reaction. Therefore, one possibility is that low levels of oxygen decrease PHD activity because ROS decrease the availability of the PHD co-factor Fe(II) (15, 42). A second possibility is that ROS activate signaling pathways that catalytically make PHDs inactive. Multiple signaling pathways have been implicated in hypoxic stabilization of HIF (9, 20, 40, 52). Finally, it could be that the low oxygen levels decrease PHD activity, and the ROS produced during hypoxia further decrease PHD activity to prevent hydroxylation of HIF$\alpha$ protein (Fig. 4). Further studies must address the relation between mitochondrial ROS and PHDs.

Recent reports indicate that levels of TCA intermediates can regulate the ability of the PHDs to hydroxylate HIF$\alpha$ (21, 29, 42, 48). Because hydroxylation of HIF$\alpha$ protein is the main determinant of HIF$\alpha$ activity *via* regulation of protein stability, it is plausible that hypoxia regulates the levels of some of these intermediates to control the ability of the PHDs to hydroxylate HIF$\alpha$. Interestingly, genetic disruption of either succinate dehydrogenase or fumarate hydratase increases succinate and fumarate levels, respectively, and promote tumorigenesis (21, 48). It has been proposed that increases in these metabolites inhibit PHD activity to stabilize HIF$\alpha$ protein, thereby promoting the formation of tumors. The effect

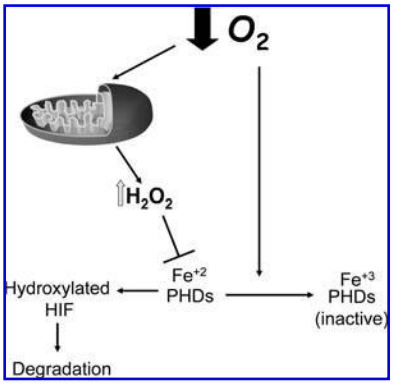


**FIG. 4. Hypoxia could directly decrease the PHD activity coupled with an increase in ROS generation, which depresses PHD activity to inhibit hydroxylation of HIF$\alpha$ protein.**

of hypoxia on the relative levels of metabolites that regulate PHD activity must be explored.

## MITOCHONDRIAL TARGETED THERAPIES FOR THE REGULATION OF HIF-MEDIATED PATHOLOGIES

That HIF is an important player in tumorigenesis and tumor progression, and that mitochondria regulate hypoxic activation of HIF, make mitochondria an attractive target for designing cancer therapeutics. MitoQ, which attenuates HIF activation, is promising as a potential therapeutic agent for targeting HIF-mediated cancers, whether by inhibiting respiration or through the ablation of ROS generated from the mitochondria. Determining exactly how mitochondria function to initiate the hypoxic response will help to develop new targeted therapies. Developing therapies that target oxygen consumption, production of ROS, or alteration of metabolite levels by the mitochondria independent of each other will allow therapies to target hypoxic tumor cells selectively without having systemic side effects. It is important to separate the potential roles of various mitochondrial functions on HIF activation to develop future therapies that target HIF-mediated tumorigenesis.

## ACKNOWLEDGMENTS

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Address reprint requests to:
*Navdeep Chandel*
*Northwestern University*
*Department of Medicine*
*240 East Huron Avenue*
*McGaw M-334*
*Chicago, IL 60611*

*E-mail:* nav@northwestern.edu

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