

Review

The updated biology of hypoxia-inducible factor

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Oxygen is essential for eukaryotic life and is inextricably linked to the evolution of multicellular organisms. Proper cellular response to changes in oxygen tension during normal development or pathological processes, such as cardiovascular disease and cancer, is ultimately regulated by the transcription factor, hypoxia-inducible factor (HIF). Over the past decade, unprecedented molecular insight has been gained into the mammalian oxygen-sensing pathway involving the canonical oxygen-dependent prolyl-hydroxylase domain-containing enzyme (PHD)–von Hippel-Lindau tumour suppressor protein (pVHL) axis and its connection to cellular metabolism. Here we review recent notable advances in the field of hypoxia that have shaped a more complex model of HIF regulation and revealed unique roles of HIF in a diverse range of biological processes, including immunity, development and stem cell biology.

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The canonical HIF pathway

Oxygen-dependent regulation of HIF signalling

Hypoxia-inducible factor (HIF) is a DNA-binding transcription factor that associates with specific nuclear cofactors under hypoxia to transactivate a myriad of genes to trigger various adaptive responses to compromised oxygen tension. HIF is a heterodimer comprising an oxygen-labile α -subunit (HIF α) and a constitutively expressed β -subunit (HIF β or ARNT). Both subunits are part of the basic Helix–Loop–Helix PER–ARNT–SIM (bHLH–PAS) family of transcription factors. Three genes encoding distinct HIF α isoforms exist in humans: *HIF1A*, encoding HIF1 α ; *EPAS1*, encoding HIF2 α ; and *HIF3A*, which is expressed as multiple HIF3 α splice variants (Wang *et al*, 1995; Makino *et al*, 2002; Maynard *et al*, 2003). HIF1 α , HIF2 α , and HIF3 α splice variants 1–3 possess an oxygen-dependent degradation domain (ODD)

and an N-terminal transactivation domain (NTAD), while HIF1 α and HIF2 α possess a C-terminal transactivation domain (CTAD; Jiang *et al*, 1997; Pugh *et al*, 1997; Gu *et al*, 1998; Huang *et al*, 1998; Ema *et al*, 1999; O'Rourke *et al*, 1999; Hara *et al*, 2001; Maynard *et al*, 2003). In this review, HIF isoforms will be specified where appropriate, while 'HIF α ' will be used to denote either HIF1 α or HIF2 α .

Under normoxia (normal oxygen tension), HIF α is hydroxylated on at least one of two conserved proline residues within the ODD (Ivan *et al*, 2001; Jaakkola *et al*, 2001; Masson *et al*, 2001) by prolyl-hydroxylase domain (PHDs)-containing enzymes (Epstein *et al*, 2001). Hydroxy-HIF α is recognized by the β -domain of von Hippel-Lindau tumour suppressor protein (pVHL) and is subsequently ubiquitinated by the Elongin BC/Cul2/pVHL ubiquitin–ligase complex assembled via the pVHL α domain, thereby marking HIF α for degradation by the 26S proteasome (Maxwell *et al*, 1999; Ohh *et al*, 2000). PHDs require oxygen for catalytic activity; thus, prolyl hydroxylation of HIF α is abrogated under hypoxia, allowing HIF α to escape recognition by the pVHL ubiquitin–ligase complex and accumulate in the nucleus. Similarly, biallelic inactivation of *VHL* has a profound stabilizing effect on HIF α protein levels, thereby implicating pVHL as a predominant HIF α antagonist. Notably, another hydroxylase-domain protein termed factor inhibiting HIF (FIH) participates in the negative regulation of HIF α by hydroxylating asparagine-803 in the CTAD in the presence of oxygen, which sterically inhibits interactions between HIF α and transcriptional coactivators (Lando *et al*, 2002).

Activation of HIF in hypoxia

Hypoxia is defined in the context of tumours as having an internal partial pressure of oxygen of less than 10–15 mm Hg (Brizel *et al*, 1999; Khan *et al*, 2012). In hypoxic conditions or in *VHL*^{−/−} cells, stabilized HIF α dimerizes with HIF β before binding to hypoxia-response elements (HREs) comprising a core 5'-[A/G]CGTG-3' consensus sequence and highly variable flanking sequences in the promoters of HIF-responsive genes (Wenger *et al*, 2005). FIH-mediated asparagine-803 hydroxylation is also reduced when oxygen is depleted, thereby allowing HIF α to interact with the transcriptional coactivators p300/Creb-binding protein (CBP; Arany *et al*, 1996). This transcriptional complex transactivates a specific subset of genes that regulate the cellular adaptive response to hypoxia, including, but not limited to, *SLC2A1* (glycolysis), *VEGFA* (angiogenesis), and *EPO* (erythropoiesis).

Expanding the canonical HIF pathway

SIRT3 is a novel HIF1 α antagonist

Regulation of HIF α stability via ROS. Oxygen tension and the functional status of pVHL are just two of many factors governing HIF α stability. HIF α protein levels are in part a

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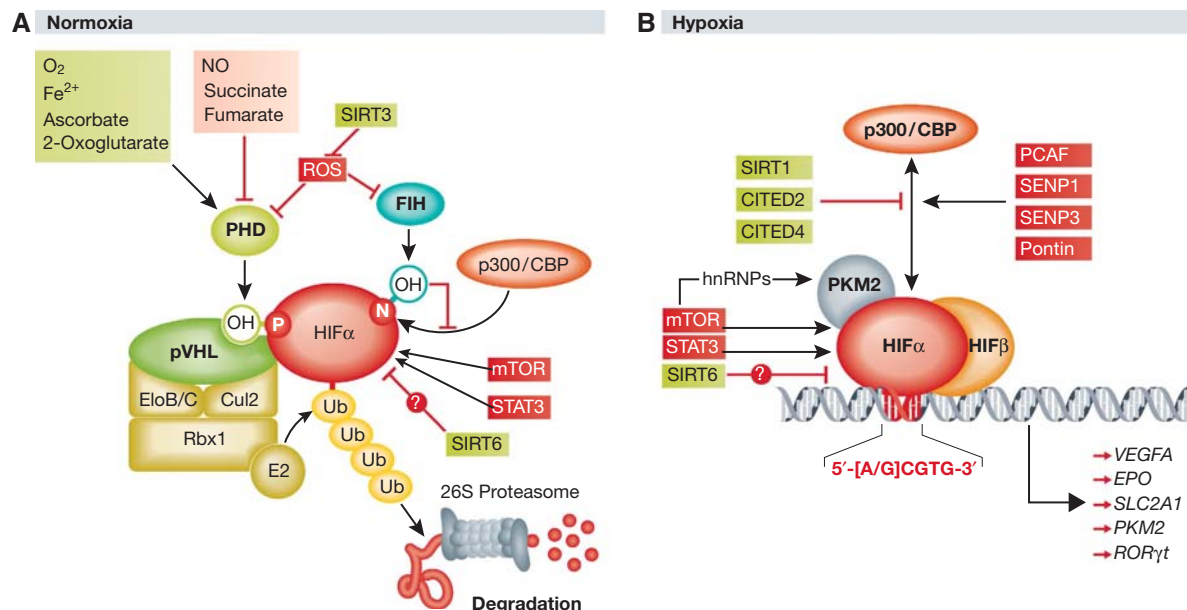


Figure 1 Expanded model of canonical HIF α regulation. **(A)** Under normal oxygen tension, HIF α is subject to oxygen-dependent prolyl hydroxylation by PHDs, which allows for substrate recognition and ubiquitylation by pVHL and its associated ubiquitin–ligase complex. Polyubiquitylated HIF α is degraded by the 26S proteasome. The prolyl-hydroxylase activity of PHDs is regulated by a number of intracellular factors, including ROS, which are in turn negatively modulated by SIRT3. Binding of the HIF α coactivator p300/CBP is inhibited by asparaginyl hydroxylation by FIH. HIF α is upregulated at the mRNA level by mTOR and STAT3, while SIRT6 negatively regulates HIF α protein levels. **(B)** Under low oxygen tension HIF α escapes prolyl hydroxylation by PHDs and associates with nuclear HIF β . The heterodimer binds to a core consensus sequence at the promoters of HIF-responsive genes, and upon binding to the coactivators p300/CBP and PKM2, initiates transcription. The interaction between HIF α and p300 may be regulated by a variety of factors that sterically impede binding or add/remove PTMs to influence the transcriptional activity of HIF α . See text for details (PHD, prolyl-hydroxylase domain-containing enzyme; NO, nitric oxide; SIRT1/3/6, sirtuin 1/3/6; FIH, factor inhibiting HIF; CBP, Creb-binding protein; OH, hydroxyl group; mTOR, mammalian target of rapamycin; STAT3, signal transducer and activator of transcription 3; ub, ubiquitin moiety; EloB/C, elongins B and C; Cul2, cullin 2; Rbx1, RING-box protein 1; pVHL, von Hippel-Lindau protein; ROS, reactive oxygen species; HIF, hypoxia-inducible factor; CITED2/4, CBP/p300 interacting transactivator with ED-rich tail 2/4; PCAF, p300/CBP-associated factor; SENP1/3, sentrin-specific protease 1/3; PKM2, pyruvate kinase isoform M2; hnRNPs, heterogeneous nuclear ribonucleoproteins).

function of HIF α mRNA stability, which can be negatively regulated by miRNAs (Bruning *et al*, 2011; Taguchi *et al*, 2008) and mRNA-destabilizing proteins (Chamboredon *et al*, 2011). Post-translational modifications (PTMs) of HIF α , such as small ubiquitin-like modifier (SUMO)ylation (Carbia-Nagashima *et al*, 2007; Cheng *et al*, 2007) and acetylation (Xenaki *et al*, 2008; Dioum *et al*, 2009; Lim *et al*, 2010), have been reported to affect HIF α stability in a proteasome-dependent manner. PHDs negatively regulate HIF α at the protein level via oxygen-dependent prolyl hydroxylation as described above, but the catalytic activity of PHDs is in turn governed by a variety of inhibitors and/or cofactors in the cellular environment (Figure 1A).

In addition to oxygen, PHDs require Fe²⁺, 2-oxoglutarate, and ascorbate for prolyl-hydroxylase activity (Schofield and Ratcliffe, 2004). In contrast, the enzymatic function of PHDs has been reported to be inhibited by nitric oxide, several metabolic intermediates of the tricarboxylic acid (TCA) cycle such as succinate and fumarate, and reactive oxygen species (ROS; Kaelin and Ratcliffe, 2008). The inverse relationship between prolyl-hydroxylated HIF α and intracellular ROS had been reported by independent groups (Brunelle *et al*, 2005; Mansfield *et al*, 2005) prior to the demonstration that peroxide-derived ROS directly inhibited PHD catalytic activity, presumably by oxidizing PHD-bound Fe²⁺ (Pan *et al*, 2007). However, the relationship between ROS production and HIF α activity has become increasingly

complex as FIH was recently demonstrated to have significantly greater sensitivity to oxidative stress than PHDs (Masson *et al*, 2012), and respiring mitochondria have been reported to produce significantly lower ROS during hypoxia than under normoxia (Hoffman *et al*, 2007). Thus, the specific role and relative significance of ROS in mediating the hypoxic response remain unclear.

SIRT3 destabilizes HIF1 α in a PHD-dependent manner. The mitochondrial deacetylase sirtuin-3 (SIRT3) was reported to have a tumour suppressor function when SIRT3^{-/-} cells were observed as having aberrant intracellular metabolism, decreased mitochondrial integrity, and a tumourigenic phenotype relative to wild-type cells (Kim *et al*, 2010). However, the specific signalling axis through which these phenomena were occurring remained unclear. Finley *et al* (2011a) recently reported that SIRT3 destabilizes HIF1 α by inhibiting ROS production, thereby promoting maximal prolyl-hydroxylase activity of PHDs. The net effect of SIRT3-mediated HIF α destabilization was a shift away from glycolytic metabolism and unchecked proliferation. Specifically, SIRT3-knockout mouse embryonic fibroblasts (MEFs) demonstrated increased glucose uptake, lactate production, and glucose-dependent proliferation rates relative to wild-type cells. Gene set enrichment analysis revealed a hypoxic signature in SIRT3-knockout MEFs, prompting an investigation into the role of SIRT3 in HIF α -mediated transactivation of proglycolytic

genes. Stable knockdown or knockout of SIRT3 resulted in an intracellular increase in ROS production, which appeared to have a stabilizing effect on HIF1 α protein levels under normoxia. This effect was shown to be due at least in part to a decrease in PHD hydroxylase activity, although direct inhibition of PHDs by ROS in this context was not clearly demonstrated. Importantly, stable knockdown of HIF1 α in both SIRT3^{-/-} and wild-type MEFs diminished the metabolic differences previously observed between them. Finally, SIRT3 deletions were found to occur in 20% of all human cancers and 40% of all breast and ovarian cancers. Together, these findings confer a specific tumour suppressor function of SIRT3 that counteracts the switch to anaerobic metabolism under normoxia, referred to as the Warburg effect, promoted by ROS generation and HIF1 α stabilization.

An independent study supports the conclusion that loss of SIRT3 stabilizes HIF1 α , perhaps through ROS production, and also demonstrates that expression of some non-metabolic HIF1 α -target genes, such as *VEGFA*, increases with knockdown of SIRT3 (Bell *et al*, 2011). This suggests that SIRT3 may oppose additional HIF1 α -driven cancer phenotypes beyond the Warburg effect, such as angiogenesis, apoptotic resistance or cellular migration/invasion.

SIRT3 suppresses ROS production. SIRT3 is a predominant mitochondrial deacetylase and is responsible for regulating the activities of a significant subset of mitochondrial proteins, many of which are strongly implicated in cellular metabolism (Lombard *et al*, 2007). SIRT3 likely suppresses ROS production through several proposed mechanisms, including but not necessarily limited to deacetylation and activation of the antioxidant enzyme superoxide dismutase (SoD; Qiu *et al*, 2010; Tao *et al*, 2010), modulation of certain electron transport chain (ETC) components (Ahn *et al*, 2008; Kim *et al*, 2010), and activation of several TCA cycle enzymes (Bell and Guarente, 2011). Specifically, deacetylation and activation of isocitrate dehydrogenase 2 (IDH2) by SIRT3 was reported to promote the production of NADPH, which is requisite for proper function of mitochondrial ROS detoxifying enzymes (Someya and Yu, 2010). In addition, SIRT3 was shown to deacetylate and activate succinate dehydrogenase (SDH), a component of the ETC, which could potentially affect ROS production (Cimen *et al*, 2010; Finley *et al*, 2011b). SDH also catalyses the oxidation of succinate to fumarate in the TCA cycle; thus, SIRT3 may negatively regulate intracellular succinate levels (Finley *et al*, 2011b). Succinate, like ROS, has been reported to inhibit PHDs (Koivunen *et al*, 2007), which suggests that intracellular accumulation of succinate in SIRT3^{-/-} MEFs contributes to the stabilization of HIF1 α via PHD inhibition (Figure 1A) as observed by Finley *et al* (2011a), although this possibility has yet to be directly addressed.

Sirtuins and the HIF pathway. SIRT3 is not the first sirtuin to be implicated in HIF α regulation (Figure 1B). Sirtuins are NAD⁺-dependent protein deacetylases and/or ADP-ribosyl-transferases known to modulate longevity and metabolism (Imai *et al*, 2000; Tissenbaum and Guarente, 2001). SIRT1 has been reported to deacetylate both HIF1 α (Lim *et al*, 2010) and HIF2 α (Dioum *et al*, 2009), causing a decrease and, unexpectedly, an increase in transcriptional activity, respectively. SIRT1, in turn, was shown to be upregulated

by both HIF1 α and HIF2 α under hypoxia, which suggests that SIRT1 may play a role in negative- and positive-feedback loops that govern HIF α activity (Chen *et al*, 2011). In contrast, SIRT6 may negatively regulate HIF1 α stability and protein synthesis via yet-defined mechanism, and may also serve as a HIF1 α corepressor to impede the induction of HIF1 α -responsive genes (Zhong *et al*, 2010). Thus, SIRT3 is currently the only sirtuin family member that has been demonstrated to destabilize HIF1 α through a PHD-dependent mechanism.

PKM2 is a novel HIF1 α coactivator

HIF α -mediated transactivation of target genes is modulated by the relative abundance of transcriptional coactivators and/or corepressors, as well as PTMs that affect the physical accessibility of, or affinity between, HIF α and its cofactors (Figure 1B). For example, de-SUMOylation of p300 by sentrin-specific protease (SEN)3 under conditions of oxidative stress enhances binding between p300 and HIF1 α , thereby increasing HIF1 α transcriptional activity (Huang *et al*, 2009). Acetylation of lysine-674 on HIF1 α by p300/CBP-associated factor (PCAF) also enhances binding between p300 and HIF1 α , while deacetylation of the same residue by SIRT1 impedes p300 recruitment and transactivation of HIF1 α -target genes (Xenaki *et al*, 2008; Lim *et al*, 2010). CBP/p300 interacting transactivator with ED-rich tail (CITED)2 and CITED4 have been shown to bind p300/CBP at the same domain required for binding to HIF α , effectively blocking the HIF1 α /p300 interaction and diminishing HIF1 α activity (Bhattacharya *et al*, 1999; Fox *et al*, 2004). Recently, the chromatin remodelling factor Pontin was demonstrated to be a HIF1 α coactivator by mediating the binding between p300 and HIF1 α under hypoxia (Lee *et al*, 2011). Together, these reports demonstrate that the transcriptional activity of HIF α can be efficiently regulated by modulating recruitment of p300 and other cofactors to the HIF α transcriptional complex.

PKM2 enhances HIF1 α transcriptional activity. Recently, Luo *et al* (2011) identified a novel role for the M2 isoform of pyruvate kinase (PKM2), the enzyme that catalyses the final step in glycolysis, as a coactivator of HIF1 α and HIF2 α (Figure 1B). PKM2 expression was induced by HIF1 α , but not HIF2 α , suggesting PKM2 and HIF1 α participate in a positive-feedback loop that increases their respective activities under hypoxia. PKM2 was demonstrated to associate directly with HIF1 α and HIF2 α , enhance binding of HIF1 α and HIF2 α to HREs, and promote HIF1 α - and HIF2 α -mediated transactivation of hypoxia-inducible genes without affecting total HIF α levels. This effect was purportedly enhanced by prolyl hydroxylation of PKM2 by PHD3, and PHD3 was shown to coprecipitate with exogenous HIF1 α /PKM2 complex. While the notion that PHD3, a negative regulator of HIF α protein levels, may be implicated in the positive regulation of HIF α activity is certainly interesting, further study is necessary to more clearly define the function of PHD3 in PKM2-mediated coactivation of HIF1 α . Importantly, PKM2 also appeared to bind p300 (although perhaps indirectly), and stable knockdown of PKM2 reduced p300 occupancy of HREs, suggesting PKM2 enhances recruitment of p300 to the HIF α transcriptional complex at the promoters of HIF α -responsive genes. These results indicate that PKM2 promotes a shift toward anaerobic metabolism by augmenting

HIF α -mediated transactivation of proglycolytic genes such as *LDHA* and *SLC2A1*.

An independent report corroborated the finding that HIF α upregulates PKM2, and that this appears to promote anaerobic metabolism in tumour cells (Sun *et al*, 2011). It was also suggested that mammalian target of rapamycin (mTOR) upregulates PKM2 via two distinct mechanisms: mTOR-mediated upregulation of HIF α may lead to transactivation of PKM2, while concomitantly, mTOR-mediated upregulation of c-myc may lead to c-myc-mediated upregulation of heterogeneous nuclear ribonucleoproteins (hnRNPs), which are reported to favour splicing of PKM2 mRNA over PKM1 mRNA (David *et al*, 2010; Sun *et al*, 2011). Thus, in addition to upregulating HIF α , mTOR may modulate HIF α signalling by upregulating the HIF α coactivator PKM2 (Figure 1B).

Role of PKM2 in the Warburg effect. PKM2 has been previously implicated in the Warburg effect observed in cancer cells (Christofk *et al*, 2008), although its specific role in promoting aerobic glycolysis has remained unclear. Recent work suggests, perhaps counter-intuitively, that PKM2 promotes glycolysis in tumour cells in a manner that is independent of its biochemical function as a glycolytic enzyme. Phosphorylation of PKM2 at tyrosine-105 (Y105) inhibits its glycolytic activity, and PKM2 was found to be phosphorylated at Y105 in several cancer cell lines (Hitosugi *et al*, 2009). Furthermore, cells expressing a PKM2 mutant defective in Y105 phosphorylation—that is, a PKM2 mutant possessing greater glycolytic activity than wild-type PKM2—exhibited increased oxidative phosphorylation, decreased lactate production, and slowed tumour growth relative to cells expressing wild-type PKM2 (Hitosugi *et al*, 2009). These data imply that the enzymatic function of PKM2 actually opposes the Warburg effect. Indeed, Luo *et al* (2011) demonstrate that a catalytically inactive form of PKM2 promotes anaerobic metabolism via HIF α transactivation as efficiently as wild-type PKM2. Together, these studies conjure a potential model of metabolic reprogramming in tumour cells whereby PKM2 undergoes a functional shift from glycolytic enzyme to HIF α coactivator.

PKM2 as a transcriptional coactivator. PKM2 has also been reported to act as a transcriptional coactivator for β -catenin (Yang *et al*, 2011). Specifically, activation of epidermal growth factor receptor (EGFR) was shown to trigger nuclear translocation of PKM2, where it appeared to bind phosphorylated β -catenin and subsequently potentiate β -catenin-mediated transactivation of cyclin D1 (Yang *et al*, 2011). Thus, while PKM2 purportedly promotes glycolysis as a HIF α coactivator (Luo *et al*, 2011), it may also promote cellular proliferation and cell cycle progression via β -catenin-mediated upregulation of cyclin D1 (Yang *et al*, 2011). Therefore, the tumour phenotype to which PKM2 contributes may depend upon the specific transcription factor being activated and its particular subset of target genes. Given the findings reported by Yang *et al*, (2011) and considering that PKM2 must be cytoplasmic to participate in glycolysis, it would seem likely that a nuclear translocation of PKM2 is necessary and precedes coactivation of HIF α . Future work on the role of PKM2 in HIF α -mediated transcription might address this possibility.

Expanding roles of HIF in cell biology

The role of HIF α as a transcription factor in the context of oxygen sensing and tumorigenesis has been extensively characterized. While the aforementioned reports serve to extend our current understanding of HIF α regulation in cell signalling, emerging evidence has implicated a larger role for HIF α in cell biology.

Role of HIF in embryonic development

HIF1 α drives a metabolic shift to glycolysis during ESC–EpiSC transition. Analogous to the Warburg effect observed in cancer cells, Zhou *et al* (2012) have recently shown that pluripotent embryonic stem cells (ESCs) also undergo a metabolic shift towards glycolysis during development and this process is regulated by HIF1 α . ESCs and epiblast stem cells (EpiSCs) represent two distinct pluripotency stages in embryonic development with unique gene expression profiles and cell morphologies, but a common ability to self-renew and differentiate into diverse cell types (Tesar *et al*, 2007). Mouse (m)ESCs isolated from the inner cell mass (ICM) of pre-implantation blastocysts represent a naive state, while EpiSCs isolated from the post-implantation epiblast represent a more developmentally advanced or ‘primed’ state (Brook and Gardner, 1997; Evans and Kaufman, 1981; Tesar *et al*, 2007; Nichols and Smith, 2009). Notably, unlike mESCs, human (h)ESCs, despite their pre-implantation origin, are not considered a naive state and are similar to EpiSCs (Tesar *et al*, 2007). Recent work by Zhou *et al* (2012) revealed that EpiSCs and hESCs have lower mitochondrial respiration and higher levels of glycolysis in comparison to mESCs, which switch between both oxidative phosphorylation and glycolysis (Figure 2). Strikingly, inhibition of glycolysis with the glucose analogue 2-deoxy-glucose (2-DG) led to cell death in EpiSCs and hESCs, exemplifying their dependence on this metabolic pathway for survival (Zhou *et al*, 2012). Similarly, haematopoietic stem cells (HSCs) have been recently shown to utilize glycolysis instead of mitochondrial respiration, suggesting that HIF α may regulate the metabolism of multiple stem cell populations (Simsek *et al*, 2010).

HIF1 α has previously been shown to promote a metabolic switch to glycolysis in response to chronic hypoxia through the transcriptional regulation of key metabolic enzymes such as pyruvate dehydrogenase kinase-1 (PDK1) and lactate dehydrogenase A (LDHA) (Kim *et al*, 2006; Seagroves *et al*, 2001). PDK1 phosphorylates and inactivates pyruvate dehydrogenase (PDH), which inhibits the conversion of pyruvate to acetyl coenzyme A (AcCoA) for entry into the citric acid cycle (Semenza, 1996). In addition, HIF1 α -mediated upregulation of LDHA increases the conversion of pyruvate to lactate to promote glycolysis (Semenza *et al*, 1996). Consistent with the reported role of HIF1 α in regulating metabolism, Zhou *et al* (2012) found that HIF1 α expression and several HIF-regulated glycolytic genes such as *PDK1*, *LDHA*, and *PYGL* were significantly higher in EpiSCs compared with mESC (Zhou *et al*, 2012). Furthermore, despite their more developed mitochondria and increased mitochondrial content, EpiSC had lower mitochondrial respiration that was attributed to downregulated expression of mitochondrial *cytochrome c oxidase* (COX) genes and their regulators peroxisome proliferator-activated receptor- γ coactivator-1 β (PGC-1 β) and oestrogen receptor-related

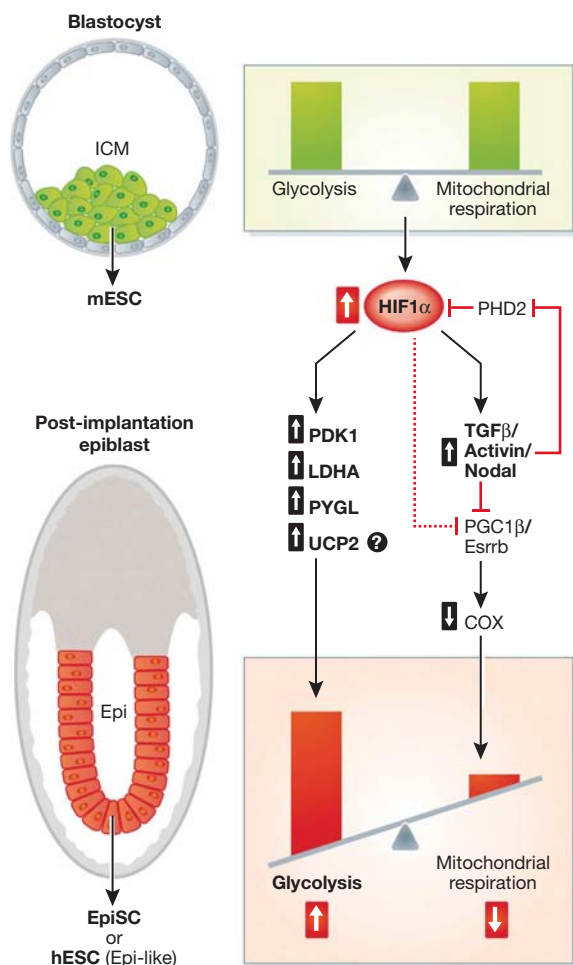


Figure 2 HIF1 α regulates the metabolic and phenotypic transition from ESC to EpiSC. mESC isolated from the inner cell mass (ICM) of the blastocyst are metabolically and phenotypically distinct from post-implantation EpiSC isolated from the epiblast (Epi) or hESC (Epi-like). HIF1 α expression regulates the metabolic and phenotypic transition from ESC to EpiSC. Increased HIF1 α expression in EpiSC/hESC promotes a highly glycolytic metabolism via increased expression of glycolytic genes such as *PDK1*, *LDHA*, and *PYGL* and downregulation of mitochondrial *COX* gene expression via TGF β /Activin/Nodal signalling.

receptor- β (*Esrrb*) (Zhou *et al*, 2012). HIF1 α has previously been shown to downregulate PGC-1 β through inhibition of c-Myc transcriptional activity (Zhang *et al*, 2007), suggesting that HIF1 α may actively suppress mitochondrial respiration while promoting glycolysis through the regulation of key metabolic genes (Figure 2). In support of this notion, Zhou *et al* (2012) demonstrated that overexpression of HIF1 α was sufficient to suppress oxidative phosphorylation, enhance glycolysis, and promote the transition from ESC to EpiSC.

Similarly, independent studies have shown that in comparison to differentiated cells, undifferentiated mESC have decreased mitochondrial respiration concomitant with increased glycolytic metabolism, which is necessary to maintain pluripotency (Mandal *et al*, 2011; Zhang *et al*, 2011). While Zhang *et al* suggest that expression of the inner mitochondrial protein, uncoupling protein 2 (UCP2), is responsible for the glycolytic shift in undifferentiated hESC, several reports have demonstrated a positive correlation between HIF α and UCP2 expression (Zhang *et al*, 2011;

Deng *et al*, 2012). In addition, putative HIF binding sites have been identified in the *UCP2* promoter (Oberkofler *et al*, 2005), suggesting that HIF α may control the expression of UCP2 and several other glycolytic and mitochondrial proteins to promote a universal metabolic shift.

Significance of glycolysis in EpiSC/hESC. Several theories have been proposed to explain the preference for glycolysis in EpiSC/hESC. Although glycolysis is energetically unfavourable in comparison with oxidative phosphorylation (yielding a mere 2 ATP in comparison with 36 ATP per glucose), this metabolic shift helps to promote cell proliferation through increased production of metabolic intermediates for amino acid, protein, and membrane synthesis. This may allow the developing blastocyst to acquire the necessary building blocks for rapid proliferation and differentiation. Second, oxidative phosphorylation is associated with the production of ROS, a known DNA mutagen. Thus, a shift to glycolytic metabolism may be a protective mechanism for the cell to maintain genomic integrity within pluripotent cells. Third, glycolysis promotes extracellular acidification through increased production of lactic acid, which may aid in invasion and implantation of the blastocyst into the uterine wall.

HIF maintains pluripotency in ESC through TGF β /Activin/Nodal signalling. Analogous to undifferentiated HSCs, which have recently been shown to regulate quiescence and long-term maintenance through the regulation of HIF α expression levels, ESC may maintain pluripotency through a HIF-dependent mechanism (Takubo *et al*, 2010). TGF β /Activin/Nodal signalling has previously been shown to drive the ESC to EpiSC transition and maintain pluripotency in EpiSC and hESC by preventing spontaneous differentiation into neuroendoderm (Vallier *et al*, 2004; James *et al*, 2005; Camus *et al*, 2006). Activin B is a direct transcriptional target of HIF α , and hypoxia has recently been shown to promote an ESC to EpiSC transition, suggesting a role for HIF α in not only the metabolic changes, but also the phenotypic changes that occur during ESC development (Wacker *et al*, 2009; Takehara *et al*, 2011). In support of these previous findings, Zhou *et al* (2012) found that inhibition of Activin signalling was sufficient to block the HIF-induced transition from ESC to EpiSC. Furthermore, Activin signalling has been shown to decrease PHD2 and stabilize HIF1 α , suggesting that a positive-feedback loop may exist to further amplify signalling (Wiley *et al*, 2010; Figure 2). In support of this notion, Zhou *et al* (2012) found that activation of Activin signalling increased both HIF1 α expression and glycolytic genes, while downregulating mitochondrial genes. Interestingly, PGC-1 β has been shown to be directly downregulated by Activin/Nodal signalling, providing additional evidence to support a role for Activin/Nodal signalling in the active suppression of mitochondrial metabolism (Li *et al*, 2009). These results infer a novel mechanism by which HIF regulates Activin/Nodal signalling to alter the expression of key metabolic genes, promoting a highly glycolytic metabolism to promote the ESC to EpiSC transition (Figure 2).

Regulation of HIF during early embryonic development. Both the pre-implantation and post-implantation blastocysts exist in the hypoxic environment of the uterus (Fischer and

Bavister, 1993). However, Zhou *et al* (2012) observed considerable differences in the expression level of HIF between these two pluripotent states. Despite the ability of the post-implantation blastocyst to access the vasculature of the surrounding tissue, the EpiSC had higher HIF1 α expression. This apparent contradiction warrants further research into the mechanisms underlying HIF expression in pre- and post-implantation ESCs. Does the post-implantation blastocyst experience lower oxygen tension in comparison to the pre-implantation ESC or are there other unique or non-canonical mechanisms of HIF regulation at play during early embryonic development? Earlier work by Fischer and Bavister (1993) demonstrated that hESC utilize glycolysis for metabolism even when removed from the hypoxic environment of the blastocyst. These results indicate that factors other than oxygen tension may regulate HIF α levels (discussed previously) to alter glucose metabolism and promote ESC to EpiSC transition.

The metabolism of HSCs was recently shown to be controlled by the haematopoietic transcription factor, Meis1, which directly activates HIF1 α expression (Simsek *et al*, 2010). These findings suggest that the high expression of HIF1 α and shift to glycolytic metabolism is not just a result of the hypoxic environment of the bone marrow, but is tightly regulated by haematopoietic stem-cell-associated transcription factors. Whether or not unique ESC-associated transcription factors regulate HIF α in ESC remains to be determined.

Role of HIF in immunity

Inflamed tissues are often hypoxic as a result of decreased perfusion, oedema, vascular insult and/or influx of oxygen-consuming immune cells or pathogens (Nizet and Johnson, 2009). Thus, the role of HIF α in host immune responses and inflammation has become an important research focus. HIF α expression is stabilized through a variety of mechanisms in a range of immune cell types (Nizet and Johnson, 2009). For instance, recognition of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) by toll-like receptors (TLRs) in human monocytes was reported to activate the mitogen-activated protein kinase (MAPK) pathway and ultimately NF- κ B, thereby transactivating *HIF1A* and producing an abundance of HIF1 α mRNA and protein under normal oxygen tension (Frede *et al*, 2006). In addition to canonical stabilization of HIF α protein levels, hypoxia was demonstrated to prohibit PHD-mediated inhibition of I κ B kinase- β (IKK- β) (Cummins *et al*, 2006), thereby leading to activation of NF- κ B and subsequent upregulation of *HIF1A* as demonstrated in murine macrophages (Rius *et al*, 2008). The mechanism of HIF α stabilization in T cells appears to be analogous to that in myeloid cells, as engagement of T cell receptors (TCRs) by foreign antigens upregulates HIF1 α via phosphoinositol-3-kinase (PI3K)/mTOR signalling pathway (Nakamura *et al*, 2005). Together, these reports demonstrate that stabilization of HIF α in the context of inflammation and immunity can occur through multiple signalling cascades in both normoxic and hypoxic environments.

Following stabilization, HIF1 α plays an important role in executing the immune response. Metabolic reprogramming in myeloid cells by HIF1 α was shown to increase the production of glycolysis-derived ATP, a critical energy source for macrophage aggregation, invasion, and motility (Cramer

et al, 2003). In addition, HIF1 α -mediated upregulation of β -integrin was reported to enhance neutrophil adhesion to activated endothelium (Kong *et al*, 2004). Neutrophil apoptosis was shown to be significantly attenuated in hypoxia by HIF1 α (Walmsley *et al*, 2005) and more recently, PHD3 (Walmsley *et al*, 2011), although whether this latter effect occurs in a HIF1 α -dependent or -independent manner remains unclear. HIF α also contributes to the production of a number of proinflammatory cytokines such as TNF α , interleukin (IL)-1 α , IL-1 β , IL-6, and IL-12, which are necessary for macrophage activation and phagocytic function, as well as lymphocyte proliferation (Peyssonnaud *et al*, 2005, 2007; Jantsch *et al*, 2008; Nizet and Johnson, 2009). In some cases, such as TNF α , HIF α -mediated upregulation occurs as a result of direct transactivation. While the aforementioned studies might suggest that HIF α acts predominantly as a proinflammatory factor, it is worth noting that HIF1 α has been demonstrated to have a robust anti-inflammatory effect in hypoxic gastrointestinal mucosa and epithelium as a result of transactivation of specific genes that have a 'protective-barrier' effect, such as mucins (Furuta *et al*, 2001; Comerford *et al*, 2002; Louis *et al*, 2006). This effect has also been successfully demonstrated in relevant animal models (Karhausen *et al*, 2004; Cummins *et al*, 2008; Robinson *et al*, 2008). Together, these roles indicate that HIF α depends upon its transcriptional activity in directing the immune response in a wide range of cell types.

HIF1 α promotes differentiation of T_H17 cells by several distinct mechanisms. Interestingly, HIF α also appears to regulate the differentiation of multiple leukocyte lineages. *Hif1a*^{-/-} mice exhibit impaired B cell maturation, and HIF1 α has been shown to modulate this process by initiating glycolysis at specific stages of B cell differentiation (Kojima *et al*, 2002, 2010). Similarly, mTOR-mediated upregulation of HIF1 α was reported to induce differentiation of naive T cells to proinflammatory T_H17 cells, as opposed to anti-inflammatory T_{reg} cells, by promoting glycolysis (Shi *et al*, 2011). These reports further support the notion that HIF1 α contributes to B and T cell differentiation through its role as a transcription factor.

A recent report from Dang *et al* (2011) demonstrates that the effect of HIF1 α on T cell differentiation extends beyond its transcriptional activity. In T_H17-skewing conditions (IL-6 with low levels of TGF- β), signal transducer and activator of transcription 3 (STAT3) was shown to transactivate *HIF1A*. STAT3 was previously reported to positively influence T_H17 cell differentiation, presumably by upregulating the key T_H17 transcriptional regulator ROR γ t (Harris *et al*, 2007), but HIF1 α had not been previously implicated in this mechanism. Dang *et al* (2011) observed decreased expression of T_H17 signature genes such as *IL-17* in murine *Hif1a*^{-/-} T cells relative to wild-type T cells, even after culturing in T_H17-skewing conditions. In addition, the proportion of *Hif1a*^{-/-} T cells that stained positive for Foxp3, the predominant transcriptional regulator of T_{reg} cells, was significantly greater than in wild-type T cells. Furthermore, incubation of T_H17-skewed wild-type T cells in hypoxia increased the IL-17⁺ population, but hypoxic treatment had no effect on *IL-17* expression in T_H17-skewed *Hif1a*^{-/-} T cells. These results imply that HIF1 α supports T_H17 cell differentiation by promoting expression of T_H17 signature genes.

Dang *et al* (2011) elucidated the precise mechanism by which HIF1 α upregulates *IL-17* by demonstrating direct transactivation of ROR γ t by HIF1 α . ROR γ t then directly induces expression of *IL-17A* and *IL-17R* in response to TGF- β and IL-6, thereby promoting differentiation of naive CD4 $^{+}$ T cells to T_H17 cells (Ivanov *et al*, 2006). Thus, these results place HIF1 α at a central point in the IL-6/TGF- β -STAT3-ROR γ t signalling cascade that drives T_H17 cell differentiation.

Upregulation of ROR γ t by HIF1 α is dependent upon the transcriptional activity of HIF1 α (Figure 3A), a mechanism that is analogous to the aforementioned modes of HIF1 α -mediated inflammation and differentiation of lymphocyte lineages. However, one key observation—that ROR γ t overexpression in *Hif1a* $^{-/-}$ T cells did not significantly increase the IL-17 $^{+}$ population—suggested that HIF1 α might have additional roles in T_H17 cell differentiation downstream of ROR γ t transactivation. Intriguingly, HIF1 α and ROR γ t were shown to directly interact with one another, and induction of *IL-17* by HIF1 α did not require a functional DNA-binding domain (Dang *et al*, 2011). In fact, the binding site for ROR γ t was mapped to amino acids 1–80 on the N terminus of HIF1 α , a site that is normally required for HIF β dimerization and binding to HREs (Jiang *et al*, 1996; Dang *et al*, 2011). Dang *et al* (2011) propose a model (Figure 3B) in which HIF1 α transactivates the ROR γ t gene prior to physically associating with its protein product at the promoters of T_H17 signature genes. The purpose of HIF1 α in this transcriptional complex appears to be recruitment of p300 through its CTAD, which improves the accessibility of ROR γ t target gene promoters through p300-mediated acetylation of histones H3 and H4 (Dang *et al*, 2011).

Finally, it is proposed that HIF1 α actively suppresses T_{reg} differentiation by targeting Foxp3 for ubiquitin-mediated degradation (Figure 3C). While the identification of a possible degradation complex remains to be elucidated, downregulation of Foxp3 by HIF1 α appears to be a post-translational, proteasome-dependent phenomenon requiring direct interaction between Foxp3 and the N terminus of HIF1 α . Expression of hydroxylation-defective HIF1 α mutants, as well as siRNA-mediated knockdown of PHD2, abrogated Foxp3 degradation (Dang *et al*, 2011), suggesting prolyl hydroxylation of HIF1 α necessarily precedes Foxp3 degradation. However, ubiquitylation and degradation of Foxp3 was also shown to

significantly increase under hypoxia, a state in which HIF1 α levels increase but prolyl hydroxylation of HIF1 α by PHD2 is impeded. Therefore, further studies are required to clarify the role of prolyl-hydroxylated HIF1 α in Foxp3 degradation.

This work is particularly interesting in that it demonstrates HIF1 α promoting T_H17 cell differentiation via three unique and functionally distinct roles: a DNA-binding transcription factor, a transcriptional coactivator for ROR γ t, and a direct mediator of Foxp3 degradation (Figure 3). Notably, all three roles require the same region of HIF1 α : the HLH domain,

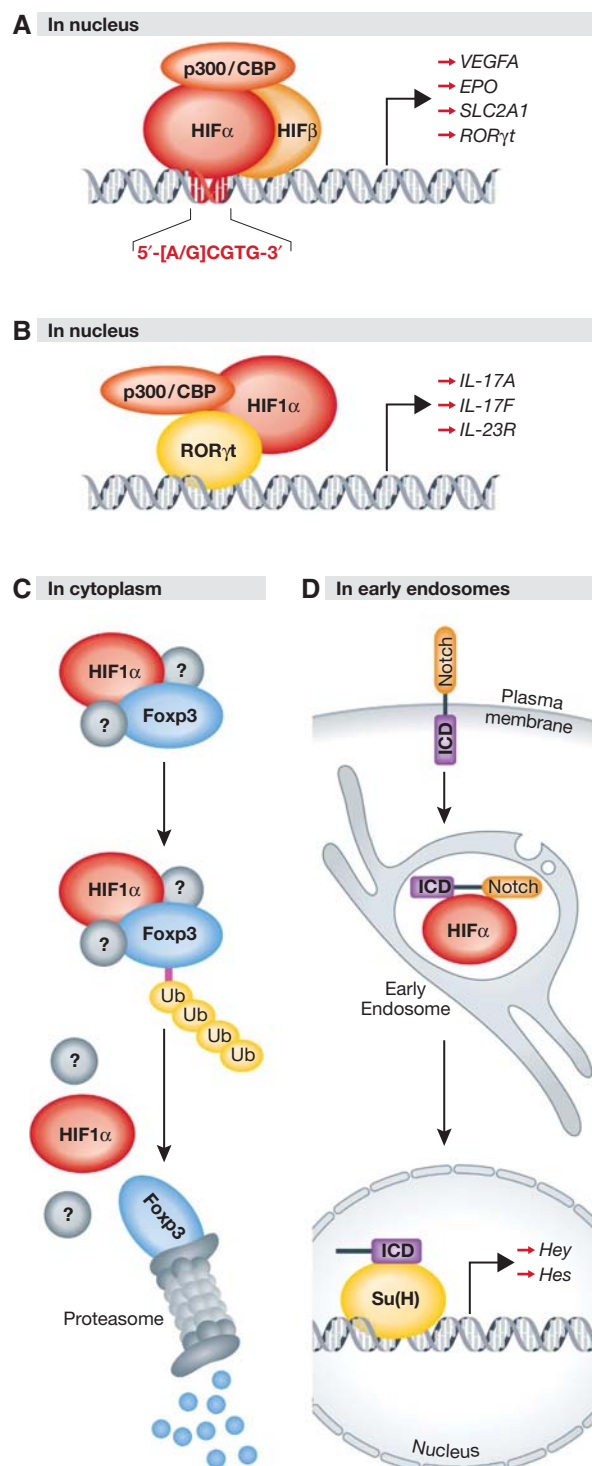


Figure 3 Canonical and non-canonical regulation by HIF α . (A) Classical DNA-binding transcription factor: HIF α associates with HIF β and transcriptional coactivators in the nucleus, binds conserved hypoxia-response elements in the DNA sequence of target promoters, and initiates transcription of HIF-target genes. (B) Transcriptional coactivator: HIF1 α binds ROR δ t, but not DNA, at the promoters of ROR δ t-responsive genes in the nucleus. Recruitment of p300 by HIF1 α leads to deacetylation of histones H3 and H4 thereby facilitating transcription. (C) Director for proteasome-mediated degradation: HIF1 α binds to Foxp3, presumably in the cytoplasm, and in association with an unidentified degradation complex mediates its ubiquitylation and subsequent proteasomal degradation. (D) Stabilizing force for endocytosed receptors: HIF α (Sima) colocalizes with and stabilizes full-length Notch that has been internalized from the plasma membrane in early endosomes. See text for details (HIF, hypoxia-inducible factor; CBP, Creb-binding protein; IL, interleukin; Ub, ubiquitin moiety; ICD, intracellular domain; Su(H), suppressor of Hairless).

which is necessary for HIF heterodimerization and DNA binding (Jiang *et al*, 1996), and was shown in this report to bind ROR γ t and Foxp3. Therefore, it is formally possible that these three functions are mutually exclusive. To better understand the dynamic shifts in HIF1 α function in T cell differentiation, it may be informative to elucidate the relative binding affinities of HIF β , ROR γ t, and Foxp3 to the HLH domain of HIF1 α .

This report also implicates HIF1 α in the T cell-mediated inflammatory response. *Hif1a*^{-/-} T cells failed to develop experimental autoimmune encephalitis in response to myelin oligodendrocyte glycoprotein peptide, whereas wild-type T cells exhibited a robust inflammatory reaction. This observation provides a rationale for investigating the use and efficacy of HIF α inhibitors in the treatment of T cell mediated autoimmune disorders.

Together, these findings outline a positive-feedback mechanism by which TGF- β and IL-6 upregulate *HIF1A* in a STAT3-dependent manner, leading to transactivation of ROR γ t and subsequently HIF1 α /ROR γ t/p300-mediated transactivation of T_H17 signature genes. Upregulation of *IL-17*, in addition to HIF1 α -mediated degradation of Foxp3, appears to favour T_H17 cell differentiation and impede the development of T_{reg} cells, thereby enhancing the inflammatory response. Ultimately, an increase in local inflammation brings about a decrease in oxygen tension, and the resulting stabilization of HIF1 α would presumably act to amplify the entire cascade.

Role of HIF in Notch-dependent developmental pathways

HIF α modulates human leukocyte differentiation via canonical and non-canonical pathways, as described above. However, HIF α can induce or suppress the differentiation of a multitude of cell types, including but not limited to adipocytes, medulloblastoma precursor cells, neuroblastoma cells, glioblastoma stem cells, and neural crest progenitors (Jogi *et al*, 2002; Yun *et al*, 2002; Sainson and Harris, 2006; Pistollato *et al*, 2010; Qiang *et al*, 2012). A number of these reports have implicated Notch, a well-established mediator of cellular differentiation processes, as an important effector.

The Notch receptor is activated upon engagement by a membrane-bound ligand, such as Serrate or Delta, on an adjacent cell (Rebay *et al*, 1991). Ligand binding triggers proteolytic cleavage of Notch, which is mediated in part by γ -secretase (De Strooper *et al*, 1999). The cleaved intracellular domain (ICD) of Notch translocates to the nucleus, and upon binding to cofactors such as p300/CBP, Suppressor of Hairless (Su(H)), and/or mastermind (MAML1), transactivates Notch-responsive genes such as *HEY* and *HES* (Fortini, 2009).

Collaboration between HIF α and Notch occurs at the intersection between the oxygen-sensing and developmental pathways. Notch has been reported to potentiate HIF α -mediated transactivation of hypoxia-inducible genes in the context of both tumourigenesis and cellular differentiation (Sahlgren *et al*, 2008; Zheng *et al*, 2008). Conversely, HIF α has been demonstrated to enhance Notch signalling through several distinct mechanisms. HIF1 α has been shown to stabilize Notch ICD in both normoxia and hypoxia in a manner that is independent of its transcriptional activity, although the precise mechanism by which this occurs remains unclear (Gustafsson *et al*, 2005). Additionally, HIF1 α transactivation of *anterior pharynx-defective 1A* (*APH-1A*), a component of

the γ -secretase complex, has been reported to lead to an increase in intracellular levels of Notch ICD (Wang *et al*, 2006). HIF1 α interaction with Notch at the *HEY2* and *HES1* promoters has also been reported, and this association augments Notch-mediated transactivation under hypoxia in a manner that appears to depend upon a functional CTAD but does not require DNA binding by HIF1 α (Gustafsson *et al*, 2005; Chen *et al*, 2010). Interestingly, Notch that is impaired in its ability to bind p300/CBP still retains the ability to transactivate target promoters under hypoxia, suggesting that the function of HIF1 α in the Notch transcriptional complex is to recruit p300/CBP (Gustafsson *et al*, 2005). This is reminiscent of the aforementioned T_H17 cell differentiation model, whereby HIF1 α recruits p300 to the ROR γ t transcriptional complex, but does not bind the *IL-17* promoter directly to induce transcription. In addition, FIH, another important component of the oxygen-sensing pathway, was reported to negatively regulate the transcriptional activity of Notch, although this effect appeared to be independent of FIH catalytic activity (Coleman *et al*, 2007; Zheng *et al*, 2008). Interestingly, FIH has a significantly higher affinity for Notch than for HIF1 α (Wilkins *et al*, 2009); thus, sequestration of intracellular FIH by Notch may mediate transcriptional repression of HIF1 α (Zheng *et al*, 2008), thereby delineating another mechanism by which Notch might augment the adaptive response to hypoxia.

Crystal cell development requires direct stabilization of endosomal Notch by HIF α . A recent report from Mukherjee *et al* (2011) proffers an entirely novel context for HIF α and Notch interaction, with each factor functioning in a starkly non-canonical manner (Figure 3D). Development and differentiation of crystal cells, a *Drosophila* blood cell subtype that is involved in wound healing, was shown to depend upon stabilization of Notch by HIF α within early endocytic vesicles. The *Drosophila* HIF α orthologue, Sima, promotes crystal cell expansion in the lymph gland, and was demonstrated to be crucial for the survival of committed crystal cell precursors. Similarly, Notch was shown to be required for initial commitment to the crystal cell lineage as well as expansion, maintenance and survival of committed crystal cell precursors. In addition, cleavage of the Notch ICD was vital for crystal cell survival, indicating an important role for Notch signalling in crystal cell development.

Sima overexpression stabilized Notch protein levels, a finding that is in keeping with previous observations regarding positive regulation of Notch signalling by HIF α . However, rather than observing an increase in nuclear levels of Notch ICD, Mukherjee *et al* (2011) found that the majority of intracellular Notch colocalized with Sima in early endocytic vesicles within crystal cells. Live endocytic trafficking assays performed on wild-type *Drosophila* lymph glands revealed that internalization of full-length Notch (Notch^{fl}) from the plasma membrane preceded its rapid degradation in all non-crystal cell types. Intriguingly, activation of Notch in committed crystal cell precursors did not appear to require ligand engagement by either Serrate or Delta, suggesting that Sima-mediated activation of Notch was occurring in a non-canonical, ligand-independent manner. This mechanism seems sensible from an evolutionary standpoint, as Notch signalling in crystal cells appears to be vital for their differentiation and survival, but given that they circulate

freely in the bloodstream it is not feasible for them to continuously engage the Notch ligands of neighbouring cells.

The function of Sima in crystal cells did not appear to include transactivation of hypoxia-responsive genes. Overexpression of the *Drosophila* HIF β orthologue Tango failed to induce expression of a Sima/Tango-responsive reporter plasmid, and hypoxic treatment only augmented crystal cell expansion. It should be noted, however, that following Notch cleavage and nuclear translocation of ICD there is a possible transcriptional role for Sima in enhancing Notch-mediated transactivation of Notch-target genes through recruitment of, and association with, other cofactors in the Notch transcriptional complex as described previously (Gustafsson *et al*, 2005; Chen *et al*, 2010).

Another non-canonical aspect of Sima function in this report involves its unusually high degree of activity in normoxic conditions. Mukherjee *et al* (2011) demonstrate elevated synthesis of nitric oxide synthase-1 (NOS1) in crystal cells, which appeared necessary for Sima stabilization under normal oxygen tension. This unique feature of crystal cells likely explains why internalized Notch is rapidly degraded in other cell types—intracellular Sima levels may not be sufficiently high under normoxia to stabilize Notch in early endosomes.

Augmentation of Notch signalling by HIF1 α was previously shown to require both hypoxic conditions and ligand (Serrate) engagement (Gustafsson *et al*, 2005). The report by Mukherjee *et al* (2011) suggests that the sole function of HIF α in crystal cells is augmentation of Notch signalling, and HIF α appears to accomplish this in large part by stabilizing Notch in a ligand-independent manner in a normoxic environment (Mukherjee *et al*, 2011). Indeed, the proposed role of HIF α in this context represents an entirely new mechanism by which it regulates differentiation and development. Future studies aimed at further characterizing this mechanism should attempt to do so in other species. *Drosophila* crystal cells are reportedly most similar to mammalian myeloid cells, thus it would be interesting to see if stabilization of Notch by HIF α occurs in the endosomes of myeloid precursors. Additional roles for HIF α in the stabilization of endosomal Notch^{fl} are also worth investigating: Mukherjee *et al* (2011) report that overexpression of Rab5 suppresses Sima/Notch-mediated crystal cell expansion by increasing endocytic turnover of Notch^{fl}. HIF α has been previously demonstrated to downregulate the Rab5 effector rapabtin-5 (Wang *et al*, 2009), thereby prolonging the endocytic pathway. However, it is unknown whether HIF α -mediated attenuation of early endosome fusion serves as a secondary mode of Notch^{fl} stabilization.

One important question left in the wake of this study pertains to the apparent lack of HIF α transcriptional activity

at HIF-target genes in crystal cells, particularly under hypoxia. Crystal cells are involved in wound healing and mediating the humoral immune response (Meister, 2004; Bidla *et al*, 2007), and are therefore likely to be presented with significant hypoxic challenge in necrotic or inflamed tissues. Given that HIF α is the key transcriptional regulator of the cellular adaptive response to hypoxia, how do crystal cells overcome the metabolic stresses that inevitably occur in low oxygen tension? One possibility is that they are not meant to. Crystal cells rupture in response to injury or inflammation and release significant amounts of prophenoloxidase, which is crucial for clotting and defence against pathogens (Meister, 2004; Tang *et al*, 2006; Bidla *et al*, 2007). Perhaps the lack of an adaptive response to hypoxic challenge is an evolutionarily-developed strategy to ensure vulnerable crystal cells rupture in inflammatory or necrotic conditions. If this is indeed the case, then the mechanism(s) responsible for attenuating the transactivation of HIF α -responsive genes in *Drosophila* crystal cells under hypoxia warrant future study.

Concluding remarks

Recent work regarding the oxygen-sensing pathway and its predominant effector, HIF α , has expanded our current understanding of HIF α regulation and transactivation, the role of HIF α and underlying genetic aberrations in health and disease, and the multifaceted nature of HIF α in a variety of physiological and pathophysiological contexts. Unexpected new players have entered into the 'canonical' model of HIF α regulation and transactivation, and have shown demonstrable influence on the hypoxic response by modulating PHD function or enhancing HIF α transcriptional activity. Furthermore, the traditional dogma regarding the function of HIF α as a hypoxia-inducible, DNA-binding transcription factor is challenged by new reports that demonstrate its capacity as a transcriptional coactivator in developmental and inflammatory contexts, a director for proteasome-mediated degradation, and a stabilizing force for internalized receptors within the endosomes. These notable studies not only provide additional research avenues, but also questions, the answers to which will undoubtedly enhance our appreciation for the increasing complexity and biological relevance of the HIF pathway in humans and other organisms.

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Conflict of interest

The authors declare that they have no conflict of interest.

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