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Oxygen sensing and hypoxia signalling pathways in animals: the implications of physiology for cancer

Peter J. Ratcliffe

Henry Wellcome Building for Molecular Physiology, Old Road Campus, University of Oxford, Oxford OX3 7BN, UK

Abstract Studies of regulation of the haematopoietic growth factor erythropoietin led to the unexpected discovery of a widespread system of direct oxygen sensing that regulates gene expression in animals. The oxygen-sensitive signal is generated by a series of non-haem Fe(II)- and 2-oxoglutarate-dependent dioxygenases that catalyse the post-translational hydroxylation of specific residues in the transcription factor hypoxia-inducible factor (HIF). These hydroxylations promote both oxygen-dependent degradation and oxygen-dependent inactivation of HIF, but are suppressed in hypoxia, leading to the accumulation of HIF and assembly of an active transcriptional complex in hypoxic cells. Hypoxia-inducible factor activates an extensive transcriptional cascade that interfaces with other cell signalling pathways, microRNA networks and RNA–protein translational control systems. The relationship of these cellular signalling pathways to the integrated physiology of oxygen homeostasis and the implication of dysregulating these massive physiological pathways in diseases such as cancer are discussed.

Introduction

All large organisms face the fundamental physiological challenge of matching oxygen supplies to the needs of respiring tissues. In many animal species, both large size and the capacity for rapid movement enhance the difficulty of this task. In higher animals, specialized organs evolved to facilitate oxygen delivery, and the lungs, heart, blood and vascular system are all devoted to this purpose.

The co-ordination of these complex homeostatic systems requires robust mechanisms for detecting and responding to error signals in oxygen homeostasis.

A direct oxygen sensing system that regulates gene expression in animals

In principle, detection systems that 'sense' an 'error signal' in oxygen homeostasis might operate at many different

Peter Ratcliffe is Nuffield Professor of Clinical Medicine at the University of Oxford. He trained as a Nephrologist, researching the pathophysiology of renal injury in shock, before switching fields to study the regulation of erythropoietin by the kidneys. The work on erythropoietin led to the discovery of a widespread system of gene regulation by oxygen and to the elucidation of the underlying oxygen sensing process. He has directed the hypoxia biology laboratory at Oxford for more than 20 years and has won many awards for this work including the Louis-Jeantet Prize for Medicine, the Canada Gairdner International Award and the Grand Prix Lefoulon-Delalande of the Institut de France.

levels. For instance, inadequate oxygen levels might be detected either indirectly, through the compromise of metabolism, or directly, through changes in the availability of oxygen itself. The central role of oxygen in energy metabolism makes these possibilities difficult to distinguish. However, early observations on one highly dynamic response, the increased production of the haematopoietic growth factor erythropoietin (Epo) by the kidneys, when blood oxygen is reduced, strongly suggested the existence of a process that was responding to the lack of oxygen itself (for review see Jelkmann, 1992; Bunn & Poyton, 1996). Notably, the demonstration that cobalt intoxication produces striking increases in erythropoiesis without obvious metabolic compromise (Berk *et al.* 1949) and that metabolic inhibitors were unable to mimic this effect (Necas & Thorling, 1972) pointed to a distinct oxygen sensing process. Nevertheless, the prevailing view was that this oxygen sensing system was restricted to Epo and not of general relevance to other cellular and systemic responses that defend oxygen homeostasis, a perspective that was reinforced by the distinctive, high-amplitude dynamics of the Epo response. This initially led to major efforts to identify and isolate the erythropoietin producing cells from kidney. Somewhat contrary to expectations, these cells were shown to be a population of interstitial fibroblasts without obvious specialized features (Bachmann *et al.* 1993; Maxwell *et al.* 1993*a*).

In parallel with this, and again contrary to expectations, early studies of the transcriptional control of the erythropoietin gene revealed that the underlying oxygen sensing system that regulated control DNA sequences at the erythropoietin locus was in fact not restricted to erythropoietin producing cells. A key oxygen-regulated control element, termed the erythropoietin 3′ enhancer, was shown to manifest activity that was inducible by both hypoxia and cobaltous ions (thus mimicking the characteristics of endogenous erythropoietin) when introduced by transfection into a very wide range of cells (Maxwell *et al.* 1993*b*). Many of these cells were entirely unrelated to the kidney or to the production of erythropoietin, indicating that the system must be widespread and involved in other responses. In subsequent work, it became clear that the key transcription factor, hypoxia-inducible factor (HIF), originally identified in erythropoietin producing hepatoma cells (Semenza & Wang, 1992), is indeed expressed very widely (probably universally) in mammalian cells (Wang & Semenza, 1993; Firth *et al.* 1994) and that the HIF system is conserved in primitive animal species that lack erythropoietin, red blood cells or even any specialized oxygen-delivery apparatus (Nagao *et al.* 1996; Loenarz *et al.* 2011). In human cells, pan-genomic analyses of HIF binding to DNA have now revealed the existence of in excess of 500 direct transcriptional targets of HIF in a given cell line (Mole *et al.*

2009; Xia *et al.* 2009; Schödel *et al.* 2012*b*). Recent work has demonstrated that HIF also interacts with microRNA networks (Kulshreshtha *et al.* 2007), engages in cross-talk with other signal pathways (Koshiji *et al.* 2004; Gustafsson *et al.* 2005) and acts as a non-transcriptional regulator of gene expression, for instance by affecting translation of specific genes (Uniacke *et al.* 2012). These primary activities will initiate secondary cascades of pathway activation, so that the overall complexity of activating the HIF response is enormous.

Hypoxia-inducible factor binds to DNA as an α/β heterodimer; both α and β subunits belonging to the basic helix-loop-helix PAS protein family (Wang *et al.* 1995). The β subunits are constitutively expressed and form heterodimers with several other members of this family to mediate other transcriptional responses, whilst the α subunits are specifically involved in the response to hypoxia. Early work that aimed at defining the upstream pathways connecting HIF to the signalling of oxygen levels defined discrete domains within the HIF polypeptide that could independently convey oxygen sensitivity on heterologous transcription factors (Jiang *et al.* 1997; Pugh *et al.* 1997; Huang *et al.* 1998). This work suggested a dual process of regulation, because some of these domains conveyed oxygen-dependent instability, whereas some appeared to affect transcriptional activity independent of stability (Fig. 1; Pugh *et al.* 1997). All domains shared the property of responding both to hypoxia and to cobalt stimulation, suggesting that they interacted independently with the same, or a closely similar, oxygen sensing signal. It was widely believed that, in common with most intracellular signalling pathways, protein phosphorylation would be involved in signal transduction. However, mutation of every phospho-acceptor residue in one of the isolated regulatory domains of HIF failed to ablate its oxygen sensitivity, suggesting that this signal process could operate independently of protein phosphorylation, thus excluding many known transduction mechanisms (Pugh *et al.* 1997).

An important insight into the upstream processes regulating HIF was gained through the recognition that the von Hippel-Lindau tumour suppressor (pVHL) was essential for proteolytic regulation of HIF-α, functioning as a ubiquitin ligase, targeting each of two internal HIF-α domains to mediate oxygen-dependent instability (Maxwell *et al.* 1999; Cockman *et al.* 2000; Ohh *et al.* 2000; Tanimoto *et al.* 2000; Masson *et al.* 2001). Detailed biochemical, mutational and mass spectrometric analysis of this interaction revealed the regulatory modification that governs the binding of HIF-α to pVHL to be oxygen-dependent *trans*-4-hydroxylation at specific prolyl residues within each of two regulatory domains in HIF-α (Pro-402 and -564 in human HIF-1α; Ivan *et al.* 2001; Jaakkola *et al.* 2001; Masson *et al.* 2001). A combination of structurally informed bioinformatic prediction and testing of candidate enzymes then led to the

identification of a single non-redundant prolyl hydroxylase in *Caenorhabditis elegans* and to the recognition that the human genome encodes three closely related HIF prolyl hydroxylases, which we termed PHD (prolyl hydroxylase domain) 1, 2 and 3 (also known as Egln 2, 1 and 3, respectively; Bruick & McKnight, 2001; Epstein *et al.* 2001). These enzymes are dioxygenases, and their absolute requirement for molecular oxygen as cosubstrate immediately suggested a mechanism for oxygen sensing. Though all three enzymes contribute to the regulation of HIF, in most cells PHD2 (Egln1) is the most abundant enzyme and therefore has a dominant role in the oxygen sensing process (Berra *et al.* 2003; Appelhoff *et al.* 2004). Prolyl hydroxylated HIF-α is recognized by the β-domain of pVHL through a hydrogen bonding network to the hydroxyproline residue, which cannot be formed by proline (Hon *et al.* 2002; Min *et al.* 2002). Added specificity to this 'molecular switch' is achieved by stereoelectronic effects that alter the bias of the pyrrolidine ring of the prolyl residue from the C4-endo to the C4-exo conformation upon hydroxylation. Specific binding of pVHL to the C4-exo conformation of the hydroxyprolyl residue results in a >1000-fold increase in affinity of HIF-α for pVHL upon hydroxylation, thus promoting the destruction of HIF-α in the presence of oxygen (Loenarz *et al.* 2009).

Similar biochemical and mass spectrometric analysis of the third (C-terminal) sequence, which had been shown to mediate oxygen-dependent transcriptional activity independently of any change in protein stability, revealed a third site of HIF-α hydroxylation (Lando *et al.* 2002*b*).

In this case, the target of hydroxylation is a specific asparaginyl residue (Asn-803 in human HIF-1α). This residue is buried in a hydrophobic region that is formed when C-terminal HIF-α sequences are bound by the CH1 domain of the p300 coactivator (Dames *et al.* 2002). Hydroxylation at the asparaginyl residue reduces transcriptional activity of HIF-α, at least in part by preventing this interaction with p300 (Fig. 1). Further bioinformatic analyses identified the HIF asparaginyl hydroxylase as a molecule termed FIH (factor inhibiting HIF; Hewitson *et al.* 2002; Lando *et al.* 2002*a*), a protein that had previously been identified in a screen for HIF interacting proteins and shown to inhibit HIF activity (Mahon *et al.* 2001).

The HIF hydroxylases (three HIF prolyl hydroxylases and the HIF asparaginyl hydroxylase) are all members of the 2-oxoglutarate (2-OG)-dependent dioxygenase super-family (for review see Loenarz & Schofield, 2008). These enzymes are non-haem iron-dependent enzymes that bind the catalytic iron using a 2-histidine-1-carboxylate 'facial triad' that occupies three of the six iron co-ordination sites (Fig. 2). The enzymes use the Krebs cycle intermediate 2-OG (α-ketoglutarate) as cosubstrate; as 'dioxygenases', they split molecular oxygen and incorporate both atoms directly into their reaction products (for review see Loenarz & Schofield, 2011). Thus, oxidization of the prime substrate (HIF-α) is coupled to the oxidative decarboxylation of 2-OG to succinate. Based on mechanistic studies of other 2-OG dioxygenases, it is likely that the reaction proceeds via the creation of
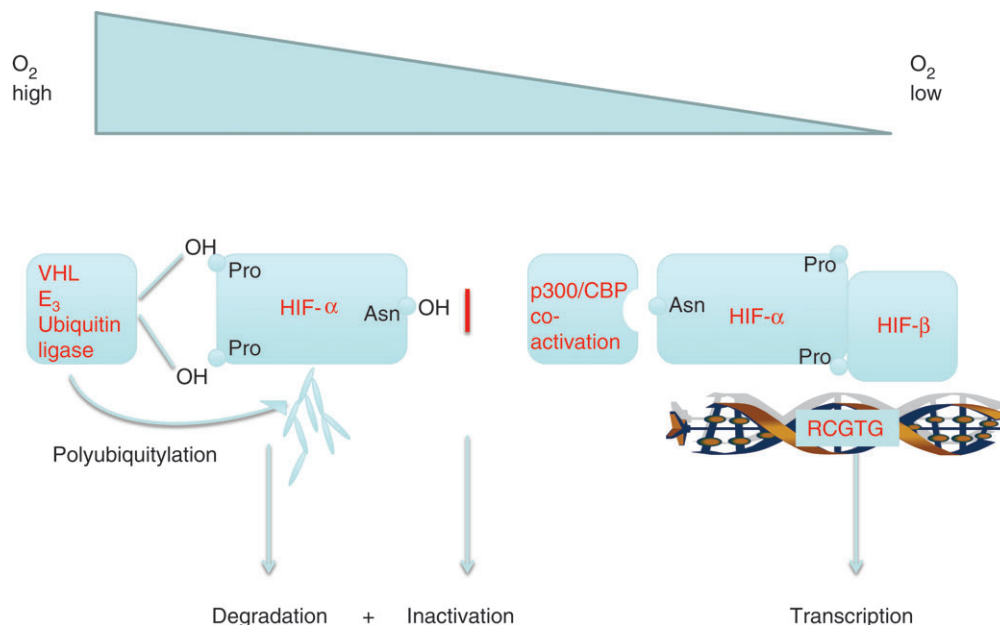
Figure 1. Schematic diagram illustrating the dual regulation of hypoxia-inducible factor (HIF) by oxygen-dependent prolyl and asparaginyl hydroxylation.
Pro, proline; OH Pro, hydroxyproline; Asn, asparagine; Asn OH, hydroxyasparagine; p300/CBP, E1A binding protein p300/CREB-binding protein.

a highly reactive [e.g. Fe(IV) = O (ferryl iron)] intermediate at the catalytic site (Price *et al.* 2003). Failure of the coupling process can leave the enzyme in an inactive oxidized state, and reduction of the catalytic iron centre is then required for activity. This process is considered to be the basis of the dependence of many members of this class of enzyme (including the HIF hydroxylases) on the reducing agent ascorbate. Direct utilization of molecular oxygen confers sensitivity to hypoxia, underpinning the role of these enzymes as intracellular oxygen sensors. Interestingly, whilst 2-OG oxygenases are prevalent in both eukaryotic and prokaryotic species (Loenarz & Schofield, 2008), and many primitive organisms exhibit well-defined programmes of gene regulation by oxygen (Bunn & Poyton, 1996), including systems that use 2-OG oxygenases (West *et al.* 2007; Hughes & Espenshade, 2008), the HIF hydroxylase (PHD/pVHL/HIF) system appears to be specific to (and universal within) the animal kingdom (Loenarz *et al.* 2011). Most vertebrate organisms express multiple PHD and HIF-α isoforms as a result of gene duplication, PHD2 and HIF-1α being the most universally expressed (Loenarz *et al.* 2011).

In the following sections, some of the biochemical and physiological characteristics of this system of oxygen sensing are reviewed, the challenges faced in linking molecular mechanisms to integrated physiology are highlighted, and the implications of dysregulation of these massive 'hard-wired' HIF hydroxylase pathways in disease are considered, particularly in relation to solid tumours, which commonly manifest tissue hypoxia and upregulation of HIF.

Biochemical aspects of oxygen sensing by HIF hydroxylases

It is estimated that the human genome encodes as many as 60–70 predicted or biochemically assigned 2-OG oxygenases with diverse roles in biology (Loenarz & Schofield, 2008). This has raised an important question as to whether the oxygen sensing function of the HIF hydroxylases is associated with some special biochemical property of these particular enzymes or whether they are biochemically similar to other 2-OG dioxygenases, but derive the oxygen sensing property from the physiological context in which they operate.

Values for K_mO_2 (the concentration of oxygen required for half-maximal initial catalytic rate) have been reported for the HIF prolyl hydroxylases to be in the range 200–250 μM (Hirsilä *et al.* 2003). These values are unusually high, but were obtained using recombinant enzymes and short HIF-α peptide substrates. Subsequent studies using longer HIF polypeptides have given lower values, in the region of 100 μM, which are similar to those reported for other 2-OG dioxygenases (Koivunen *et al.* 2006; Ehrismann *et al.* 2007). In any case, physiological tissue P_{O_2} levels are generally found to be substantially below even the lower K_mO_2 values (Vaupel *et al.* 1991), and intracellular gradients probably create even lower values in the intracellular microenvironment. Therefore, even enzymes with a much lower K_mO_2 would have the potential to act as oxygen sensors if their catalytic action was rate limiting for the overall physiological function of the pathway. Thus, it appears unlikely that the unusually high K_mO_2 values are relevant to the function of the HIF hydroxylases as oxygen sensors.

A different characteristic that has attracted considerable interest is the possibility that the multiple cofactor requirements that are common to this class of enzyme provide additional means of regulating activity that assist the complex task of maintaining oxygen homeostasis (Schofield & Ratcliffe, 2004; Kaelin & Ratcliffe, 2008). For instance, 2-oxoglutarate is a Krebs cycle intermediate which (via transamination and deamidation reactions) also occupies a pivotal position in amino acid metabolism.
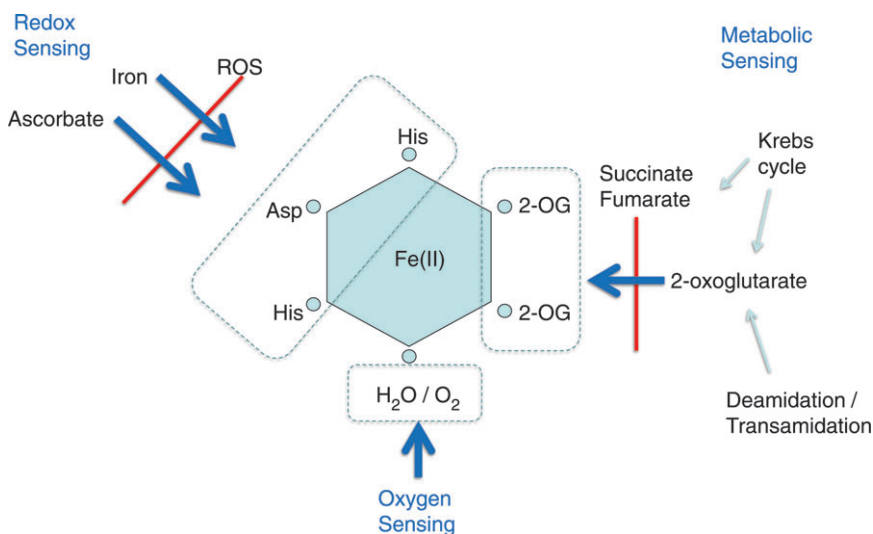
Figure 2. The catalytic iron centre of a typical 2-oxoglutarate (2-OG)-dependent dioxygenase
Of six co-ordinate positions, three are used to bind the 2-histidine-1-carboxylate 'facial triad' of the apo-enzyme, two are used to bind the cosubstrate 2-oxoglutarate and the sixth is occupied by a water molecule or (upon activation of the enzyme) molecular oxygen. Potentially, these interactions provide signalling interfaces with molecular oxygen, redox stresses and metabolism.

Thus, the use of both molecular oxygen and 2-oxoglutarate in the hydroxylation reaction might provide a link between hypoxic and metabolic signals (Isaacs *et al.* 2005; Hewitson *et al.* 2007; Koivunen *et al.* 2007). To date, however, it is unclear whether and in what circumstances 2-oxoglutarate levels are limiting for HIF hydroxylase activity. Perhaps of greater interest to oxygen sensing is the requirement for Fe(II) as cofactor. The binding of Fe(II) by the 2-histidine-1-carboxylate facial triad of the apo-enzyme is relatively labile. For instance, in contrast with haem enzymes, the catalytic Fe(II) can readily be chelated, thus inactivating the enzyme (Hirsilä *et al.* 2005). This lability (and the need for ascorbate to maintain the iron centre in a reduced and active form) could provide an inter-face both with cellular iron availability and with cellular redox signals. Redox signals are linked to the availability of oxygen. Thus, the possibility that such processes could provide a second oxygen-sensitive signal inter-acting with regulation by molecular oxygen availability, or even dominate the regulation of HIF hydroxylase activity, has attracted considerable interest. Substantial evidence indicates that the HIF hydroxylases can indeed be inhibited by oxidant stress.

In cultured cells, supplementation with both iron and ascorbate reduces the levels of HIF-α that are observed in standard (oxygenated) conditions (Knowles *et al.* 2003, 2006; Kuiper *et al.* 2010), implying that the availability of these cofactors can be limiting for full enzyme activity even in the presence of oxygen. Exogenous sources of reactive oxygen species inhibit HIF hydroxylases both *in vitro* and in cells (Gerald *et al.* 2004; Pan *et al.* 2007). Taken together, these findings strongly suggest that 'oxygen sensing' hydroxylase activity is modulated by redox signals, at least in pathophysiological conditions. What is less clear is how this relates to the physiological signalling of hypo-xia. While excess oxygen (hyperoxia) can cause cell injury through the excess production of reactive oxygen species, and some investigators have reported that hypoxia reduces the production of reactive oxygen species (Hoffman *et al.* 2007), other investigators have assembled considerable evidence that inhibition of electron transport in hypoxia leads to increased production of reactive oxygen species and have proposed that this, rather than lack of molecular oxygen itself, is responsible for the reduction in HIF hydro-xylase activity that signals hypoxia (Chandel *et al.* 1998; Bell *et al.* 2007). Of relevance to this, researchers in my laboratory recently performed comparative analyses of HIF prolyl and asparaginyl hydroxylation in cells. These studies revealed that whilst HIF prolyl hydroxylation is more sensitive to hypoxia than HIF asparaginyl hydro-xylation, the reverse was true when cells were exposed to hydrogen peroxide to mimic oxidant stress (Tian, 2011; Masson *et al.* 2012). Hypoxia-inducible factor asparaginyl hydroxylation was found to be strongly inhibited at very low concentrations of hydrogen peroxide that had

little, if any, effect on HIF prolyl hydroxylation (Masson *et al.* 2012). Furthermore, reoxygenation of hypoxic cells promotes immediate hydroxylation and degradation of HIF, whereas recovery from oxidant inactivation, at least for the HIF asparaginyl hydroxylase FIH, was found to be delayed (Tian, 2011; Masson *et al.* 2012). These findings suggest that hypoxia and oxidant stress represent separate regulatory inputs to the HIF hydroxylase pathways.

What is not currently clear is whether differential sensitivity of HIF prolyl and asparaginyl hydroxylation to peroxide represents unusual sensitivity of FIH or unusual resistance of PHD2 (among the 2-oxoglutarate dioxygenase enzyme family). Interestingly, biophysical analyses have revealed two unusual properties of PHD2 (the major HIF prolyl hydroxylase) that could be relevant; firstly, the enzyme binds to Fe(II) unusually tightly (McNeill *et al.* 2005); and secondly, in the absence of HIF substrate, the enzyme complex is unusually stable, even in the presence of oxygen (Flashman *et al.* 2010; Loenarz *et al.* 2011). Furthermore, structural studies of the HIF prolyl hydroxylase PHD2 and the HIF asparaginyl hydroxylase FIH complexed to their respective HIF peptide substrates show major differences that might relate to the functional findings. Whilst FIH binds its substrate in an open cleft (Elkins *et al.* 2003), the binding of target HIF-α peptides to PHD2 is more closed and results in a conformational change in which a loop of PHD2 shifts to form a clamp-like structure over the substrate (Chowdhury *et al.* 2009). Both the tight binding of Fe(II) and the stability of the PHD2 enzyme complex in the absence of substrate (reduced susceptibility to uncoupled turnover) may be predicted to reduce sensitivity to inactivation by oxidant stress. It is thus tempting to speculate that these properties are important for the function of PHD2 as a sensor that responds most specifically to molecular oxygen.

Nevertheless, it is clear that all HIF hydroxylases are, to different degrees, sensitive to oxidant injury (Pan *et al.* 2007; Diebold *et al.* 2010). Moreover, given that hypoxia does not suppress the sensitivity to peroxide, it is likely that the two stimuli interact (Masson *et al.* 2012). This raises the intriguing possibility that oxidant stress, by inactivating a proportion of the expressed HIF hydroxylase enzyme, acts as a 'range finding' mechanism that alters the effective oxygen sensing 'set point'. In this model, the rate of hydro-xylation at any particular oxygen tension is reduced by oxidant stress, making the system more sensitive to hypo-xia and switching on HIF pathways that may defend against the stress.

## Range finding mechanisms; linking biochemistry to physiology

Such 'range finding' effects are likely to be of funda-mental importance in linking the biochemistry of HIF

hydroxylases to integrated physiology. Thus, a key principle for operation as 'oxygen sensors' is that oxygen-dependent rates of hydroxylation are matched to the rates of other processes involved in the regulation of HIF (Fig. 3). For instance, if rates of HIF-α translation and/or rates of HIF-α degradation downstream of the hydroxylation step were low, then hydroxylation of HIF-α might readily run to completion even in very severe hypoxia. The system would then not be oxygen regulated. Therefore the biochemical process of oxygen-dependent hydroxylation must be 'on range' to convey oxygen sensitivity on the physiological pathway.

The need for 'range finding' mechanisms is emphasized by the diversity of the integrated physiology that is connected to HIF. This includes not only well-characterized aspects of oxygen homeostasis, such as cardiopulmonary and ventilatory control (Smith *et al.* 2008; Prabhakar & Semenza, 2012), but also diverse aspects of cell physiology, such as lymphocyte, neutrophil and macrophage functions that are not classically associated with hypoxia signalling but are dysregulated in animals bearing inactivating alleles of HIF-α isoforms (Cramer *et al.* 2003; Walmsley *et al.* 2005; Takeda *et al.* 2010; Dang *et al.* 2011). Although the exact role of tissue hypoxia in many of this processes is not known,

it is clear that in the intact organism oxygen-dependent regulation of HIF can be observed in diverse tissues operating at strikingly different 'set points'. This is well illustrated by studies in the kidney, which harbours particularly large oxygen gradients (Leichtweiss *et al.* 1969). Immunohistochemical studies of the induction of HIF-α protein in rodents subjected to anaemic/hypoxic stimulation reveal similar regulatory characteristics for HIF in tubular segments that are operating at tissue P_{O_2} levels that differ by an order of magnitude (Leichtweiss *et al.* 1969; Rosenberger *et al.* 2002). Although the integrative mechanisms that define tissue/cell-specific 'range finding' are not understood, many analyses have defined properties (both of the hydroxylases and connected pathways) that could contribute to such 'range finding' processes.

Abundance and location of the HIF hydroxylases. Any process that alters the effective activity of the hydroxylase enzyme has the potential to change the oxygen sensing 'set point'. Thus, it is of interest that transcriptional induction of the HIF prolyl hydroxylases by HIF itself is a highly conserved feature of the pathway, observed for at least one PHD enzyme in all species examined to date, from the 'basal' animal *Trichoplax adherens* to
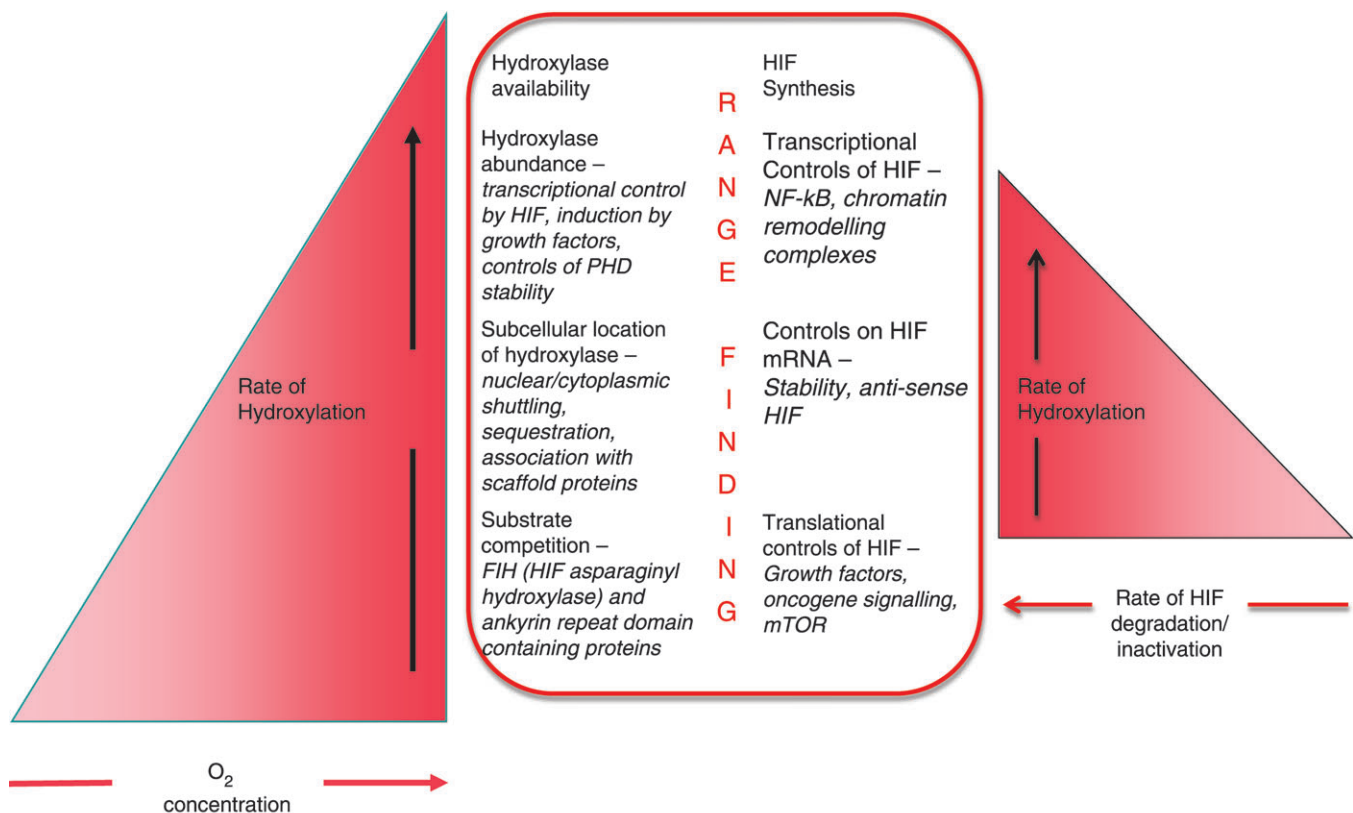
Figure 3. The principle of 'range finding' processes that match HIF prolyl hydroxylation to the rate of synthesis of HIF and to the capacity of HIF degradation pathways downstream of hydroxylation.
NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; mTOR, mammalian target of rapamycin.

man (Epstein *et al.* 2001; Stiehl *et al.* 2006; Loenarz *et al.* 2011). Although, experimentally, these responses are usually analysed with respect to time (hence, they are described as a dynamic feedback response, which limits HIF activation over a period of time), they would be equally well fitted for a physiological role, perhaps in 'range finding' across cells operating at different oxygen tensions; i.e. in tissues operating at low oxygen levels, induction of PHD expression would lower the operating range for regulation by hydroxylation and match this range more closely to tissue oxygen tension.

Other controls over effective hydroxylase activity are likely to be exerted by intracellular processes that affect the subcellular location of the enzyme and/or its access to HIF substrate. For instance, binding of the principal HIF prolyl hydroxylase enzyme PHD2 to the peptidyl-prolyl *cis-trans* isomerase FKBP38 is proposed to limit PHD2 activity by a process that involves membrane anchoring and proteasomal degradation but is independent of the isomerase activity (Barth *et al.* 2009). It has also been proposed that PHD enzymes operate in a spatially restricted context through association with scaffold proteins (Foxler *et al.* 2012). Cellular compartmentalization may provide another control. For instance, it has been shown that PHD2 undergoes nuclear–cytoplasmic shuttling and that the deletion of PHD2 sequences required for nuclear entry is associated with a greatly impaired capacity to downregulate HIF (Pientka *et al.* 2012). Whether and how such processes are used to tune the PHD/pVHL/HIF system physiologically is unclear, but biological control at this level is supported by the observation of associations between nuclear localization of PHD2 and aggressive cancer phenotypes (Jokilehto *et al.* 2010).

Yet another possible means of regulating enzyme activity has been raised by recent studies of the HIF asparaginyl hydroxylase, FIH. This enzyme catalyses the hydroxylation of many other substrates, in particular asparaginyl residues that form part of the consensus 'ankyrin repeat' in the family of 'ankyrin repeat domain' containing proteins (Cockman *et al.* 2006). These proteins show a high affinity for FIH and are abundant in cells, so that they might be predicted to compete with HIF for hydroxylation (Coleman *et al.* 2007; Wilkins *et al.* 2009). Given that competition from ankyrin substrates will be dependent on the hydroxylation status of those substrates (itself determined by oxygen availability), this type of 'endogenous competition' could increase the complexity of the interface with oxygen levels and contribute to 'range finding'.

Components of the VHL/HIF system. In addition to control of the 'oxygen sensing' hydroxylase enzymes, the integrated function of the PHD/pVHL/HIF pathway

could potentially be tuned by multiple feedback controls operating at other steps in the pathway, which effectively downregulate the pathway after a period of HIF activation. Although these processes have generally been viewed as temporal feedback responses, they also have the potential to act as range finding mechanisms. They include the upregulation of an antisense HIF-1α transcript, which downregulates the HIF-1α coding mRNA (Rossignol *et al.* 2002; Uchida *et al.* 2004), induction of HIF-3α, an isoform that can antagonize HIF transcription by forming transcriptionally inactive complexes with other HIF-α proteins (Makino *et al.* 2001, 2007), and the induction of Rbx-2 (Tan *et al.* 2008), a component of the pVHL ubiquitin E3 ligase that promotes more rapid destruction of HIF-α proteins. In all of these processes, the upregulated 'antagonist' is a direct transcriptional target of HIF itself. Interestingly, given that they impinge differently on different HIF-α isoforms (HIF-1α and HIF-2α), which have different spectra of transcriptional targets, these mechanisms also have the potential to alter the character of the transcriptional output.

Finally, one crucial property of the PHD/pVHL/HIF system is that bidirectional signalling is achieved by the rapid resynthesis of HIF protein. Although the signalling pathways that regulate HIF protein synthesis have not been studied as intensively as pathways of degradation, it is clear that steady-state levels will reflect a balance between degradation and synthesis. Thus, at any given oxygen concentration (and rate of hydroxylation) the steady-state level of HIF (hence, the 'oxygen sensing poise' of the system) will be affected by the synthetic rate (Fig. 3). In this context, many growth promoting stimuli enhance HIF translation, presumably reflecting the need to entrain mechanisms for modulating basal oxygen homeostasis as demand is increased (Zundel *et al.* 2000; Laughner *et al.* 2001; Brugarolas & Kaelin, 2004).

Overall, whilst many mechanisms have been defined that could potentially provided 'range finding' controls for the oxygen sensing HIF hydroxylases, understanding whether and precisely how these operate to maintain oxygen homeostasis in large animals remains far from being completely understood. Nevertheless, genetic data clearly indicate that genes and physiological processes operating with different dynamics and at different oxygen tensions are regulated by the same HIF hydroxylase oxygen sensing system. This has some general implications for understanding interfaces between molecular and integrative physiology. In particular, it implies that inferring the existence of distinct molecular mechanisms from the quantitative properties of individual integrated physiological responses may be misleading (as witnessed by the erroneous view that erythropoietin was regulated by a 'private' oxygen sensing system in kidney cells). As a corollary, it suggests that precise predictions of effects on integrated physiology from a simple understanding

of the molecular process of signal generation will also be difficult. This is now of fundamental importance in assessing the therapeutic potential of HIF hydroxylase inhibitors (Hewitson & Schofield, 2004; Fraisl *et al.* 2009); careful empirical analysis through experimental medicine and integrated physiological approaches will probably be required for this to succeed.

Hypoxia and cancer

Molecular analysis of the HIF hydroxylase system and hypoxia signalling pathways has revealed enormous complexity. In addition to the challenge of understanding the operation of these pathways in integrated physiology, this has major implications for the understanding of disease. In this final section, the importance of these physiological considerations is illustrated by reference to hypoxia and cancer.

It has long been recognized that solid tumours frequently contain regions of profound hypoxia and that tumour hypoxia is associated with adverse prognosis. This association has been reported to be independent of treatment modality (surgery, radiotherapy or chemotherapy) and to be observed in many different types of tumours (Höckel *et al.* 1993; Brizel *et al.* 1996). In keeping with these early observations, numerous studies have reported that activation of HIF is associated with aggressive tumour behaviour and adverse prognosis (Chi *et al.* 2006; Winter *et al.* 2007; for review see Semenza, 2003, 2012). These associations are extremely robust and, indeed, enhanced glucose uptake (in part, a consequence of transcriptional upregulation of specific glucose transporters and glycolytic enzymes by HIF; Semenza, 2010) underpins 18-fluorodeoxyglucose positron emission tomographic scanning in the diagnosis of cancer. Therefore, a central question has emerged concerning the role played by the activation of HIF signalling pathways in cancer and whether this drives the malignant phenotype.

Two classes of observation have lent support to this possibility. Firstly, as successive HIF targets have emerged from investigation of the molecular physiology of the system, it has become clear that many classical 'cancer-associated' properties (such as invasion and angiogenesis) involve the activation of HIF target genes (for review see Semenza, 2003). Secondly, many oncogenic pathways have been found to be mechanistically linked to the activation of HIF (Maxwell *et al.* 1999; Zundel *et al.* 2000; Laughner *et al.* 2001; Brugarolas & Kaelin, 2004; Bernardi *et al.* 2006). Most strikingly, mutation of the von Hippel-Lindau tumour suppressor (pVHL) disables the key ubiquitin E3 ligase complex that is necessary for degradation of HIF, leading to constitutive stabilization of HIF-α proteins and activation of the pathway (Maxwell *et al.* 1999). Other tumour suppressor and oncogenic

pathways are linked to HIF activation through different mechanisms. For instance, classical tumour suppressor mutations in PTEN, promyelocytic leukaemia gene (PML) and TSC activate HIF by promoting translation of HIF-α proteins (Zundel *et al.* 2000; Brugarolas & Kaelin, 2004; Bernardi *et al.* 2006). Even unusual and poorly understood oncogenic pathways, such as those activated by mutations in the Krebs cycle enzymes fumarate hydratase and succinate dehydrogenase activate HIF (Isaacs *et al.* 2005; Pollard *et al.* 2005; Selak *et al.* 2005). In these conditions, HIF activation is a consequence of inhibition of the HIF prolyl hydroxylases by accumulated fumarate and/or succinate, which act as 2-oxoglutarate analogues (Hewitson *et al.* 2007; O'Flaherty *et al.* 2010). If oncogenic mutations activate HIF, HIF activation is associated with aggressive cancer, and HIF target genes include many with established oncogenic associations, it is therefore tempting to infer the existence of a simple connection whereby general activation of HIF causes cancer or at least causes cancer progression.

Oddly, however, despite the mechanistic links between oncogenic mutation and upregulation of HIF, activating mutations of HIF itself (for instance deletions or mutations in the degradation domain) have not been described in cancer genome sequencing programmes. Moreover, although in experimental systems genetic inactivation of HIF usually has a negative effect on tumour growth (reviewed by Semenza, 2003), this is not always the case. For instance, HIF activation is clearly observed in fumarate hydratase-associated human kidney cysts and cancer (Isaacs *et al.* 2005; Pollard *et al.* 2005). However, in a mouse model of the disease caused by kidney-specific inactivation of fumarate hydratase and characterized by hyperplastic renal cyst development, combined inactivation of HIF-1α in association with inactivation of fumarate hydratase exacerbates rather than suppresses disease (Adam *et al.*). The effect of inactivating a particular component of the HIF system also appears to be highly context specific. For instance, genetic inactivation of HIF-1α in transformed murine astrocytes has positive or negative effects on tumour growth depending on the microenvironment in which the cells are grown (Blouw *et al.* 2003).

In other situations, inactivation of different components of the HIF pathway has different or even opposing effects on tumour growth. This has been most clearly established in pVHL-defective clear cell renal cancer (CCRC). Inactivation of the pVHL ubiquitin E3 ligase leads to constitutive activation of the entire HIF pathway (Maxwell *et al.* 1999). However, genetic studies on CCRC cell lines grown as xenografts have clearly indicated that oncogenic drive is restricted to specific components of the HIF pathway. Thus, whilst HIF-2α overexpression promotes tumour growth, HIF-1α overexpression has the reverse effect (Maranchie *et al.* 2002; Kondo *et al.*

2003; Raval *et al.* 2005; Shen *et al.* 2011). Interestingly, in support of an antitumourigenic action of HIF-1α in this context, human VHL-defective CCRC is associated with a high prevalence of copy number reduction for a region of chromosome 14q containing HIF-1α, amongst other genes (Shen *et al.* 2011). Furthermore, cancer DNA sequencing has revealed a small but significant number of inactivating mutations in HIF-1α in VHL-defective CCRC (Morris *et al.* 2009; Dalgliesh *et al.* 2010). Large-scale studies of human CCRC predisposition have revealed polymorphisms at the HIF-2α locus and a HIF-2α-dependent transcriptional enhancer of cyclin D1 as CCRC susceptibility determinants (Purdue *et al.* 2010; Schödel *et al.*). Although it has been argued that HIF-2α is generally more protumourigenic than HIF-1α (Keith *et al.* 2012), and this is strongly supported by data in CCRC, protumourigenic effects of HIF-2α are again inconsistent, with inactivation of HIF-2α promoting tumour growth in other contexts (Acker *et al.* 2005; Majmundar *et al.* 2010).

Thus, despite extraordinarily strong and consistent associations between hypoxia, general activation of the HIF system and aggressive cancer, a simple causal association is not consistently supported by genetic evidence. Rather, these studies suggest that specific components (as opposed to general activation of the HIF system) are responsible for protumourigenic actions, that some components of the HIF system are antitumourigenic, and that pro- *versus* antitumourigenic actions of HIF are highly context specific. This review concludes with an outline of how consideration of the massive complexity of physiological hypoxic signalling may explain this paradox and consideration of the implications for the understanding of cancer and its treatment.
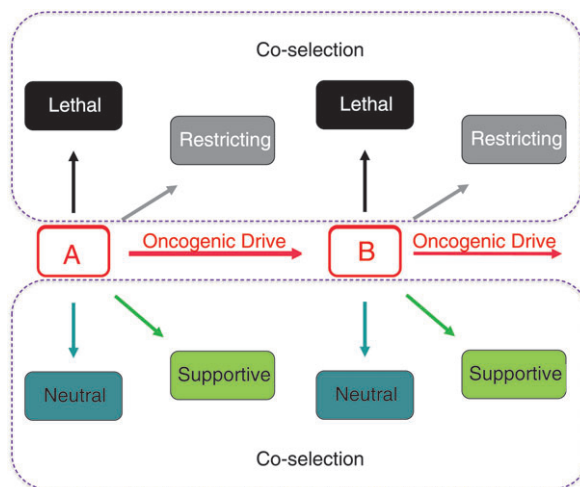
Figure 4. Coselection of extensive hard-wired physiological pathways in cancer
Oncogenic mutations (A) and (B) provide a cell with autonomous advantage, hence oncogenic drive, but are physiologically linked to pathways whose activation is supportive, neutral, restrictive or even lethal.

Given that increases in tissue mass create a demand for oxygen, growth pathways (which are dysregulated in cancer) are physiologically linked to the activation of HIF pathways in order to maintain oxygen homeostasis. However, physiological homeostatic responses to hypoxia may achieve their purpose by diverse mechanisms, encompassing both enhancement of oxygen delivery (e.g. angiogenesis, erythropoiesis) and restriction of oxygen demand (e.g. cytostasis, altered energy metabolism). As outlined above, recent analyses of HIF hydroxylase pathways have revealed that they are extraordinarily complex. When activated by oncogenic mutations or even by microenvironmental hypoxia in cancer, these massive pathways, which evolved to be fit for a physiological (not oncogenic) purpose, will be activated in their entirety. Whether they are promoting oncogenesis, neutral or even restricting the oncogenic process, all components of the pathway will be activated in the cancer as a consequence of the physiological 'hard wiring' of the network (Fig. 4). What are the implications of this?

The first implication is simple; that tight associations may arise simply because of physiological 'hard wiring' and do not necessarily imply causation, explaining the discrepancies between the robust association of HIF activation with cancer and the variable findings in genetic intervention studies. Other implications are less obvious. For instance, coselection provides a potential explanation for the appearance in cancer of properties that are difficult to rationalize on the basis of the standard genetic model of cancer, which is classically considered to be a multistep process involving clonal selection of cells on the basis of cell autonomous advantage (Vogelstein & Kinzler, 1993). For instance, it is difficult to understand how such selection could operate to generate an 'angiogenic switch' (Hanahan & Folkman, 1996) to improve the blood supply to the tumour, because the advantage would be equally available to neighbouring cells in clonal competition. Coselection of physiologically hard-wired pathways connecting growth to oxygen supply (angiogenesis) would provide a more credible explanation (Fig. 5).

However, the most profound implications arise from consideration of the penalty that is entrained by the switching of massive physiological pathways, such as the hypoxia response, where not all components of the pathway are protumourigenic (Fig. 5). The balance between penalty and oncogenic advantage of dysregulating a physiological pathway is likely to be specific to the cellular context; in the case of hypoxia signalling pathways, reflecting cell-specific strategies for restoration of oxygen homeostasis. Such mechanisms would explain the contextual specificity of genetic interventions on the HIF system and (more generally) the striking tissue specificity of cancer predisposition that is often manifested by ubiquitously expressed tumour suppressors. For instance, the PHD/pVHL/HIF system is ubiquitous in animal cells,

but VHL-associated cancer is highly tissue specific, being largely confined to renal cancer, phaeochromocytoma and ocular/CNS haemangioblastoma (reviewed by Kaelin, 2008). Moreover, across the mutational spectrum of VHL disease there is a strong correlation between the quantitative level of HIF dysregulation and the type of tumour predisposition (Clifford *et al.* 2001; Hoffman *et al.* 2001; Li *et al.* 2007; Shen *et al.* 2011). High-level HIF dysregulation appears to be inconsistent with the development of phaeochromocytoma but consistent (or even required) for renal clear cell carcinoma development. Thus, it appears that whether the HIF pathway is driving cancer or simply needing to be compatible with cancer development, the extent of activation needs to be 'just right', reflecting the need to balance the penalty incurred by coselection of 'hard-wired' physiological pathways against the oncogenic drive. Assuming this, one might expect alterations in the physiological pathway to become apparent during the course of cancer development. This is clearly illustrated in VHL-associated CCRC. Following VHL inactivation in kidney tubules, the HIF system shifts from being dominantly represented by HIF-1α (the normal renal tubular epithelial isoform) to being dominantly represented by HIF-2α (Mandriota *et al.* 2002; Rosenberger *et al.* 2002). The mechanisms behind the switch are largely unclear, but the shift appears to be associated with an alteration in the balance of activated HIF targets towards a more oncogenic profile (Raval *et al.* 2005; Keith & Simon, 2007).

I would like to conclude with two further speculations on the implications of these arguments for cancer therapy. Firstly, the need to avoid or limit the coselection penalty entrained by the activation of physiological pathways provides strong selective pressures for plasticity and heterogeneity. Given that multiple microenvironmental stresses (not limited to hypoxia) will be entrained as a solid tumour grows, it seems likely that the need to evade the penalty of the dysregulated activation of massive physiological pathways will select for the plasticity and heterogeneity that are characteristic of solid tumours and are considered to underlie escape from targeted chemotherapeutics. Secondly, the penalty of coselecting massive physiological pathways, such as the hypoxia response, makes cancer cells intrinsically vulnerable. For this reason alone, they may therefore manifest enhanced susceptibility to further stress, such as chemotherapeutic agents; i.e. the chemotherapeutic selectivity of many agents may be due (at least in part) to general cellular sensitivity of cancer cells, as much as to intrinsic selectivity of drug action. Thus, the penalty (and means of escape) from the anti-tumourigenic components of activating massive physiological pathways in cancer might account for much of the recent positive and negative experience in targeted cancer therapeutics; useful chemotherapeutic selectivity from agents that might otherwise be predicted to affect both neoplastic and non-neoplastic cells, and rapid escape from control in tumours that initially appear very sensitive to such agents.
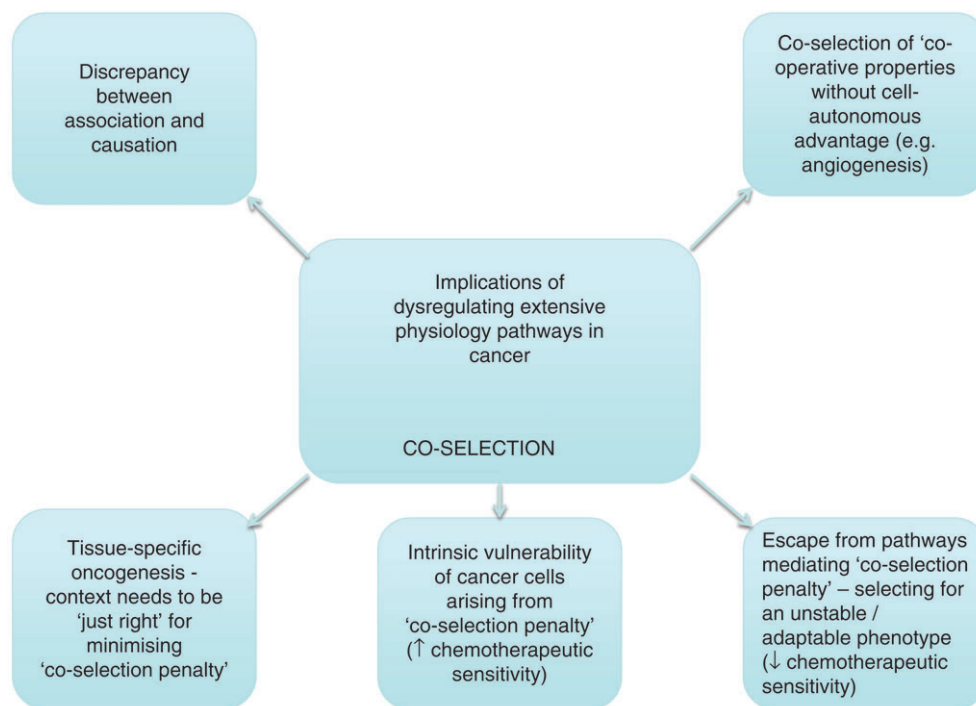
Figure 5. Implications of the physiological 'coselection penalty' in cancer

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