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Understanding complexity in the HIF signaling pathway using systems biology and mathematical modeling.

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Abstract

Hypoxia is a common micro-environmental stress which is experienced by cells during a range of physiologic and pathophysiologic processes. The identification of the hypoxia-inducible factor (HIF) as the master regulator of the transcriptional response to hypoxia transformed our understanding of the mechanism underpinning hypoxic response at the molecular level and identified HIF as a potentially important new therapeutic target. It has recently become clear that multiple levels of regulatory control exert influence on the HIF pathway giving the response a complex and dynamic activity profile. These include positive and negative feedback loops within the HIF pathway as well as multiple levels of crosstalk with other signaling pathways. The emerging model reflects a multi-level regulatory network that affects multiple aspects of the physiologic response to hypoxia including proliferation, apoptosis and differentiation. Understanding the interplay between the molecular mechanisms involved in the dynamic regulation of the HIF pathway at a systems level is critically important in defining appropriate new therapeutic targets for human diseases including ischemia, cancer and chronic inflammation. Here, we review our current knowledge of the regulatory circuits which exert influence over the HIF response and give examples of *in silico* model-based predictions of the temporally dynamic behaviour of this system.

Introduction

The development of molecular machinery capable of utilizing atmospheric oxygen for bioenergetic purposes (oxidative phosphorylation) was a key event in the evolution of life on Earth. This, along with other processes such as the development of membrane compartmentalisation, allowed eukaryotic organisms to greatly increase their metabolic efficiency. This enabled the development of a range of biochemical processes which provided the bioenergetic capacity to permit the evolution of more complex life forms such as metazoans [1]. However, this also led to a high degree of dependence of most eukaryotic cells upon a constant supply of oxygen in order to maintain metabolic homeostasis and cell, tissue and organism survival. Hypoxia (which occurs when oxygen demand exceeds supply) is a relatively common occurrence in health and disease [2]. It may occur in response to physiologic stimuli such as ascension to high altitude or exercise or as a result of diseases such as cancer, chronic inflammation or vascular disease. The critical dependence on oxygen for metabolic homeostasis and survival led to the early evolution of molecular mechanisms that enabled cells, tissue and organisms to adapt to hypoxia. These adaptive responses include fundamental changes in cellular metabolism primarily orchestrated by the helix-loop-helix (HLH) transcription factor family member termed hypoxia inducible factors (HIF).

To date, three HIF variants have been identified in mammals (HIF-1, HIF-2 and HIF-3) of which the best characterized is the HIF-1 [3]. HIF-1 is composed of discrete alpha and beta subunits (HIF-1 α and - β , respectively) both of which are ubiquitously expressed in human tissues [4, 5]. While HIF-1 β (also known as the aryl hydrocarbon nuclear translocator, ARNT) is stably expressed, the HIF- α subunits are continuously degraded by the 26S proteasome under physiologic conditions thus preventing HIF activity in normoxia. In hypoxia, degradation of HIF- α is inhibited, the α and β subunits dimerize, translocate to the nucleus and activate the expression of a family of genes which facilitate cellular adaptation to hypoxia.

The mechanism underpinning the activation of HIF in hypoxia was first elucidated in 2002 and involves the inhibition of the oxygen-dependent hydroxylation of HIF- α subunits by a family of HIF-hydroxylases. To date, three HIF-regulating prolyl-4-hydroxylases (PHD1, -2 and -3) have been identified in mammalian cells [6]. They utilize molecular oxygen and the Krebs cycle (TCA cycle) intermediate α -ketoglutarate as co-substrates to hydroxylate HIF- α subunits at conserved prolyl residues [7, 8]. This increases the α subunit's affinity for the von Hippel-Lindau protein (pVHL), a member of an E3 ubiquitin ligase complex [9]. The pVHL-

mediated ubiquitylation leads to the constitutive proteasomal degradation of the HIF- α subunit. In hypoxia, PHD activity is reduced due to the lack of available oxygen resulting in stabilization and formation of the active HIF heterodimer. A second level of hydroxylation-dependent regulation of HIF is mediated by the asparagine hydroxylase termed factor inhibiting HIF (FIH) which prevents HIF's interaction with the transcriptional co-activator p300/CBP [10-12]. The adaptive programmes activated by HIF include the metabolic switch from the oxygen-dependent oxidative phosphorylation to glycolysis, increased angiogenesis and erythropoiesis. Therefore, HIF represents a key regulator of the metazoan transcriptional response to hypoxia which plays a key role both in physiology and disease. Appreciating the dynamic nature of this pathway is critical to our future understanding of adaptation to hypoxia and targeting the underlying pathways for therapeutic benefit.

While the canonical pathway regulating the activity of HIF (as outlined above) is the key link between hypoxic sensing and the activation of an adaptive transcriptional response, a range of other inputs are also involved in shaping the spatial and temporal nature of the HIF response (Figure 1). It is likely that such inputs confer upon HIF a richly dynamic activity profile allowing a high degree of selectivity in terms of context-dependent gene expression profiles. These inputs include feedback loops within the HIF pathway as well as crosstalk with other signaling pathways representing a high degree of complexity.

The intricate nature of these regulatory systems necessitates novel analytic measure like mathematical modeling. *Since it was done recently, reviewing the mathematical modeling efforts on HIF signaling in its entirety is beyond the scope of this work [13, 14].* Instead here, we review those sufficiently characterized HIF interactors that are considered to play key roles in the regulation of the HIF-orchestrated hypoxic response. In addition, we would like to demonstrate the potential power of the model-based approach in disentangling HIF complexity by attempting to answer a series of questions using simplified mathematical models. Through these practical examples, we hope to highlight the type of questions one can ask and how one may go about interrogating the models to reach answers.

Feedback loops in the HIF pathway

Physiologic Feedback

Upon experiencing systemic hypoxia, co-ordinated multi-organ measures are deployed to survive the hypoxic insult and restore oxygen homeostasis. These include attempts to increase the oxygen supply through elevated erythropoiesis and/or neovascularization both governed

by HIF-inducible genes. At a systemic level, hypoxia-activated HIF induces *erythropoietin* expression in interstitial kidney and liver cells that subsequently increases erythropoiesis in the bone marrow [15, 16]. In addition, HIF-driven increase of the serum erythropoietin levels represses the *hamp* gene that encodes the hepatocyte-specific iron homeostasis regulator hepcidin [17-19]. The subsequent drop of serum hepcidin results in elevated iron release from the intestinal epithelium supplying the increased iron demand of the expanded erythropoiesis [20]. The consequently elevated oxygen and iron supply, however, may also provide prolyl-4-hydroxylases their co-substrates thus promoting a systemic physiological negative feedback loop in the HIF system [21].

At the tissue level, another negative feedback loop is mediated by the vascular endothelial growth factor (VEGF) that may limit the HIF response. HIF-induced VEGF and their cognate receptors act on the vascular endothelial cells to promote angiogenesis that further supports oxygen supply to the hypoxic tissues [3, 22, 23]. These HIF-orchestrated adaptive measures ultimately lead to increased tissue oxygenation which overcomes the causative hypoxic insult resulting in physiologic negative feedback loops (Figure 2A).

Metabolic Feedback

At the cellular level, hypoxia-activated HIF rewires metabolism and renders it less oxygen dependent by regulating a cluster of metabolic enzyme-encoding genes [24]. Some of these have also been found to regulate HIF activity forming metabolic feedback loops in the HIF system. A prototype of such a loop is pyruvate dehydrogenase kinase-1 (PDK-1) which phosphorylates pyruvate dehydrogenase (PDH). PDH fuels the mitochondrial TCA cycle via conversion of pyruvate to the TCA cycle substrate acetyl-coenzyme A (ac-CoA) [25]. PDK-1 mediates inactivating phosphorylation of PDH shutting down the ac-CoA supply of the TCA cycle. This leads to fundamental changes in mitochondrial functions including the accumulation of TCA cycle intermediates [26]. Since HIF-regulating prolyl-4-hydroxylases utilise α -ketoglutarate as a co-substrate and produce succinate during their catalytic activity, it is not surprising that the accumulation of succinate blocks these hydroxylases [27]. Indeed, loss-of-function mutations in succinate dehydrogenase were found to be inhibitory towards PHDs leading to the stabilisation of HIF- α subunits and succinate was identified as the mediator of this effect [28, 29]. Thus, PHDs can also integrate metabolism-dependent stimuli in the regulation of HIF- α which, in return, induces a panel of adaptive genes forming a classical feedforward loop.

Glycerol-3-phosphate dehydrogenase 1 like (GPD1-L), another metabolic enzyme indirectly regulated by HIF, has also been suggested to act on the prolyl hydroxylase-mediated limitation of HIF function. GPD1-L has enzymatic activity similar to the mitochondrial glycerol-3-phosphate dehydrogenase that catalyses the redox conversion of glycerol-3-phosphate (G3P) to dihydroxyacetone phosphate (DAP) and is believed to connect oxidative phosphorylation to glycolysis and lipogenesis via the regulation of the amount of the cytosolic G3P [30]. The microRNA-210 (miR-210) mediated silencing of GPD1-L was found to decrease the rate of PHD-mediated HIF-1 α degradation and this effect was reversed by pharmacological inhibition of the proteasome or PHD activities [31]. GPD1-L, however, is not the only metabolic enzyme that affects HIF stability via its microRNA-mediated regulation. In hypoxia, miR-183 targets isocitrate dehydrogenase, the TCA cycle mediator that produces α -ketoglutarate from isocitrate. Due to the α -ketoglutarate-dependent nature of the prolyl-4-hydroxylases, the miR-183-mediated blockade of the α -ketoglutarate production favours HIF- α stabilisation via the inhibition of PHDs [32]. Although the mechanism of hypoxic upregulation of miR-183 is yet to be determined, miR-210 has already been shown to be HIF-1-inducible. Thus, both the HIF-inducible PDK-1 and miR-210 complete synergistic metabolic positive feedback loops within the HIF pathway targeting the primary HIF repressor prolyl-4-hydroxylases [33] (Figure 2A).

Which PHD isoforms control the transient dynamics of HIF-1 α ?

The transient dynamics of HIF-1 α protein expression in response to a hypoxic insult has been observed in multiple cell types [13, 14]. Since such an adaptive response profile is often underpinned by negative feedback regulation, PHD-mediated feedback loops have long been thought to be the underlying mechanisms. Yet, the fact that both PHD2 and PHD3 form negative feedback loops with HIF-1 α posed questions of whether they are functionally redundant, and if not, how different are individual isoforms in modulating HIF timing, duration and signaling amplitude? To probe these questions, we adapted a model recently developed by Bagnall *et al.* which was trained using single-cell imaging data of HIF dynamic profiles [34]. The schematic diagram of this model is given in Figure 2A, and detailed model information is presented in the Supplementary Materials (SM). A major advantage of having a model is that one can analyse and predict how a particular signaling output depends on changes of certain biological factors in the modelled system. Techniques such as sensitivity analysis allow us to do just that.

We first theoretically vary the parameter controlling PHD2 mediated feedback strength and simulate the corresponding HIF-1 α response to hypoxia. The model predicts that strong feedback induces not only a lower steady-state level of HIF-1 α response, but also a lower peak amplitude and shorter peak duration (Figure 2B). A similar analysis for the PHD3-mediated feedback however shows a much less pronounced effect. While varying, the feedback strength mildly affects the response peak, such change does not seem to influence steady-state level of HIF-1 α (Figure 2D). This observation is further supported by dose-response simulations showing that the steady-state level of HIF-1 α exponentially depends on the PHD2 mediated feedback strength while it is relatively insensitive to change in feedback strength mediated by PHD3 (Figure 2F). These simulations are in agreement with the conclusions in previous reports suggesting PHD2 to be the more dominant isoform in driving HIF-1 α transient response [34, 35]. Further questions could be asked. For example, how would the turnover rate of PHD2 and PHD3 differentially affect HIF response? These simulations could be readily performed as shown in Figure 2C and E, which predict that less stable PHD3 imparts a more pronounced effect on steady-state level of HIF-1 α .

Transcriptional Feedback

As a transcription factor, HIF actively contributes to its own regulation. Most importantly, HIF trans-activates the *PHD-2* and *-3* genes adding a direct negative feedback arm to the PHD-mediated HIF regulation [36, 37]. Despite their oxygen dependency which prevents them from functioning under hypoxic conditions, experimental data indicate that the enzymatic activity of both PHD-2 and -3 remains detectable in hypoxic cells maintaining the reactivity of the HIF system for additional hypoxic insults [37]. This feedback loop is possibly further supported by oxygen redistributed from mitochondria during the HIF-driven metabolic switch. Indeed, upon the inhibition of the mitochondrial respiration by nitric oxide, O₂ redistribution was observed leading to the inactivation of HIF [38]. Thus, one can speculate that the HIF-mediated induction of the PHDs may allow setting the PHD-HIF system to a new steady state at lower pO₂ levels (Figure 2A).

A distinct type of transcriptional feedback is mediated by one of the HIF-3 α isoforms termed inhibitory PAS domain protein (IPAS). IPAS is an alternative splicing product of the HIF-3 locus that lacks the C-terminal transactivation domains of the HIF-1 and -2 α [39]. As such, it competes with HIF-1 β for binding the α subunits acting as a dominant negative regulator of HIFs [40]. The IPAS-specific splicing product of the HIF-3 α locus is hypoxia-inducible and

HIF-1 binds to the hypoxia-responsive cis-element of the IPAS promoter representing a classic negative feedback that restricts HIF-mediated gene expression in hypoxia (Figure 2A) [39, 41]. Interestingly, however, the IPAS-specific mRNA splicing was still observed in the absence of the HIF-1-binding site of the IPAS promoter indicating that HIF-independent factors may also be involved in the production of the dominant negative isoform [41]. The uncoupled nature of IPAS expression and splicing suggest additional control mechanisms in this regulation. Indeed, since the normoxic expression of the IPAS is apparently restricted to corneal epithelial cells and some neuronal elements in mice, the HIF-independent control mechanisms may contribute to the tissue-specific nature of the IPAS-mediated regulation of the HIF activity.

HIF-1 α is not only under feedback control by the hydroxylases or IPAS but an increasing body of experimental evidence shows that it is also negatively regulated by miRNAs. These include miR-210 that not only indirectly facilitates HIF activity by targeting GPD1-L but also silences MNT, a member of the MYC/MAD/MYX transcription factor family, which antagonizes the trans-activating function of MYC [42]. This enables MYC-mediated induction of genes involved in the resolution of HIF-induced cell cycle arrest as well as cellular metabolism. The latter includes PDK1 that, as mentioned above, may play a role in the metabolic inhibition of PHDs so one can speculate that the miR-210-mediated upregulation of MYC represents a mechanism to amplify HIF activity [43]. Since MYC has also been reported to support HIF-1 α directly by interfering with the VHL-dependent degradation of HIF-1 α , these findings suggest the existence of MYC-mediated feedforward loops in the HIF pathway [44]. Intriguingly, however, HIF and MYC are traditionally considered to have antagonistic effects in the hypoxic cell. Indeed, HIF was found to counteract MYC by various underlying mechanisms including the induction of MXI1, another MYC antagonist, competition with MYC for promoter binding or promoting its proteasomal degradation [45, 46]. This paradox may reflect the different models studied so the biological relevance of the miR-210-promoted MYC functions in the regulation of HIF requires further clarification. Additional targets of miR-210 including the mitochondrial iron-sulfur cluster scaffold protein or the transferrin receptor, elements of the intracellular iron homeostasis, add another level of complexity to the regulation of the HIF pathway by merging physiologic, metabolic and transcriptional feedback loops [47, 48].

Besides miR-210, the hypoxia upregulated, HIF-inducible miR-155 also seems to be critical in the regulation of HIF. While (as we discuss later) miR-155 targets multiple elements related to the mTOR pathway adding further complexity to the interplay between HIF and

other signaling pathways (Figure 3A), it also mediates the degradation of the HIF-1 α transcript itself establishing a hypoxia-responsive negative transcriptional feedback loop within the HIF pathway [49] (Figure 2A).

Do microRNAs contribute to shaping HIF dynamics?

The miRNA-mediated negative feedback loops pose the question if they have any role in shaping HIF systems dynamics? Having different mechanistic details, miRNA-mediated feedbacks generally operate on a shorter time-scale than feedbacks induced by the PHDs and are, thus, expected to have differential roles. Nevertheless, our understanding regarding their functional redundancy remains poorly defined.

In order to examine this question, we constructed a core mathematical model of HIF regulation that accounts for the mechanistic differences between a miRNA and PHD-mediated feedback. For simplicity, the model considers only one feedback of each type, assumed to be driven by PHD2 and miR-155 (model scheme is given in Figure 2A and model description is in the SM). We calibrate the model so that it could capture the typical transient HIF-1 α profile under a hypoxic insult based on previously obtained parameter values [14]. Model simulations revealed unexpected differences between the feedbacks. While altering the PHD2-mediated feedback (by changing HIF-1 induced PHD2 transcription rate) does not really affect HIF-1 α rising time (Figure 2H), strengthening the miR-155-induced feedback instead accelerates the reaction of HIF-1 α to hypoxia with a faster response rate and a quicker return to steady-state level (Figure 2G). This suggests the negative feedback induced by miR-155 provides additional control to the reaction rate, duration and amplitude of HIF-1 α response to hypoxia that are not easily achieved with hydroxylase-induced feedback alone. Instead of increasing miR-155 transcriptional rate, we next ask the model whether a tighter association between miR-155 and HIF-1 α mRNA would affect the system's dynamics, and if so, how? Interestingly, comparative simulations of HIF-1 α dynamic response show that when miR-155 binds weakly to HIF-1 α mRNA, increasing the rate of HIF-1 α degradation triggered by PHD2 lowers HIF-1 α steady-state level as expected (Figure 2I). However, the opposite is predicted when miR-155 binds tightly to HIF-1 α mRNA (Figure 2J). Moreover, when we simulate the steady-state HIF-1 α level fold-change in response to hypoxia allowing both miR-155-HIF-1 α binding rate and HIF-1 α degradation rate to change, we found that HIF-1 α level responds in a biphasic manner to its degradation rate at weak miR-155-HIF-1 α association, while it increases monotonically with its degradation rate at strong miR-155-HIF-1 α

association (Figure 2K). These predictions together imply that miR-155 does not just add another redundant feedback to HIF control, but serves to fine-tune HIF signaling dynamics in an intricate and nonlinear manner. Modulating how miR-155 regulates HIF-1 α transcription could significantly influence how HIF-1 α responding to factors related to the hydroxylases under both normal and hypoxic conditions.

What controls HIF-1's oscillation?

While a primary function of negative feedback regulation is to enable signal adaptation, it is well known to underlie oscillatory behaviours [50, 51]. Under concurrent control of multiple negative feedbacks, it is expected that HIF-1 may oscillate in specific cellular contexts [51]. Although it remains debatable what physiological function an oscillatory HIF-1 profile may bring, accumulating evidence suggest that an oscillatory dynamics may provide a way to encode more signaling information than transient dynamics [52]. We use our simplified model to ask how oscillation may arise, and what may control HIF oscillatory dynamics (see model details in the SM)? Exploration of the model parameter space shows that spontaneous oscillation could robustly arise in the system under both normoxic and hypoxic conditions. Interestingly, we observed four typical scenarios from simulations when cells are switched from equilibrium in normoxia to hypoxia, as displayed in Figure 3A. In response to hypoxia, pre-existing oscillation occurring in normoxia may be completely abolished, maintained, or changed to damped oscillation depending on the parameter regimes. In case where oscillation persists when cells are switched to hypoxia, we frequently observed change in the oscillatory pattern with lower or higher amplitude, but generally the oscillation period is preserved. While examining the role of miR-155- and PHD2-mediated feedbacks in the emergence of oscillation, model simulations of feedback knock-out predict that breaking the miR-155 feedback only mildly affect oscillation while breaking the PHD2 feedback completely eliminates oscillation (Figure 3B). This suggests that negative feedbacks induced by the PHDs play a determining role in triggering oscillation. Moreover, varying the strength of this feedback loop could robustly modulate the amplitude of the oscillatory profile (Figure 3C).

Crosstalk between the HIF and other regulatory pathways

The above examples demonstrate the intricate nature of the HIF regulation by various feedback loops and how these individual circuits create regulatory networks to provide contextual opportunities for the precise temporo-spatial regulation of the HIF-mediated

responses. However, additional layers of complexity also exist in the form of crosstalk between HIF and other signaling pathways.

The HIF-mediated hypoxic induction of the PHDs has been hypothesized to form a link between the HIF and the mammalian target of rapamycin (mTOR) pathways as well. In this model, elevated levels of PHDs represents an adaptive step to match the stabilized HIFs and, thus, limit the effect of the mTOR pathway on HIF-1 α [53]. The involvement of the mTOR in the regulation of HIF-1 was first suggested by independent studies of the oncogene-related activation of VEGF [54-58]. Although mTOR is believed to act as a hub for various signaling pathways, the PKB/Akt pathway was found to be the dominant upstream regulator of the mTOR-mediated HIF-1 activity in hypoxia [59-61]. It became also clear that mTOR enhances HIF-1 transcriptional activity without affecting its mRNA levels or degradation rate [56, 61]. Current data suggest that mTOR may up-regulate HIF-1 by multiple mechanisms possibly depending on the cell type and/or physiological context Figure 4A.

mTOR is a known regulator of the translation by phosphorylating the eukaryotic initiation factor 4E-binding protein 1 (eIF4E-BP1), a suppressor of the 5' CAP-dependent translation [62]. As this has been shown to alter the protein expression pattern in hypoxic cells, the mTOR-mediated enhancement of the *HIF- α* translation is a widely accepted explanation for the negative effects of rapamycin on HIF-1 [63]. Indeed, down-regulation of the mTOR complex 2 (mTORC2), a redox-sensitive activator of the PKB/Akt pathway, leads to decreased abundance of the *HIF-2 α* transcripts in the polysomal fractions (Figure 4A) [64].

In recent years, it has become appreciated that reactive oxygen species (ROS) may also play an important role in cellular signaling pathways. A number of studies have proposed that ROS can regulate hydroxylase activity and promote the stabilization of HIF [65-68]. A good example for the ROS-mediated HIF regulation is the HIF-inducible lysyl oxidase (LOX) [61]. The *LOX* gene encodes a copper-dependent amine oxidase that catalyzes the cross-linking of collagen and elastin in the extracellular matrix while producing hydrogen peroxide (H₂O₂). Current data indicate that, following its HIF-dependent up-regulation in hypoxia, LOX-generated H₂O₂ activates the PKB/Akt-mTOR axis leading to the up-regulation of the HIF-1 α translation forming another positive feedback loop between mTOR and HIF [61]. These data also raise the question of whether the mTORC2 receives and transmits the signals of the reactive oxygen species to the HIF pathway via the PKB/Akt-mTORC1 axis.

Besides the mTOR-mediated translational regulation, PKB/Akt has also been reported to be involved in the proteasomal degradation of HIF-1 α [69]. This effect seems to involve the

glycogen synthase kinase 3 β (GSK3 β) mediated phosphorylation of HIF-1 α that facilitates its binding to the FBW7, an E3 ubiquitin ligase that recognizes GSK3 β -phosphorylated proteins and targets them for proteasomal degradation [70]. Since the inactivating phosphorylation of GSK3 β is primarily mediated by PKB/Akt, activation of the PKB/Akt pathway not only elevates the translational rate of the *HIF-1 α* transcript via mTOR but also mimics the effect of hypoxia as it also blocks proteasomal degradation of HIF-1.

mTOR has additional effects on HIF-1 α that are also independent of the up-regulation of its translation. It was found to associate with HIF-1 α via the mTOR complex 1 member RAPTOR and a putative TOR motif of HIF-1 α [58]. Although mTOR possesses serine/threonine kinase activity and phosphorylation of HIF-1 α has been reported as well, consequences of the physical association of mTOR and HIF-1 remain to be determined [58, 71]. More recently, however, mTOR has been shown to phosphorylate MINT3, a regulator of the membrane-type matrix metalloproteinases (MT-MMPs), at its Thr5/Ser7 residues [72]. This modification promotes MINT3 binding and inactivating FIH-1 [73]. By sequestering the HIF-1 suppressor FIH-1 to the Golgi membrane in cooperation with the MT1-MMP, the mTOR/MINT3/MT1-MMP axis could efficiently support the transcriptional activity of HIF-1 independently of the rate of its translation. Interestingly, in independent studies, *MT1-MMP* has been found to be a target gene of HIF-2 in renal cell carcinoma cells raising the question if the mTOR-regulated MINT3/MT1-MMP/FIH-1-mediated positive feedback loop is a general mechanism in the regulation of HIFs (Figure 4A) [74].

The rapamycin-sensitive up-regulation of the HIF-1 activity has been observed in various experimental settings leading to the induction of a wide range of HIF-1 targets. Many of these target genes have been found to form feedback loops via the regulation of the hypoxia-related activity of the mTOR pathway. These include REDD1 that has been reported to activate the Tuberous Sclerosis Complex 1/2 (TSC1/2) [75]. The TSC1/2 possesses GTPase activating function that renders the mTOR activator RHEB inactive [76]. BNIP3, another known HIF-1 target, has also been reported to facilitate the accumulation of the GDP-bound form of RHEB and the consequent down-regulation of mTOR in hypoxia [77]. In addition, the HIF-1-inducible miR-155 also targets elements of the mTOR pathway including RHEB, the mTORC2 member RICOR and the mTOR effector ribosomal protein S6KB2 [78]. Down-regulation of these targets seemingly complements the effect of REDD1 and BNIP3 and may contribute to the limitation of the mTOR activity during hypoxia thereby forming a negative feedback loop via the mTOR and HIF pathways (Figure 4A).

Crosstalk with ERK

Anabolic extracellular signals that activate the mTOR pathway often diverge and activate the ERK signaling cascade as well. Although the link between the ERK and HIF pathway was identified soon after the discovery of HIFs, the nature of their interplay has remained less clear. Stabilised HIF-1 α has been shown to be phosphorylated by p42/44 MAP kinases both under hypoxic conditions and in response to receptor-mediated ERK-activating stimuli [79-81]. The ERK-mediated phosphorylation was found to enhance the transcriptional activity of HIF-1 in various model systems, although the exact mechanism is still to be elucidated [82, 83]. Phosphorylation sites were identified within the carboxy-terminal part of HIF-1 α at the 641 and 643 positions [84]. Experimental data suggest that these modifications affect the nuclear export of HIF-1 and fundamentally alter the predicted composition of HIF-1 containing nuclear complexes [85, 86]. Hence, current data suggest that the ERK-mediated up-regulation of the HIF pathway differs from the mTOR-mediated effect and primarily acts on the transactivation function of HIFs possibly complementing the mTOR-mediated effects.

Crosstalk with NF- κ B

Although the regulation of HIF-1 α is considered mostly post-translational, recent studies revealed processes that influence its transcription as well. Intriguingly, besides its role in the mTOR-mediated HIF regulation, miR-155 is one of the identified feedbacks targeting the *HIF-1 α* message indicating its pivotal role in the regulation of the HIF pathway. In addition, besides HIF responsive elements, NF- κ B consensus sequences are also present in the miR-155 promoter indicating the capacity of NF- κ B-mediated stimuli to influence the HIF pathway via miR-155 [49]. Indeed, although the detailed mechanism is still not fully understood, it is clear that basal NF- κ B is moderately activated in hypoxia [87-89]. Experimental data suggest that the hypoxia-mediated inhibition of the PHD activity contributes to the up-regulation of the NF- κ B pathway. While active, PHDs inhibit the I κ B kinase (IKK) attenuating the dissociation of the inhibitory κ B (I κ B) from NF- κ B [90, 91]. In hypoxia, the PHD-mediated blockade of IKK is resolved leading to phosphorylation of I κ B followed by activation of NF- κ B. This mechanism is able to potentiate NF- κ B responsiveness to certain stimuli e.g. TNF α [90]. It is noteworthy, however, that under other cytokine-stimulated conditions (e.g. IL-1 β), inhibition of the prolyl-4-hydroxylases blocks NF- κ B activity suggesting a stimulus and/or cell type-specific

interaction between HIF and NF- κ B pathway [92]. Once active, NF- κ B induces *HIF-1 α* via evolutionary conserved consensus binding sites identified in the *HIF-1 α* promoter [91, 93-95]. Since NF- κ B activity is not sufficient for the accumulation of the HIF-1 α protein in the absence of hypoxia, current data suggest that the canonical NF- κ B pathway contributes to the maintenance of the *HIF-1 α* mRNA level, a pre-requisite of the PHD-mediated post-translational regulation of the HIF system [91]. An additional, possibly tissue specific arm of the NF- κ B-mediated regulation of the HIF pathway has also been identified by showing that NF- κ B can rescue HIF-2 α subunits from proteasomal degradation by directly trans-activating the *HIF-1 β* promoter [96]. Considering the HIF-inducible properties of the prolyl-4-hydroxylases, the PHD/NF- κ B/HIF axis may represent a regulatory circuit in the HIF pathway that is capable of integrating extracellular stimuli distinct from the ones mediated by the mTOR or ERK pathways (Figure 5). This hypothesis is further supported by recent findings that HIF-1 activity contributes to the down-regulation of inflammatory stimuli-activated NF- κ B pathway via kinases including IKK β that may represent the negative feedback arm of this putative regulatory loop [97].

What are the consequences of the crosstalk between the HIF-1 and other pathways?

Although most of the experimental and modeling effort to date has been centered on the *in vitro* HIF response to hypoxia with the HIF pathway being the sole focus, HIF is known to have important roles in normoxia and more complex physiological conditions as well [53]. Current data on the intensive crosstalk between the HIF and other signalling pathways depict a complex regulatory system that complements hypoxia-driven feedbacks by enabling the cell to adjust the HIF-mediated response to the status of the surrounding microenvironment. Here, we demonstrate the potential benefit of modeling by investigating the crosstalk between HIF and mTOR signaling pathways using a mathematical modeling approach.

The model's schematic diagram is given in Figure 8a and model description is presented in details in the SM. Following the experimental details reviewed in the previous sections, the model is designed to capture a positive feedback between mTORC1 and HIF-1 mediated via LOX on one hand, and a positive feed-forward loop between Akt and HIF-1 via GSK-3 β on the other. The presence of a positive feedback loop in the interaction circuit prompts us to hypothesize that the system may display bistable switch-like behavior. Nevertheless, it is not trivial under what parameter regime bistability could arise, nor what factors may control its emergence, and how one may go about in experimentally validating such hypothesis. Our

model simulations show that under permissive parameter conditions, bistability can arise in the system as illustrated in Figure 8b. Here, the steady-state level of HIF-1 α is predicted to respond in a bistable fashion to increasing Akt total (and activity, not shown).

Bistability is an interesting phenomenon in which a dynamic system could switch between two distinct stable equilibrium states in response to a single given input [98]. Long known to be widespread in physics, bistable behaviours have been identified in many cellular systems which underlie key functions, particularly decision-making in cell cycle progression, cellular differentiation, and apoptosis [98]. The occurrence of bistable behaviour has three different and related consequences. Firstly, there exists a range (i.e. bistable range) of input level (in this case Akt total), within which a given input could induce two different, a low and a high, expression levels of HIF-1 protein at steady state (Figure 4C). Whether the system settles in a low or a high state is a nontrivial matter, the answer to which depends on the system's initial condition (i.e. its previous history). However, given sufficient knowledge of the system's initial condition (i.e. expression and activity levels of its species), such prediction could be made with accuracy using model-based analysis. The second consequence, arguably more biologically relevant in many cases, is the ability of the system to generate abrupt switch-like responses to graded change in triggering cues, i.e. the ability to convert an analogue input to a digital output. Consequently, a graded increase in the Akt concentration (or activity) could result in a sudden surge of the HIF-1 α expression when the Akt exceeds a threshold level. The third consequence of bistability is termed “hysteresis”, which is the dependence of the system not only on its current input, but also on the history of the input itself. Such property could be demonstrated by visually tracing the dose-dependence curve in Figure 4B. Starting from a low level, a gradual increase of Akt will abruptly lead to a high HIF-1 level at threshold T_1 following the “low” branch of the curve. On the other hand, if the system starts at the “high” branch with high HIF-1 level gradually decreases; it will traverse the high branch and jump to the low branch at the second Akt threshold (T_2), which is lower than the first. The existence of two different input thresholds that characterise the system switching between the low and high branch is the hallmark of hysteresis, providing a “memory” for the system in response to that signal.

Since mTORC1 are often hyper-activated in cancer cells, we ask whether enhanced mTORC1 activity would affect the occurrence of bistability. Model simulations of this scenario suggests that enhanced mTORC activation induces more pronounced bistable switch, characterised by enlarged bistable range, while in contrast low mTORC1 activity could abolish bistable

behaviour (Figure 4D). We next interrogate the role of GSK-3 β . Interestingly, although without the positive feedback induced by LOX, the feed-forward governed by GSK-3 β alone is incapable of giving rise to bistability, the level of GSK-3 β profoundly affects the occurrence and profile of the bistable switch. Higher GSK-3 β promotes bistability but leads to reduced HIF-1 steady state level as expected, while loss of GSK-3 β could transform the dose-response from a bistable to a sigmoidal switch (Figure 4E).

Summary and Conclusions

As we have seen above, decades of experimental work using traditional biochemical approaches have uncovered a great deal of mechanistic understanding into hypoxia and HIF signaling. What has emerged is a complex picture that substitutes the notion of HIF pathway as a linear cascade of signal transduction with a novel network-based perspective, where complexity and non-linearity are appreciated. With this view, HIF is not merely a transducer but an integrator of multiple signals making it a processing hub in a network of connected interactions. The ability of HIF to precisely receive, integrate and mediate signals from multiple sources to maintain homeostasis and adequately respond to extracellular perturbations is enabled by complex feedback loops and crosstalk with other signaling pathways. Such intricate control mechanisms allow the timing and amplitude of each signaling response to be fine-tuned and kept within an appropriate range. Under disease conditions, genomic and epigenetic alterations leading to aberrant protein expression or function often disrupt these regulatory mechanisms. Understanding how these complex mechanisms operate under homeostasis and how they break down in pathogenesis is of great importance. Systems biology approaches where network-based mathematical modeling is employed to manage and model complexity will be increasingly needed to obtain a systems-level understanding of the basic functioning of HIF and hypoxia signaling, as well as to discover more effective therapeutic strategies with less adverse side effects.

Mathematical models provide a platform for the description, prediction and understanding of the various regulatory mechanisms in a quantitative and integrative way [98-100]. Although much has been learnt about the HIF pathway as a key sensor of hypoxia, many outstanding questions remain including, but not limited to, those exemplified above. These questions are not only of interest from a basic understanding of the HIF pathway viewpoint but also could aid in our effort to target HIF signaling for therapeutic purposes. Indeed, HIF is a key regulator of

metabolic homeostasis in health and in a range of disease states. Developing our understanding of the temporal and dynamic activity of this critical pathway will involve an appreciation of a systems biology approach to model the HIF pathway. This includes the integration of the canonical HIF pathway with the multiple feedback loops and levels of crosstalk which exist with other signaling networks. Interestingly but perhaps not surprisingly, due to the increased complexity of the system, studies aimed at providing an integrated, systems-level understanding the HIF signaling remain limited. We strongly believe that systems biology approaches, where mathematical modeling is integrated with experimentation in a systematic and iterative manner, will be particularly useful to disentangle the network complexity of HIF signaling and identify governing conditions that characterize the network behavior. Deployment of models with different levels of detail enables us to flexibly zoom-in and -out of the network structure which facilitates not only numerical simulations but also analytical studies. Importantly, model-based analyses and predictions are excellent ways to articulate novel hypotheses and design appropriate experiments to test them. Using this approach, we expect to develop a deep understanding of the nature of the HIF response in hypoxia and how this impacts on physiology and disease. This will be important in identifying appropriate pharmacological approaches to interfere with this pathway for therapeutic benefit.

Figure legends

Figure 1. Multiple interactions in the HIF pathway. Besides the canonical PHD-mediated effects, various additional signals can influence the HIF activity via extensive interactions between the HIF and other pathways. This complex regulatory network enables HIF to integrate a wide range of extracellular stimuli and the modulation of the HIF-mediated adaptive responses to hypoxia according to the demands posed by the variable extracellular milieu. Green arrows and red connectors indicate positive (e.g. induction or activation) and negative (inactivation) effects, respectively. Dashed arrows indicate indirect or putative interactions.

Figure 2. Connected feedback loops in the HIF regulation. (A) Simplified overview of the PHD-mediated regulatory feedbacks of HIF- α proteins. Binding of active HIF to its consensus sequences (HRE) is under the control of multiple, interlocked feedback loops mediated by HIF target gene products and metabolic intermediates alike. **(B-F) Role of PHD isoforms in shaping HIF-1 α transient dynamics. (B, C, D, E)** Model simulations of HIF-1 α time course in response to hypoxia exposure at time 0 for different values of the indicated parameters. The system was modelled to reach steady state in normoxia first before being exposed to hypoxia. **(F)** Dose-response simulation of dependence of steady-state HIF-1 α on increasing feedback strength of the PHD2 and PHD3 mediated feedbacks. Details of the model equations and values used for plotting are given in the Appendix (or Supplementary Information). **(G-K) miR-155 vs. PHD2 in shaping HIF-1 α transient dynamics. (G-J)** Model simulations of HIF-1 α time course in response to hypoxia exposure at time 0 for different values of the indicated parameters. The system was modelled to reach steady state in normoxia first before being exposed to hypoxia. **(K)** A 3D simulation plot showing the dependence of steady-state HIF-1 α fold-change (hypoxia vs. normoxia) on miR-155-HIF-1 α binding and HIF-1 α protein degradation rate. Details of the model equations and values used for plotting are given in the Appendix (or Supplementary Information). All species units are in nM.

Figure 3. Emergence of oscillation and controlling factors. (A) Illustrative simulations of different scenarios showing non-oscillatory dynamics, damped and sustained oscillations and

their dynamic transition when the cells are switched from normoxic to hypoxic conditions. Simulations were carried out using the model given in Figure 2. **(B)** Comparative knock-out simulations of HIF-1 α time course when the negative feedback loop mediated by PHD2 and miR-155 are broken. **(C)** Sensitivity analysis showing the effect of the PHD2 feedback strength on the oscillation pattern. Parameter alues used for plotting are given in the Appendix (or Supplementary Information). All species units are in nM.

Figure 4. Intensive crosstalk between the AKT/mTOR and HIF pathways. Schematic overview of the interplay between the AKT/mTOR and HIF pathway including the positive feedback loop via LOX and a coherent feed-forward loop via GSK-3 β . mTOR, that can integrate various stimuli, influences the HIF-mediated cellular responses directly by adjusting the translational rate of the HIF mRNA and indirectly via the modification of its transcriptional activity. In return, HIF induces a number of measures to regulate various elements of the mTOR pathway. **(B-E) Modeling Akt/mTORC1-HIF crosstalk.** **(B)** Simulated bistable dependence of HIF-1 α steady-state level on increasing total Akt. T₁ (T₂) indicates the Akt level threshold at which the system abruptly switches up (down) to a high (low) level of HIF-1 α , as a consequence of bistability-induced hysteresis. **(C)** Time-course simulations showing the system could settle in either one of two stable steady states when it resides within a bistable regime. **(C)** Predicted effect of changing mTORC1 activity on bistability. **(E)** Predicted effect of changing GSK-3 β abundance on bistability. Details of the model equations and values used for plotting are given in the Appendix (or Supplementary Information). All species units are in nM.

Figure 5. Interplay between the NF- κ B and HIF pathways. The PHD-HIF axis can sense inflammatory stimuli and modulate both inflammatory signalling and hypoxia response via multi-level crosstalk with the NF- κ B pathway. Green arrows and red connectors indicate positive (e.g. induction or activation) and negative (inactivation) effects, respectively.

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