Hypoxia: an alarm signal during intestinal inflammation

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Abstract

Intestinal epithelial cells that line the mucosal surface of the gastrointestinal tract are positioned between an anaerobic lumen and a highly metabolic lamina propria. As a result of this unique anatomy, intestinal epithelial cells function within a steep physiologic oxygen gradient relative to other cell types. Furthermore, during active inflammatory disease such as IBD, metabolic shifts toward hypoxia are severe. Studies in vitro and in vivo have shown that the activation of hypoxia-inducible factor (HIF) serves as an alarm signal for the resolution of inflammation in various murine disease models. Amelioration of disease occurs, at least in part, through transcriptional up-regulation of non-classical epithelial barrier genes. There is much recent interest in harnessing hypoxia-inducible pathways, including targeting the hypoxia-inducible factor (HIF) and the proyl-hydroxylase enzyme (which stabilizes HIF), for therapy of IBD. Here, we review the signaling pathways involved and define how hypoxia may serve as an endogenous alarm signal for mucosal inflammatory disease. We also discuss the upside and potential downsides of targeting these pathways to treat patients with IBD.

Key points

- A steep oxygen gradient exists from the anaerobic lumen of the intestine, across the intestinal epithelial barrier into the highly vascular sub-epithelium
- Loss of function of the intestinal epithelial barrier, together with hypoxia and inflammation underlie the pathology of IBD
- Hypoxia-inducible factor 1 (HIF-1) is induced in inflammed lesions, and triggers transcription of many genes that allow the intestinal epithelial cells to
act as an effective barrier, and is thought to be protective against inflammation and IBD

- Proly-hydroxylases (PHDs) are expressed in the intestinal mucosal tissue and regulate the stability of HIF

- Targeted therapies to stabilize HIF, for instance by inhibiting PHDs, are in development to treat IBD
Introduction

The intestinal epithelium lines the entire gastrointestinal tract, covering a surface area of approximately 300 m² in the adult human and forming an essential barrier to the outside world. This intestinal epithelial barrier consists of a monolayer of cells with intercellular tight junctions, a complex three-dimensional structure and a thick mucous gel layer, and provides a dynamic and regulated barrier to the flux of the luminal contents to the lamina propria. As well as having an important role in nutrient uptake and development of oral tolerance to nonpathogenic antigens, the intestinal epithelial barrier drives the daily absorption of at least 9 l of fluid. Both the absorptive and barrier functions of the intestinal epithelium are regulated by the availability of O₂³.

It is widely understood that the gastrointestinal tract functions in a state of 'low grade inflammation'. Such a state results from the constant processing of luminal antigenic material during the development of oral tolerance and the priming of the mucosal immune system for rapid and effective responses to antigens or microbes that may penetrate the barrier.

The anatomy and function of the intestine provide a fascinating oxygenation profile as, even under physiologic conditions, the intestinal mucosa experiences profound fluctuations in blood flow and metabolism. For example, less than 5% of total blood volume is present in the gut during fasting, but, following ingestion of a meal, approximately 30% of total blood volume is present in the gastrointestinal tract. Such changes in blood flow also result in marked shifts in local pO₂. Notably, there is a steep oxygen gradient from the anaerobic lumen of the intestine across the epithelium into the highly vascularized subepithelium. From this perspective, it is perhaps not surprising that the epithelium has
evolved a number of features to cope with this metabolic setting. In fact, studies comparing functional responses between epithelial cells from different tissues have revealed that intestinal epithelial cells seem to be uniquely resistant to hypoxia and that an extremely low level of oxygenation within the normal intestinal epithelial barrier (so-called “physiologic hypoxia”) may be a regulatory adaptation mechanism to the steep oxygen gradient.

Loss of epithelial barrier function with the resultant unrestricted flux of luminal antigens to the mucosal immune system underlies the pathology of IBD, and results in hypoxia within the chronically inflamed mucosa, particularly within the epithelial cell layer. This loss of epithelial barrier, together with hypoxia and inflammation underlie the pathology of IBD. Ongoing studies suggest that hypoxia-regulated pathways are highly associated with IBD and contribute particularly to the resolution of ongoing inflammation. In this review we discuss the signaling pathways involved in these processes and the possibility of developing therapies to modify the hypoxic state to treat IBD.

Hypoxia and the immune response

Sites of mucosal inflammation are characterized by profound changes in tissue metabolism, including local depletion of nutrients, imbalances in tissue oxygen supply and demand, and the generation of large quantities of reactive nitrogen and oxygen intermediates. In part, these changes can be attributed to recruitment of inflammatory cells, including myeloid cells such as neutrophils (polymorphonuclear cells; PMNs) and monocytes (Figure 1). PMNs are recruited by chemical signals, such as the chemokine interleukin 8, complement factor C5a, N-formylated peptides, platelet-activating factor and leukotriene B4, which are generated at sites of active inflammation as part of the innate host immune
response to microorganisms. In transit, these cells expend tremendous amounts of energy. For instance, large amounts of ATP are needed for the high actin turnover required for cell migration. Once at the sites of inflammation, the nutrient, energy and oxygen demands of the PMNs increase to accomplish the processes of phagocytosis and microbial killing. It has long been known that PMNs are primarily glycolytic cells, with few mitochondria and little energy produced from respiration. A predominantly glycolytic metabolism ensures that PMN can function at the low oxygen concentrations (even anoxia) associated with inflammatory lesions.

Once at the inflammatory site, PMNs recognize and engulf pathogens and activate the release of antibacterial peptides, proteases and reactive oxygen species (ROS; superoxide anion, hydrogen peroxide, hydroxyl radical and hypochlorous acid) into the vacuole, which together kill the invading microbes (Figure 1). ROS are produced by phagocytes in a powerful oxidative burst, driven by a rapid increase in oxygen uptake and glucose consumption, which in turn triggers further generation of ROS. When activated, it is estimated that PMN can consume up to 10 times more O\(_2\) than any other cell in the body. Notably, the PMN oxidative burst is not hindered by even relatively low O\(_2\) (as low as 4.5% O\(_2\))\(^8\), which is important, since it means that ROS can be generated in the relatively low O\(_2\) environments of inflamed intestinal mucosa\(^3\).

In contrast to the innate immune response associated with PMNs, adaptive immune responses to inflammation in the intestine are characterized by a combination recruitment and high rates of local T and B cell proliferation. As a result, adaptive immune responses have markedly different demands for glucose, oxygen and ATP. \(^9,10\) In the past 10 years we have begun to understand the nature of interactions between microenvironmental metabolic
changes and the generation of recruitment signals and molecular mechanisms of leukocyte migration into these areas. The metabolic changes that occur as a result of the recruitment and activation of leukocytes during inflammation provide information about the potential sources of hypoxia at the intestinal epithelial barrier (Figure 1).

IBD is an interesting disease for studying the metabolic changes associated with inflammation, particularly the development of hypoxia within inflammatory lesions. Some microvascular abnormalities that occur in patients with IBD have been associated with abnormal blood flow to the intestine, including increased production of tissue vasoconstrictor molecules and a reduced generation of nitric oxide by endothelial cells, as well as vascular endothelial growth factor-dependent angiogenesis. In addition, studies of active inflammation in mouse models of IBD have shown the intestinal epithelial cell to be a primary target for hypoxia.

Hypoxia in murine models of IBD was revealed using 2-nitroimidazole dyes, a class of compounds known to undergo intracellular metabolism depending on tissue oxygenation (Figure 2). Tissue staining with these nitroimidazole dyes revealed two profound observations. First, in the normal intestinal epithelial cells, especially in the colon, physiologic hypoxia predominates (Figure 2). Whether such low oxygen levels function to regulate basal gene expression in intestinal epithelial cells is not known. Second, the inflammatory lesions seen in these mouse models are profoundly hypoxic or even anoxic, similar to that seen in some large tumors, and penetrate deep into the mucosal tissue. It is likely that there are multiple contributing factors, such as vasculitis, vasoconstriction, edema, increased O₂ consumption; Figure 1), which predispose the inflamed intestinal epithelia to decreased oxygen delivery and hypoxia. While these 2-nitroimidazole
compounds have not been used to image inflammatory lesions, they have shown significant clinical utility in tumor imaging and in the identification of stroke regions within the brain of patients 15. As opposed to other imaging techniques, these compounds have the advantages that they image only viable tissue and are not active within apoptotic or necrotic regions 16. Likewise, studies are underway to use these compounds as adjunct radiosensitizers for enhancing chemotherapy targeting 17.

**Hypoxia-inducible factor**

The same series of studies which identified inflammation-related hypoxia also showed that expression of HIF-1 is induced in the inflammatory lesions 13. Many cell types, including intestinal epithelial cells 18, express both HIF-1 and HIF-2 and murine knockout studies suggest that these proteins have non-redundant roles 19. Some have suggested that distinct transcriptional responses mediated by HIF-1 and HIF-2 may be integrated in ways that support particular adaptations to hypoxia. For example, the transcriptional responses that coordinate the glycolytic pathways include more than 11 target genes and seem to be more selective for the HIF-1α than for the HIF-2α isoform 19. Studies addressing the selectivity of the two isoforms for erythropoietin induction have suggested a more important role for the HIF-2α isoform 19. Currently, this specificity is not well understood. Some have suggested that binding of HIF-1α or HIF-2α to other transcription factors at the site of DNA binding could determine such specificity 19, but this is not conclusive.

Several studies have shown that HIF-1 triggers the transcription of many genes that enable intestinal epithelial cells to act as an effective barrier 4,20-22. Originally shown by microarray analysis of hypoxic intestinal epithelial cells 21, these studies have been validated
in animal models of intestinal inflammation$^{13,23-27}$ and in human intestinal inflammation tissues$^{28-30}$. The functional proteins encoded by hypoxia-induced, HIF-dependent mRNAs localize primarily to the most luminal aspect of polarized epithelia. Molecular studies of these hypoxia-elicited pathway(s) have shown a dependence on HIF-mediated transcriptional responses. Notably, epithelial barrier protective pathways driven by HIF tend not to be the classical regulators of barrier function, such as the tight junction proteins occludin or claudin. Rather, the HIF-dependent pathways are more to do with overall tissue integrity, ranging from increased mucin production,$^{31}$ including molecules that modify mucins, such as, intestinal trefoil factor$^4$, to xenobiotic clearance by P-glycoprotein,$^{20}$ to nucleotide metabolism (by ecto-5'-nucleotidase and CD73)$^{21,22}$ and nucleotide signaling through the adenosine A2B receptor$^{22}$.

As an extension of the original studies identifying HIF induction within the intestinal mucosa, Karhausen, et al. generated transgenic miceexpressing either mutant Hif1-α (causing constitutive repression of Hif1-α) or mutant von Hippel-Lindau (causing constitutive overexpression of HIF) targeted to the intestinal epithelial cells$^{13}$. Loss of epithelial HIF-1α resulted in a more severe colitic phenotype than wild-type animals, with increased weight loss, decreased colon length and increased epithelial permeability, whereas constitutively active intestinal epithelial HIF was protective for these parameters. These findings may be somewhat model-dependent, since epithelial HIF-based signaling has also been shown to promote inflammation in another study$^{27}$. However, the findings confirmed that intestinal epithelial cells can adapt to hypoxia and that HIF may contribute to this.
Non-epithelial cell types within the gastrointestinal mucosa have also been studied for HIF expression and response to hypoxia. Activated T cells show increased expression of HIF-1α, which prevents them from undergoing activation-induced cell death in hypoxic settings. T-cell survival in hypoxia is, at least in part, mediated by the vasoactive peptide adrenomedullin.

Other studies using chimeric mice bearing HIF-1α-deficient T and B cells have revealed lineage-specific defects that result in increased autoimmunity, including autoantibodies, increased rheumatoid factor and kidney damage. HIF function has also been studied in some detail in myeloid cells. Cre-LoxP-based elimination of HIF-1α in cells of the myeloid lineage (lysozyme M promoter) have revealed multiple features which importantly implicate metabolic control of myeloid function. In particular, these studies have shown that PMN and macrophage bacterial killing capacities are severely limited in the absence of HIF-1α, as HIF-1α is central to production of antimicrobial peptides and granule proteases. These findings are explained, at least in part, by the inability of myeloid cells to mount appropriate metabolic responses to diminished O2 characteristic of infectious sites. Finally, compelling evidence have revealed that HIF-1α transcriptionally controls the critical integrin important in all myeloid cell adhesion and transmigration, namely the β2 integrin (CD18). Such findings are important for our current understanding of the role of functional PMN in IBD. A recent study, for example, used PMN depletion techniques to document a central role for PMN in the resolution of inflammation in several murine IBD models.
Prolyl-hydroxylases and HIF Expression

In the past 10 years, the molecular mechanisms of HIF stabilization have been clarified. Four HIF-hydroxylases termed PHD1-3 and Functional Inhibitor of HIF (FIH) have been demonstrated to be important in the hypoxic regulation of the HIF pathway. As depicted in Figure 3, each of these hydroxylases are encoded by different genes and their gene product enzymes demonstrate tissue specific expression patterns. All three PHD’s and FIH are found in the intestinal epithelium. Significantly different phenotypes in mice genetically lacking individual isoforms of the hydroxylases exist. For instance, PHD1-/- mice demonstrate a reprogrammed basal metabolic profile in normal tissue which decreases exercise performance but these animals are protected against acute liver and muscle ischemia. PHD2 homozygous knockout is embryonic lethal due to developmental angiogenesis dysfunction. PHD2 heterozygous knockout animals demonstrate enhanced tumor angiogenesis but decreased metastasis. PHD3 homozygous knockout mice demonstrate reduced neuronal apoptosis, abnormal sympathoadrenal development and reduced blood pressure. These diverse phenotypes strongly suggests distinct isoform-specific functions in vivo.

In the presence of 2-oxoglutarate, Fe$^{2+}$ and molecular oxygen, these enzymes hydroxylate HIF alpha subunits leading to directed ubiquitination and subsequent degradation. Hypoxia or pharmacologic agents (such as DMOG) inhibit HIF-hydroxylases leading to derepression of HIF. The impact of HIF hydroxylase inhibitors on epithelial cell gene expression is not restricted to regulation via HIF. For example, NF-κB has recently been reported to be regulated by HIF hydroxylases in a number of studies. The transcriptional targets of HIF hydroxylases can impact upon epithelial barrier function in a
number of ways. For example, HIF regulates the expression of a family of barrier protective factors including intestinal trefoil factor \(^4\), the mucins \(^{31}\) and actin cytoskeletal crosslinkers \(^{45}\). Likewise, NF-\(\kappa\)B is thought to be largely protective in the intestinal epithelium via the inhibition of enterocyte apoptosis leading to enhanced intestinal barrier function \(^{23}\).

**Convergence of inflammation and hypoxia**

The oxygen-dependent regulatory role of PHDs may not be restricted to the HIF pathway and may provide a means to better understand how hypoxia contributes to other aspects of inflammation. For instance, NF-\(\kappa\)B may interact with with the HIF pathway and is activated during inflammation. NF-\(\kappa\)B consists of either homodimers or heterodimers and which, on activation, translocates to the nucleus and binds with the transcriptional coactivator CBP/p300 to begin transcription or repression of various genes. The activity of NF-\(\kappa\)B is regulated by the inhibitory \(I\kappa\)B proteins \(^{46}\). The best-studied complex is \(I\kappa\)B\(\alpha\) bound to the NF-\(\kappa\)B p50-p65 dimer \(^{46}\). The interaction with \(I\kappa\)B\(\alpha\) inhibits NF-\(\kappa\)B from binding to DNA and maintains the complex in the cytoplasm. On activation by various extracellular signals, \(I\kappa\)B kinase (IKK) is activated, resulting in phosphorylation \(^{47}\) and polyubiquitylation of \(I\kappa\)B\(\alpha\) \(^{48}\). The polyubiquitinated \(I\kappa\)B\(\alpha\) is then selectively degraded by the S26 proteasome. Once dissociated from \(I\kappa\)B\(\alpha\), NF-\(\kappa\)B rapidly enters the nucleus and activates gene expression.

It is now appreciated that NF-\(\kappa\)B-dependent pathways are also be regulated by PHDs and that the usefulness PHD inhibitors for murine colitis also target the NF-kB pathway. Indeed, hypoxia has been shown to activate NF-\(\kappa\)B and this seems, at least in part to be, to be dependent on PHD-mediated hydroxylation \(^{38,49}\) of IKK\(\beta\). \(^{38}\) In normoxia, IKK\(\beta\)
activity is held in check through LXXLAP-dependent hydroxylation by PHD1 and PHD2. It is notable that conditional deletion of the NF-κB pathway in intestinal epithelial cells in mice leads to an increased susceptibility to colitis, similar to that of the mice expressing homozygous mutant HIF1α. This implicates epithelial NF-κB in a prominently protective role in colitis, probably through the expression of anti-apoptotic genes in intestinal epithelial cells and through enhanced epithelial barrier function. Some studies have suggested that both the HIF and NF-κB pathways may also be influenced by mediators found within inflammatory sites, including microbial products, cytokines and even intact bacteria. NF-κB is a classic transcriptional regulator activated by a spectrum of agonists, the activation of which drives a complex series of receptor-mediated signaling pathways. Recent studies indicate that transcription of HIF-1α is activated by NF-κB-mediated signaling. Inflammation-associated upregulation of HIF-1α mRNA occurs in an NF-κB-dependent manner. It also seems that increased NF-κB activity in hypoxia can be regulated by HIF-1, thus a cross-regulatory loop may exist between these two pathways and may involve other transcriptional regulators that bear non-redundant PHD sensitivity, including activating transcription factor-4 and Notch, both critical regulators of cell fate (see Figure 4). Given that intestinal epithelial cells are in an environment with constant exposure to potentially inflammatory stimuli, the cross regulation of HIF and NF-κB may have profound implications for intestinal epithelial cell function and survival under both homeostatic and disease conditions.
Therapies for IBD

The finding that hypoxia serves as an alarm signal to promote the resolution of inflammation in IBD holds specific promise for the development of therapies targeting hypoxia pathways. While pre-clinical studies in murine models have focused primarily on the use of PHD inhibitors in acute models of colitis, the ultimate goal should be the development of viable therapies for active and chronic intestinal inflammation.

The identification of HIF-selective PHDs has provided unique opportunities for the development of PHD-based therapies. While there is wide interest in developing HIF-1 inhibitors as potential cancer therapies, opportunities also exist to selectively stabilize HIF in an attempt to support mucosal barrier function and promote inflammatory resolution in IBD. One approach that we have taken is to stabilize HIF through the use of PHD inhibitors. For example, 2-OG analogues can stabilize HIF-α. While this approach is not selective for particular PHD isoforms, some in vitro studies suggest that marked differences in substrate specificity may exist and could be harnessed for selectivity. For example, all PHDs hydroxylate the C-ODD domain more efficiently than the N-ODD domain (see Figure 3), and PHD2 hydroxylates the N-ODD domain less efficiently on HIF-2α than on HIF-1α. In addition, PHD3 does not hydroxylate the N-ODD domain of HIF-1α. It is also likely that the protection afforded by PHD inhibitors (e.g. decreased tissue inflammatory cytokines, increased barrier function, decreased epithelial apoptosis) may involve both HIF and NF-κB activities (Figure 4).

Given the central role of HIF-mediated signaling on erythropoietin production, these drugs have been developed and are in clinical trials for the treatment of anemia. Several PHD inhibitors have been described, including direct inhibitors, analogs of
naturally occurring cyclic hydroxamates\textsuperscript{60} and antagonists of alpha-keto-glutarate\textsuperscript{54}. Each of these molecules serve as competitive inhibitors of the PHDs through substitution for alpha-ketoglutarate in the hydroxylation reaction shown in Figure 3. Within the gastrointestinal tract, the PHD inhibitors DMOG and FG-4497 \textsuperscript{23,26} have been used effectively to reduce symptoms in at least two mouse models of colitis\textsuperscript{23,26}. Indeed, these studies have shown that both DMOG and FG-4497 provide an overall beneficial influence on multiple parameters studied in chemically induced (trinitrobenzene sulfonic acid [TNBS] or dextran sodium sulfate [DSS] mouse models of colitis. In these mouse models, the drugs were well tolerated with no significant adverse side effects. In our experience, both FG-4497 and DMOG can be delivered by multiple routes of administration (intraperitoneal, oral and intravenous), and both FG-4497 are absorbed orally with only a slight loss of efficacy compared to intraperitoneal administration. To date, no trials have been initiated for the treatment of IBD.

Two notes of caution are worthwhile discussing regarding the use of HIF activators (e.g. PHD inhibitors) as potential therapies. First, this class of drugs substantially elevates hematocrit through increased erythropoietin production. In our initial studies with murine colitis, we observed that high doses of FG-4497 (>60 mg/kg) with 5 daily intervals of dosing resulted in occasional vascular occlusion within the intestine. Likely, these side effects were from erythrocyte sludging due to high hematocrits. This problem was rectified by lowering the dose and decreasing the interval of dosing. As a second precautionary note, chronic HIF-1 and HIF-2 stabilization have been significantly associated with a number of cancers. Whether pharmacological HIF stabilization could initiate or promote tumor development is not currently known but should be considered as a potential serious
side effect of these drugs. Until proven otherwise, the safest use of PHD inhibitors may be
for acute exposure or as an adjunct therapy with other drugs over a short term period.

Conclusions

The gastrointestinal mucosa is an interesting tissue in which to investigate tissue
oxygenation and disease-based metabolism. In this review, we have outlined the evidence
for hypoxia as an important alarm signal within the intestinal mucosa. Studies derived from
cultured cell systems, animal models and patient-derived materials have documented the
that hypoxia is a significant component of the inflammatory microenvironment. Likewise,
studies to date in animal models of intestinal inflammation have demonstrated an almost
uniformly beneficial influence of HIF stabilization on disease outcomes. It is notable that
the increased susceptibility to colitis following the genetic deletion of intestinal epithelial
HIF may be somewhat model-dependent and will require additional validation with PHD
inhibitors before clinical studies can be implements. Ongoing studies to define the
differences and similarities between innate and adaptive immune responses will continue to
teach us important lessons about the complexity of the gastrointestinal tract. Such
information will provide new insight into the pathogenesis disease and importantly, will
provide new targets as templates for the development of therapies for human disease.
References


**Figure Legends:**

*Figure 1: Potential sources of hypoxia in mucosal inflammation.* During episodes of inflammation, a number of factors influence the supply and demand of oxygen to the tissues, as well as influencing oxygen delivery. Noted here are edema, vasculitis and vasoconstriction, which separate epithelial cells from the blood supply and limit oxygen availability. In addition, local depletion of oxygen through processes such as the polymorphonuclear cells oxygen burst can use large quantities of oxygen and result in localized hypoxia, where red depicts normoxia and blue represents hypoxia.

*Figure 2: Detection of hypoxia in the mucosa.* a) The *in vivo* evidence for hypoxia associated with inflammation (so called “inflammatory hypoxia”) are provided using nitroimidazole-based dye retention in vitro and in mice. These molecules (R-NO₂) are taken into cells passively and reduced to highly reactive nitrogen intermediates (R-NO₂-[dot]). In the absence of adequate oxygen to regenerate the native compound, these intermediates react with cellular proteins to form adducts (R-NH₂), which can be visualized using labeled antibodies. b) In the colons of mice with no inflammation (control) small amounts of nitroimidazole adduct is detected along the luminal aspect of the colon (red), suggesting a degree of physiological hypoxia in the normal colon. c) During episodes of inflammation, such as seen here in a mouse model of colitis, intense and deep tissue hypoxia is prevalent, particularly in areas overlying lesions.

*Figure 3: Structural features of hypoxia-inducible factor (HIF) and mechanism of HIF stabilization.* A) The alpha subunit of HIF (HIF-1, HIF-2 and HIF-3) consist of a C-terminal
basic helix-loop-helix domain (bHLH, gray), a Per-Amt-SIM (PAS, purple), a C- and N-terminal oxygen-dependent degradation domain (ODD) region (orange) and a N-terminal transactivation domain (TAD, blue). Also shown are the proline (Pro) and asparagines (Asn) sites for hydroxylation by proly-hydroxylases (PHD) 1-3 and functional inhibitor of HIF (FIH), respectively. B) Depiction of HIF hydroxylation by the combination of alpha-ketoglutarate (αKG), molecular oxygen (O₂) and the PHD enzyme in normoxia. When O₂ becomes limiting (hypoxia), the alpha subunit is stabilized, binds to the HIF-1 beta subunit within the nucleus where it becomes transcriptionally active upon binding to the hypoxia-response element (HRE) consensus sequence on DNA.

Figure 4: Activation of hypoxia-inducible factor (HIF) and NF-κB in IEC’s during inflammation. Episodes of inflammation and hypoxia activate a number of pathways in intestinal epithelial cells. The transcriptional activation of both HIF (blue) and NF-κB (red) is stabilized by proly-hydroxylase enzymes (PHDs) and functional inhibitor of HIF (FIH). Activation of these pathways has been shown to promote epithelial barrier function and to decrease epithelial apoptosis, with an overall epithelial protective phenotype. As noted, the various PHD enzymes cross-regulate both HIF and NF-κB in a non-redundant manner, including the regulation of activating transcription factor-4 (ATF-4) and Notch (see text). Green arrows indicate hydroxylation reactions and black arrows indicate transcriptional activation. Activation of HIF through these hydroxylation reactions (blue) promotes barrier function while activation of NF-κB (red) diminishes epithelial apoptosis.