Review article

Maintenance of redox homeostasis by hypoxia-inducible factors

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ABSTRACT

Oxidative phosphorylation enables cells to generate the large amounts of ATP required for development and maintenance of multicellular organisms. However, under conditions of reduced O_2 availability, electron transport becomes less efficient, leading to increased generation of superoxide anions. Hypoxia-inducible factors switch cells from oxidative to glycolytic metabolism, to reduce mitochondrial superoxide generation, and increase the synthesis of NADPH and glutathione, in order to maintain redox homeostasis under hypoxic conditions.

1. Oxygen and reactive oxygen species

All human cells require a constant supply of O_2 for continued survival. O_2 is utilized by electron transport chain (ETC) complex IV (cytochrome *c* oxidase) as the final electron acceptor in the process of mitochondrial oxidative phosphorylation. O_2 levels in cells are tightly regulated to balance benefit (generation of ATP) and risk (generation of reactive oxygen species [ROS]). Under physiological PO_2 levels, ETC complex IV transfers electrons to O_2 to form water. However, when PO_2 levels deviate from the physiological range, electrons escape from the ETC at an increased rate and react with O_2 prior to complex IV, resulting in the formation of superoxide anion radical ($O_2^{\cdot-}$), which can subsequently be converted to hydrogen peroxide (H_2O_2) and other reactive molecules, including hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$) and ozone (O_3) [2,3]. At the plasma membrane, ROS are generated through the activity of NADPH oxidase (NOX) enzymes [1]. ROS are generated by many other enzymatic reactions and are also produced when cells encounter external agents such as pollutants, tobacco smoke or radiation [4].

The consequences of ROS generation in cells are dependent on context and concentration. Physiological ROS signaling involves H_2O_2-mediated oxidation of cysteine residues within proteins [5]. At nanomolar concentrations, H_2O_2 oxidizes the thiolate anion form of cysteine residues (Cys-S⁻) to the sulfenic form (Cys-SOH) [6]. This modification may cause allosteric changes to proteins that have functional consequences but is reversible. At higher H_2O_2 concentrations, the thiolate anion oxidizes to sulfinic (SO_2H) or sulfonic (SO_3H) species, which is reversed by sulfiredoxin [7].

2. Redox signaling and its biological consequences

Redox signaling is important for the maintenance of physiological functions, such as cellular differentiation and tissue regeneration, whereas loss of redox homeostasis is associated with disease states, such as cancer and autoimmunity. The innate and adaptive immune systems are critical for pathogen-specific defense, immunological memory and tissue repair. ROS signaling plays a critical role in cells of the innate and adaptive immune systems [8,9]. Physiological levels of ROS maintain a healthy immune system, whereas decreased ROS levels inhibit activation of the immune system, leading to immunosuppression, and increased ROS levels induce hyperactivation of immune system through release of inflammatory cytokines, leading to autoimmunity [10]. Redox signaling also plays an important role in tissue regeneration. Physiological ROS levels are required for stem cell maintenance, whereas high levels of ROS lead to stem cell exhaustion [11]. The same may hold true for certain cancers. At lower concentrations, ROS are important signaling molecules involved in cellular proliferation, migration, and metabolic adaptation. However, at higher concentrations, they cause damage due to irreversible oxidation of proteins, lipids and DNA, leading to cell death [12]. Several treatment approaches in cancer, including chemotherapy and radiation therapy, generate ROS at high levels, which are toxic to cancer cells. In this review, we will discuss how breast cancer cells maintain ROS homeostasis under hypoxic conditions in the tumor microenvironment.

3. Hypoxia and HIFs

Hypoxia-inducible factors (HIFs) are transcriptional activators that
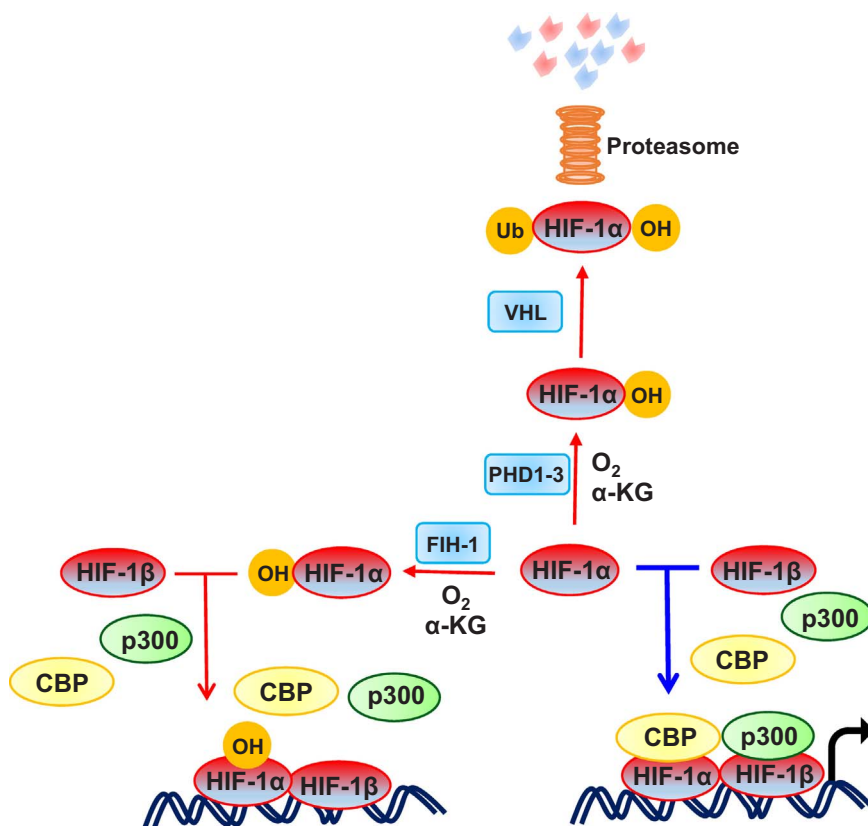
serve as master regulators of oxygen homeostasis [13]. Many solid tumors have regions of low oxygenation ($PO_2 < 10$ mmHg) due to an imbalance between O_2 supply and demand. HIFs are heterodimeric transcription factors composed of an O_2-regulated HIF-1α, HIF-2α or HIF-3α subunit and a constitutively expressed HIF-1β subunit. HIF-1α, HIF-2α or both are required for the transcriptional activation of a large battery of genes, whose protein products are required for discrete steps in cancer invasion and metastasis [14]. Under normoxic conditions, HIF-α subunits are hydroxylated at two proline residues (Pro-402 and Pro-564 in human HIF-1α). This post-translational modification is mediated by three HIF prolyl hydroxylase domain proteins (PHD1-3), which are α-ketoglutarate – dependent dioxygenases, with PHD2 playing a particularly important role [15,16]. The von Hippel-Lindau (VHL) ubiquitin ligase complex recognizes prolyl hydroxylated HIF-α subunits and marks them for proteasomal degradation (Fig. 1). The PHDs are believed to have a relatively high K_M value (Michaelis-Menten constant) for O_2, such that their hydroxylase activity falls when oxygenation is inadequate [16].

In addition to the O_2-dependent negative regulation of protein stability by PHDs, HIF-1α (and HIF-2α) transactivation function is negatively regulated by factor inhibiting HIF-1 (FIH-1) [17], which is also an O_2- and α-ketoglutarate-dependent dioxygenase. FIH-1 catalyzes asparaginyl hydroxylation (Asn-803 in human HIF-1α), which blocks binding of the coactivators CBP (CREB-binding protein) and p300 [18,19], as shown in Fig. 1. In summary, the stability and activity of the HIFs are modulated by hydroxylation, such that changes in O_2 availability are transduced to the nucleus as changes in HIF transcriptional activity. In order to maintain redox homeostasis in the presence of intratumoral hypoxia, HIFs activate the transcription of target genes encoding proteins that serve to either decrease production of mitochondrial ROS or increase production of ROS scavengers, as described in detail in the following section.
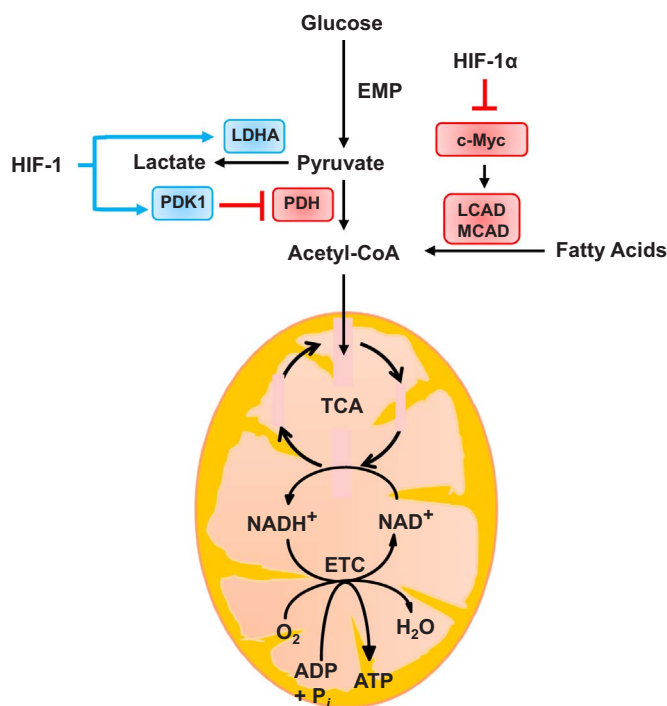
4. HIF-1 inhibits ROS production by regulating acetyl-CoA synthesis

Reduced O_2 availability has profound effects on cellular metabolism. Glucose and glutamine are considered primary metabolic substrates for many cancer cells. Glucose is converted to pyruvate through the Embden-Meyerhof (glycolytic) pathway (EMP). Under aerobic conditions, pyruvate dehydrogenase (PDH) converts pyruvate to acetyl-CoA for oxidation in the tricarboxylic acid (TCA) cycle. Glutamine is converted to glutamate and then to α-ketoglutarate, which also enters the TCA cycle. A third major energy substrate for cancer cells is fatty acids, which are oxidized to generate acetyl-CoA [20], as described in greater detail below. The further oxidation of acetyl-CoA in the TCA cycle generates reducing equivalents (NADH and $FADH_2$), which are donated to the ETC to generate a proton gradient that is used to generate ATP. Thus, the flux of acetyl-CoA metabolized by the TCA cycle determines the flux of electrons delivered to the ETC.

Previously, it was thought that cells subjected to hypoxic conditions (e.g. transfer of tissue culture cells from 20% to 1% O_2 ambient environment) utilized glycolysis to generate ATP because O_2 becomes limiting for respiration. Studies of wild-type (WT) and HIF-1α–deficient ($Hif1\alpha^{-/-}$) mouse embryo fibroblasts (MEFs) revealed that WT MEFs switched from oxidative to glycolytic metabolism when the cells were exposed to 1% O_2 or less for 48 h or more, leading to decreased ATP levels, because, compared to respiration, glycolysis generates much less ATP per mole of glucose consumed. $Hif1\alpha^{-/-}$ MEFs did not switch from oxidative to glycolytic metabolism and maintained higher ATP levels at 1% O_2 than did WT MEFs at 20% O_2, demonstrating that 1% O_2 is not limiting for oxidative phosphorylation. However, the $Hif1\alpha^{-/-}$ MEFs died due to excessive ROS production [21,22]. HIF-1 determines the balance between oxidative and glycolytic metabolism of glucose by regulating the expression of LDHA and PDK1, which promote conversion of pyruvate to lactate, relative to PDH, which converts pyruvate to acetyl-CoA [22,23] as shown in Fig. 2.

Besides glucose, fatty acid oxidation (FAO) is an additional source of

Fig. 2. Oxygen-dependent regulation of ROS production by HIF-1. In well-oxygenated cells, glucose is metabolized to pyruvate, which is converted to acetyl-CoA by pyruvate dehydrogenase (PDH) for entry into the tricarboxylic acid cycle (TCA). However under hypoxic conditions, HIF-1 decreases glucose oxidation by activating the transcription of *PDK1,* which encodes a kinase that phosphorylates and inactivates PDH; and *LDHA*, which encodes the enzyme that converts pyruvate to lactate. HIF-1 also decreases fatty acid oxidation by repressing the c-Myc-dependent expression of *MCAD* and *LCAD*. Blue rectangles indicate proteins that are products of HIF-1 target genes. Blue arrows and red blocked arrows indicate induction and inhibition, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

acetyl–CoA. FAO generates one molecule of acetyl CoA in each oxidation cycle and two molecules in the last cycle. Transcription of the *MCAD* and *LCAD* genes encoding medium-chain and long-chain acyl-CoA dehydrogenases, which catalyze the first step of FAO in the mitochondria, was repressed when hepatocellular carcinoma cells were subjected to hypoxic conditions [24]. Under hypoxia, HIF-1α suppressed the expression of C-MYC, which is a transcriptional activator of the gene encoding PGC-1β, which is in turn a coactivator of *MCAD* and *LCAD* transcription. The decrease in FAO decreases mitochondrial ROS generation, as shown in Fig. 2.

5. HIF-1 inhibits mitochondrial ROS production in hypoxic cells by regulating mitochondrial proteins and autophagy

HIF-1-dependent expression of BNIP3 in MEFs [21] and BNIP3L in cancer cells [25] was shown to stimulate mitochondrial-selective autophagy. The reduction in mitochondrial mass suppresses the oxidation of both glucose and fatty acids and thus decreases mitochondrial ROS production under hypoxic conditions, as shown in Fig. 3.

Major sites of ROS production in mammalian mitochondria are the ubiquinone reduction site of complex I and the outer quinone-binding site of the Q cycle in complex III [26]. Hypoxia modulates respiratory chain function in cancer cells by orchestrating a subunit switch (from COX4-1 to COX4-2) in cytochrome c oxidase (COX). COX catalyzes the terminal transfer of electrons to O_2. In hypoxic cells, HIF-1 activates the transcription of the *COX4l2* and *LON* genes. LON is a mitochondrial protease that is required for COX4-1 degradation. The subunit switch from COX4-1 to COX4-2 may increase the efficiency of electron transfer to O_2 and allow continued respiration without increased ROS levels

when O_2 availability is modestly reduced [27].

Under hypoxic conditions, HIF-1 activates transcription of the *NDUFA4L2* gene, which encodes NADH dehydrogenase [ubiquinone] 1α subcomplex, 4-like 2. ROS levels were increased in hypoxic cells lacking NDUFA4L2, which may act by inhibiting complex I activity [28]. Thus, *NDUFAL2* gene expression represents another adaptive mechanism that reduces flux through the ETC, thereby reducing ROS production, as shown in Fig. 3.

HIFs also transactivate genes encoding microRNAs (miRs), which are small RNAs that bind to mRNAs in a sequence-specific manner to either inhibit their translation or induce their degradation [29]. Hypoxia induces miR-210 expression in many cell types in a HIF-dependent manner [30]. miR-210 represses *ISCU* which encodes iron-sulfur cluster assembly factors that are required for the biogenesis of iron-sulfur-dependent enzyme complexes including complex I, complex III, and aconitase, which are critical for electron transport [31]. Inhibition of ISCU1/2 expression reduces mitochondrial ROS generation.

Finally, hypoxia also induces the transformation of complex I from the active to 'deactive' form in endothelial cells and brain tissue, which involves a switch from NADH-ubiquinone oxidoreductase activity to Na^+/H^+ antiporter activity, which suppresses ROS production [32].

6. HIF regulates antioxidant defenses in hypoxic cells

Cellular redox homeostasis represents a balance between oxidants (principally, ROS) and antioxidants (principally, reduced glutathione). Antioxidant defense is dependent on the generation of NADPH, which is used to maintain glutathione in a reduced form, and also on the de novo synthesis of glutathione. In human breast cancer cells, HIFs function as master regulators that orchestrate the generation of NADPH and glutathione under hypoxic conditions.

There are two major pathways in the cell that generate NADPH. One is the pentose phosphate pathway (PPP), which generates NADPH in the cytoplasm. In the first step of the PPP, glucose-6-phosphate dehydrogenase (G6PD) converts the glycolytic intermediate glucose-6-phosphate and $NADP^+$ to 6-phosphogluconolactone and NADPH. The other major pathway for NADPH production is one-carbon metabolism (1CM; also known as the folate cycle). Serine is the major donor of one-carbon units to the folate cycle. The serine synthesis pathway (SSP) provides a mechanism for glucose-derived carbons to be diverted from glycolysis for de novo serine synthesis [33]. Serine is required for a number of biosynthetic and signaling pathways, including synthesis of amino acids, such as glycine and cysteine, both of which are required for the synthesis of glutathione. As shown in Fig. 4, in the SSP, phosphoglycerate dehydrogenase (PHGDH) converts the glycolytic intermediate 3-phosphoglycerate (3-PG) to 3-phosphopyruvate (3-PHP). The next enzyme in the SSP, phosphoserine aminotransferase (PSAT1), converts 3-PHP into phosphoserine (P-Ser) and, finally, phosphoserine phosphatase (PSPH) converts P-Ser into serine. Serine and $NADP^+$ are then utilized for 1CM, either in the cytosol or mitochondria, which generates glycine and NADPH. In the mitochondria, 1CM is catalyzed by serine hydroxymethyltransferase 2 (SHMT2), methylene tetrahydrofolate dehydrogenase 2 (MTHFD2) or MTHFD2-like (MTHFD2L), and MTHFD1L. In the cytosol, SHMT1 and MTHFD1 carry out the same reactions (MTHFD1 catalyzes the reactions performed by both MTHFD2/MTHFD2L and MTHFD1L) [33].

In breast cancer cells, analysis of gene expression revealed that, irrespective of the estrogen receptor (ER) status, hypoxia induced the expression of the three enzymes of the SSP (PHGDH, PSAT1 and PSPH) and three enzymes required for mitochondrial 1CM (SHMT2, MTHFD2, and MTHFD1L) in a HIF-dependent manner [34]. In contrast, the enzymes required for cytosolic 1CM (SHMT1, MTHFD1) were not hypoxia-induced in most breast cancer cell lines. Additionally, the *G6PD* gene, was repressed by hypoxia in all breast cancer cell lines analyzed [34]. These results suggest that in hypoxic breast cancer cells, glucose metabolism is reprogrammed to increase flux through the SSP and
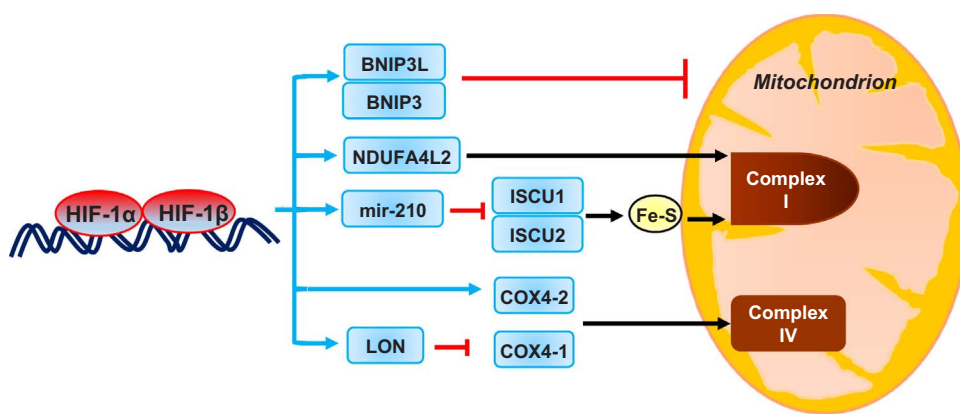
Fig. 3. Oxygen-dependent regulation of mitochondrial function by HIF-1. Under hypoxic conditions HIF-1 induces the expression of: (a) BNIP3 and BNIP3L, which trigger mitochondrial selective autophagy; (b) NDUFA4L2, which inhibits complex I activity; (c) miR-210, which represses ISCU, a protein that is required for ETC assembly; and (d) COX4-2 and LON, which orchestrate a subunit switch in complex IV that increases electron transfer efficiency. The expression of each of these HIF- regulated genes serves to decrease mitochondrial ROS production. Blue rectangles indicate mRNAs and microRNA that are products of HIF-1 target genes. Blue arrows indicate increased expression and blocked red arrows denote destruction of mitochondria and COX4-1 by autophagy and LON protease, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

decrease flux through the PPP. This conclusion was confirmed by metabolomic analysis of cell lysates. Silencing of PHGDH expression by RNA interference in either ER^+ or ER^- breast cancer cells led to decreased NADPH levels, disturbed mitochondrial redox homeostasis, and increased apoptosis, which abrogated the enrichment of breast cancer stem cells (BCSCs) under hypoxic conditions [34]. These results reveal a novel mechanism by which HIF regulates the cellular antioxidant capacity and thus regulates redox homeostasis, which in turn is required for the maintenance of BCSCs.

Glutathione (γ-L-glutamyl-L-cysteinylglycine) is a small peptide that is generated from the amino acids cysteine, glutamate, and glycine. *SLC7A11* encodes the pore-forming subunit of a plasma membrane transporter for cystine uptake. *GCLM* encodes the regulatory subunit of glutamate cysteine ligase, which catalyzes the first step of glutathione biosynthesis. In breast cancer cells, hypoxia induced *SLC7A11* and *GCLM* expression in a HIF dependent manner [35]. Thus, in response to hypoxia, HIF coordinately activates the transcription of genes encoding enzymes that regulate the production of NADPH and glutathione, which are the major antioxidants responsible for mitochondrial ROS homeostasis. As in the case of *PHGDH*, loss of *SLC7A11* or *GCLM* expression blocks hypoxia-induced BCSC enrichment. Whereas HIF-1 alone controls expression of LDHA and PDK1, both HIF-1 and HIF-2 regulate *SLC7A11*, *GCLM*, and the genes encoding SSP and mitochondrial 1CM enzymes [34,35].

7. Perspective

ROS act as a double-edged sword: low levels of ROS induce signaling pathways that support normal physiological processes, whereas high levels of ROS are toxic. Redox status might be a potential therapeutic target, but targeting ROS would have to be context dependent. For example, abrogating ROS-mediated signaling might reduce primary tumor growth, but mouse models of breast cancer and melanoma suggest that abrogating oxidative stress promotes metastasis [36,37]. An alternative therapeutic approach is to inhibit HIFs, which control redox balance. HIF inhibitors block the enrichment of BCSCs that occurs in response to hypoxia [34,38–41] or chemotherapy [34,35,42] and also block hypoxia-induced expression of many genes that play critical roles in the metastatic process [14].
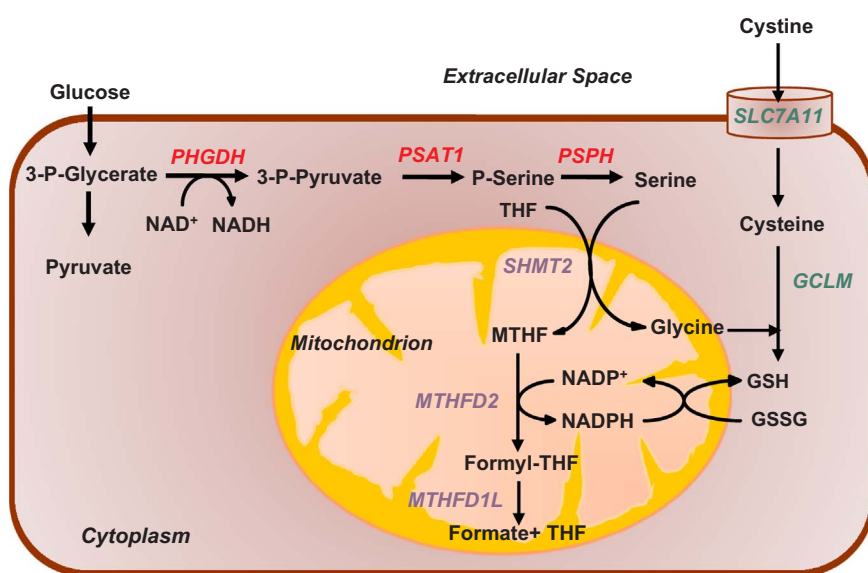
Fig. 4. Regulation of antioxidant production by HIF-1. Under hypoxic conditions, HIFs activate the transcription of *PHGDH*, *PSAT1*, and *PSPH* (red) to increase conversion of glucose to serine (serine synthesis pathway); and *SHMT2*, *MTHFD2*, and *MTHFD1L* (purple) to increase generation of mitochondrial NADPH (by mitochondrial one-carbon metabolism), which is required to convert glutathione from oxidized (GSSG) to reduced (GSH) form to protect against increased ROS generated by the ETC. Under hypoxic conditions, HIFs also activate the transcription of *SLC7A11* and *GCLM* (green) to increase glutathione production. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Disclosures

None.

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