

Genetic approaches to understand cellular responses to oxygen availability

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Running title: Genetic approaches and oxygen-sensing

Abbreviations

ccRCC, Clear Cell Renal Cell Carcinoma

CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats

FIH-1, Factor Inhibiting HIF-1

FH, Fumarate Hydratase

HIF, Hypoxia Inducible Factor

OGDHC, 2-oxoglutarate dehydrogenase complex

PHD, Prolyl Hydroxylase

SDH, Succinate Dehydrogenase

TCA, Tricarboxylic Acid

VHL, Von-Hippel Lindau

2-OG, 2-oxoglutarate

2-OG DD, 2-oxoglutarate dependent dioxygenase

Keywords

Hypoxia Inducible Factor, oxygen, genetic screens, hypoxia, VHL

Conflicts of Interest

The authors declare no competing interests

Abstract

Oxygen-sensing mechanisms have evolved to allow organisms to respond and adapt to oxygen availability. In metazoans, oxygen-sensing is predominantly mediated by the Hypoxia Inducible Factors (HIFs). These transcription factors are stabilised when oxygen is limiting, activating genes involved in angiogenesis, cell growth, pH regulation and metabolism to reset cell function and adapt to the cellular environment. However, the recognition that other cellular pathways and enzymes can also respond to changes in oxygen abundance provides further complexity. Dissecting this interplay of oxygen-sensing mechanisms has been a key research goal. Here, we review how genetic approaches have contributed to our knowledge of oxygen-sensing pathways which to date have been predominantly focused on the HIF pathway. We discuss how genetic studies have advanced the field, and outline the implications and limitations of such approaches for the development of therapies targeting oxygen-sensing mechanisms in human disease.

Introduction

The ability to sense and respond to oxygen levels is common to most organisms. While the pathways involved may differ, they all serve to maintain cell survival when oxygen is scarce, as well as drive biological processes dependent on oxygen gradients. Therefore, dissecting how cells respond to oxygen has been at the forefront of biological research. This review focuses on how genetic studies have contributed to our understanding of oxygen-sensing mechanisms.

In metazoans, Hypoxia Inducible Factors (HIFs) are the well-established core components of responses to oxygen levels [1-6]. These transcription factors are constitutively expressed but their protein levels are regulated according to oxygen availability in a post-translational manner. When oxygen is present, the HIF- α subunit (HIF-1 α or HIF-2 α) of the HIF dimeric complex is hydroxylated at two conserved proline residues by one of three prolyl hydroxylases (PHDs, or EGLNs), which act as the recruitment signal for the Von-Hippel Lindau (VHL) E3 ligase that ubiquitinates HIF- α , facilitating its rapid proteasome-mediated degradation. In oxygen limiting conditions, the PHDs are no longer catalytically active, and the HIF- α subunit is no longer hydroxylated. This stabilises HIF- α , allowing its binding to HIF1 β (also known as Aryl Hydrocarbon Receptor Nuclear Translocator, ARNT) to form a heterodimeric transcription factor which activates hundreds of genes involved in angiogenesis, metabolism, pH, inflammation and cell growth.

PHDs are members of the 2-oxoglutarate (2-OG) dependent dioxygenase (2-OG DD) family and provide the oxygen-sensing component of the HIF pathway [7]. There are approximately 60 of these enzymes in humans, which all require oxygen, iron and the rate-limiting tricarboxylic acid (TCA) cycle metabolite 2-OG (**Figure 1**). Whether all members of the 2-oxoglutarate DD family have the capacity to act as oxygen sensors depends on their binding affinity for oxygen, and in many cases the affinities are not known. However, other 2-OG DDs are involved in modulating HIF transcriptional activity, aside from the PHDs. Factor Inhibiting HIF-1, (FIH-1) is an asparagine hydroxylase of the 2-OG DD family that remains catalytically active at lower oxygen concentrations than PHDs, and modulates transcriptional activation of a subset of HIF target genes [8, 9]. 2-OG DDs are also involved in controlling histone demethylation and DNA hydroxymethylcytosine modifications, both of which have also been shown to be oxygen sensitive [10-12] (**Figure 1**).

While 2-OG DDs highlight the ability of some oxygen-dependent enzymes to sense cellular oxygen, other sensing mechanisms have also been uncovered. Rapid oxygen-sensing in the carotid body relies on a distinct mechanism to the HIF pathway, with acute hypoxia leading to cell depolarisation, calcium influx and neurotransmitter release [13]. How oxygen is actually sensed in

these cells is still debated. Oxygen-sensitive potassium channels were thought to mediate the calcium influx, but loss or inhibition of the main potassium channels in rodents does not prevent the response of glomus cells to hypoxia [14]. It is most likely that oxygen-sensing is linked to activity of the mitochondrial respiratory chain and reactive oxygen species (ROS) generation [15], although how ROS signals to the membrane channels remains to be resolved.

The evolution of distinct oxygen-sensing mechanisms in plants [16] and fission yeast [17], as well as the identification of oxygen-sensing cysteine dioxygenases in mammals [18] provides further complexity to how cells adapt and respond to oxygen availability. Dissecting the cellular mechanisms involved and relative contribution of these pathways to oxygen-sensing is challenging, but has been aided by genetic studies, particularly with respect to the HIF response. Here, we summarise the genetics of the HIF pathway, consider the contributions that forward and reverse genetics have made to the field, and outline where genetic approaches may be of benefit in understanding other cellular responses to oxygen. We also discuss advances in genome editing techniques, the merits of and potential pitfalls of genetic screens, and their implications for future studies on cellular oxygen-sensing.

Evolutionary, molecular and clinical genetics in oxygen-sensing pathways

Von Hippel Lindau Disease and HIFs

The VHL E3 ligase is named from its identification as the gene mutated and inactivated in VHL disease [19]. This autosomal dominant familial syndrome is characterised by the development of renal cell carcinomas, pheochromocytomas, and angiogenic tumours [20] (**Table 1**). Tumours do not typically develop until adulthood, and renal cell cancers and cerebral haemangioblastomas are major causes of morbidity [21]. The tumour suppressor nature of VHL is supported by the finding that nearly 90% of clear cell renal cell carcinomas (ccRCCs) have loss of VHL function [22]. The trajectory of VHL loss in these sporadic renal cell cancers is fascinating, whereby recent multi-region genomic profiling of primary tumours has mapped their clonal evolution [23]. *VHL* mutations are clearly the most significant drivers in renal cell cancer, but these clonal mutations can occur early in life and take several decades to lead to the formation of tumours (median 28 years) [23]. The addition of one or more other driver mutations (e.g *BAP1*, *SETD2*, *PBRM1* or *PTEN*) reduces the time from identification of the clonal ancestor to tumour diagnosis to 2-5 years [23].

Tumours in VHL disease are hypervascular and associated with increased red cell production, and it was observed that VHL deficiency led to the induction of genes activated in hypoxia, such as *VEGF* [24, 25]. These observations suggested that VHL may be regulating the then recently identified HIFs by the Semenza group [26, 27]. Work pioneered by the Kaelin and Ratcliffe groups subsequently demonstrated that VHL was an E3 ligase, and responsible for the ubiquitination and proteasome-mediated degradation of HIF- α [28-31].

Phenotypic genetic analysis of oxygen-sensing in metazoans: from prolyl hydroxylation to erythrocytosis

Biochemical assays of VHL in association with HIF- α subunits demonstrated that prolyl hydroxylation must be enzymatic, and defined the oxygen-sensitive domains of HIF-1 α and HIF-2 α . However, the genes involved were not identified. *HIF-1 α* orthologues had been identified in worms (*Caenorhabditis elegans*) [32], which displayed the same sensitivity to oxygen concentrations as observed in humans. The ability of worms to be easily genetically manipulated provided the ideal tool to define the enzymes involved. Epstein et al first examined the main 2-OG DDs known at that time, the collagen prolyl hydroxylases, but these had no effect on HIF stability [33]. They then analysed the worms for other potential hydroxylases, identifying that *Egl-9* showed all the structural requirements for 2-OG DD activity. The presence of a single *Egl-9* gene (analogous to PHD2) in worms and flies accounted for the regulation of HIF. However, there has been an expansion in this group of enzymes in mammals, with humans encoding three PHD genes. PHD2 accounts for the main hydroxylase regulating HIFs in most human cells, but all the enzymes are involved, and have been implicated in regulating HIFs and other substrates in different conditions [7, 34].

Since the initial characterisation of the HIF pathway in *C. elegans*, this organism and other less diverse metazoans have continued to provide a useful tool to understand the HIF response. Worms are viable without *hif-1*, *vhl* and *egl-9*, and this has enabled analysis of genes regulated by HIF-1, as well as phenotypic studies into the role of the HIF pathway in ageing [35]. The HIF response is also well conserved in *Drosophila Melanogaster*, with Similar (Sima) and Tango (Tgo) bHLH-PAS proteins forming an analogous HIF-1 α /HIF β heterodimer [36, 37]. The ease of genetic manipulation in *Drosophila* has helped map the interactions of HIF with target of rapamycin (TOR) pathways [38], and the effects on cell growth [39]. In addition, gene silencing screens in *Drosophila* have helped identify co-activators recruited by HIF-1 for selective gene transcription, which we discuss in more detail (see activation of the HIF response).

The identification of the HIF pathway has also provided the basis for exploring the genetic causes of erythrocytosis (also known as polycythaemia). HIF regulation of *EPO* transcription prompted studies to understand congenital erythrocytosis. Chuvash polycythaemia is a rare autosomal recessive condition endemic in the Russian region of Chuvashia. Ang et al mapped the locus to a missense mutation in *VHL* (Arg200Trp) which impaired VHL ubiquitination of HIF- α and enhanced activation of HIF target genes [40]. Studies indicate that this mutation arose from a single ancestor around 12,000 years ago, explaining the occurrence of this mutation occurring in other populations via migration [41]. However, the Chuvash mutation does not lead to a tumour syndrome, and this likely relates to the residual activity of VHL that occurs, which is absent in VHL disease.

Other mutations in *VHL* have been linked to erythrocytosis, as have many mutations in *PHD2* and some in *HIF-2 α* (reviewed by Lee and Percy [42]). While a detailed discussion of the mutations is beyond the scope of this review, it is worth noting that mutations in *VHL*, *PHD2* and *HIF-2 α* have discrete phenotypes, present in different age groups, and are distinct from VHL disease. Moreover, how these monogenetic disorders relate to populations living at high altitude has been of interest. A genome-wide association study of those living in the Tibetan plateau versus the lowland Han identified single nucleotide polymorphisms (SNPs) in *HIF-2 α* resulting in lower haemoglobin levels [43]. The potential advantage of these SNPs is not fully understood, and may relate to reducing mountain sickness or relate to a role of HIF-2 α in foetal development [43, 44].

Hereditary cancer syndromes provide links between mitochondrial metabolism and oxygen-sensing

Aside from VHL disease, rarer hereditary cancer syndromes have provided insights into the HIF pathway. Familial mutations in two TCA enzymes, succinate dehydrogenase (*SDH*) and fumarate hydratase (*FH*) result in cancer syndromes that include the development of renal cell carcinomas, as well as several other tumours (e.g. paragangliomas, pheochromocytomas with *SDH*, and leiomyomas with *FH*) [45-48] (**Table 1**). The activation of genes regulated by HIFs and the vascular nature of these tumours prompted studies exploring whether metabolites may alter PHD activity, potentially independently of HIFs [47].

Germline *SDH* mutations in subunits -B, -C, and -D all give rise to paragangliomas and pheochromocytomas [49, 50], with *SDHB* being rarely linked to renal cell cancers [51]. It was hypothesised that the accumulation in succinate would impair PHD activity, and consequently shown

in *SDHD* depleted immortalised cell lines that HIF- α prolyl hydroxylation was reduced, resulting in HIF stabilisation and activation of HIF target genes [52]. Biochemical and structural studies subsequently confirmed that TCA cycle intermediates, and fumarate in particular, could competitively inhibit PHDs and other 2-OG DDs [53]. However, the relative importance of HIF in driving the *SDH* mutant tumours is still debated, as ROS have been implicated [54], and succinate accumulation will also impair TET driven 5-hmc of DNA [55], which by altering DNA demethylation, may also be a major driver of the tumour development [56].

A similar picture has been noted with FH familial tumours [57, 58]. Here, *FH* mutations can again lead to HIF activation through the accumulation of fumarate and PHD inhibition. Fumarate is in fact a more potent inhibitor of PHD activity than succinate [53]. However, it should not be concluded that HIF activation is solely responsible for tumour formation, and the current paradigm involves metabolic adaptation with reductive carboxylation, epigenetic modifications, and haem biosynthesis, along with a contribution by HIF transcriptional programming [59, 60].

Aside from these familial cancer syndromes there is considerable evidence that somatic mutations in TCA enzymes drive other tumours, and modulate oxygen-sensitive enzymes. Isocitrate dehydrogenase (IDH) catalyses the formation of 2-OG from isocitrate, and driver somatic mutations in *IDH* (e.g. *IDH-1* R132H) have been identified in glioblastomas and acute myeloid leukaemia (**Table 1**). These mutations alter enzyme function to produce the D enantiomer of 2-hydroxyglutarate (D-2-HG). This metabolite inhibits a number of 2-OG DDs, particularly KDMs and TETs [61, 62], contributing to tumour formation by altering the epigenetic landscape [63-65]. However, HIF stabilization is not observed, and D-2-HG can actually potentiate HIF degradation, although the mechanism involved is debated [64-66].

While hereditary cancer syndromes have informed the identification of key components of the HIF pathway, reciprocal, cell based genetic screens have helped identify genes responsible for germline cancers with unclear aetiology. For example, the identification that mutations in a further key component on the TCA cycle, the 2-oxoglutarate dehydrogenase complex (OGDHc), lead to recurrent paragangliomas and pheochromocytomas [67], arose from observations with a forward genetic screen uncovering that OGDHc loss causes L enantiomer accumulation of 2-HG [68] (**Table 1**) (see also genetic screening section).

Evolutionary genetics and comparative biology define other oxygen-sensing mechanisms

While the HIF pathway is conserved in metazoans, distinct oxygen-sensing mechanisms exist in yeast and plants. Understanding how oxygen is sensed in these organisms has not only uncovered interesting biology, but helped define other oxygen-sensing pathways that occur in humans, alongside the HIF response.

Fission yeast (*Schizosaccharomyces pombe*) have an intriguing system that relies on the sterol sensing pathway, which involves several oxygen dependent steps. The SREBP orthologue, *Sre1* is a transcription factor that is unusually retained in the ER when cholesterol is present, but on cholesterol depletion is cleaved, allowing the release of an N-terminal fragment that translocates to the nucleus (Sre1N). In *S pombe*, Sre1N also stimulates the transcription of genes required for hypoxic adaptation [39]. Hughes et al showed that Sre1N is stabilised when oxygen levels are low, and is degraded by the proteasome. Remarkably, this oxygen-sensitivity is also regulated by a 2-OG DD, termed *Ofd1* [69]. However, an analogous combined sterol and oxygen-sensing pathway has not been identified in higher metazoans. Although three mammalian orthologues of *Ofd1* exist (*OGFOD 1-3*), they do not seem to regulate the mammalian orthologue of *Sre1*, SREBP2, and are instead involved in ribosomal hydroxylation (OGFOD1)[70].

In plants, oxygen-sensing is governed by ubiquitin enzymes of the N-end rule pathway [16, 71]. A family of cysteine oxidases act as oxygen-sensors and control hypoxic adaptation through modification and subsequent degradation of target proteins through the Cys-branch of the N-degron pathway [16, 71]. This work led Masson et al to investigate the mechanism of N-terminal cysteine oxidation in mammals [18]. They identified a thiol oxidase, ADO, as a cysteine oxidase involved in the N-end rule pathway. ADO catalyses cysteine to cysteine sulfinic acid in an oxygen-sensitive manner, regulating the ubiquitination and degradation of N-end rule substrates in G-protein signalling and calcium signalling. This comparative biological approach highlights that other oxygen-sensing pathways have evolved in mammals aside from HIF, and understanding how they provide a co-ordinated response to changes in oxygen will be important to address in future studies.

Genetic screening approaches to interrogate the HIF pathway and adaption to oxygen availability.

In considering how genetic approaches have been applied to oxygen-sensing pathways, it is helpful to outline the techniques involved, and the relative merits and disadvantages of different

systems. Firstly, it is important to acknowledge that genetic screens in organisms such as worms, fungi and flies have provided scientific advances for around the past 100 years [33, 72, 73]. However, genetic screens in mammalian cells have been more challenging, principally due to genetic complexity and manipulation of ploidy. One approach to circumvent the issue of ploidy was to find haploid or near-haploid cell lines for use in genetic screens. Chinese hamster ovary (CHO) cells are near-haploid, and early studies of the HIF pathway demonstrated the utility of chemical mutagenesis in CHO cells to help define HIF-1 dependent and independent gene expression in hypoxia [74]. A further breakthrough in performing knockout based screens in haploid human cells arose from pioneering studies by the Brummelkamp group, where they established the use of haploid (HAP1) or the near-haploid (KBM7) cells derived from a patient with chronic myeloid leukaemia (CML) to carry out unbiased genetic screening [75-77]. These insertional gene-trap mutagenesis screens have been extensively used to identify host-pathogen restriction factors [75, 76], as well as being applied to transcriptional regulation [78] and the HIF response [68].

RNA interference (RNAi) and CRISPR (clustered regularly interspaced short palindromic repeats)-Cas mutagenesis have provided the other major methodological advances to facilitate genetic screens. Both have been transformative in allowing genetic screens to be conducted in mammalian cells, and the rapid reduction in protein levels with RNAi is advantageous for genome-wide screens [79]. However, a potential limitation of RNAi is the reasonably high level of off-target effects and difficulty in achieving complete genome coverage. CRISPR-Cas gene editing typically has less off-target effects than RNAi, and the ability to target nucleotide sequences with high efficiency allows pooled sgRNA screens to be conducted rapidly. The most commonly used strategy involves pooled libraries of between 4-10 sgRNAs targeting all genes within the genome, and transducing these sgRNA into cells expressing the Cas9 nuclease, resulting in insertion-deletions (indels) and loss of gene function. Fusion of a catalytically inactive Cas9 with either a transcriptional repressor (CRISPR interference, CRISPRi) or transcriptional activator (CRISPR activation, CRISPRa) provides further versatility to the CRISPR sgRNA approach [80, 81]. While the advantages of CRISPR-Cas screens are clear, there are several limitations that should be considered. Off-target mutations can still occur and CRISPR-Cas mutagenesis is in principle binary, such that mutations will result in complete loss of gene function, which is less evident than with siRNA depletion. In addition, indel formation typically results in loss of gene function after several days, with phenotypes taking longer to develop, and the potential for confounding cellular adaptation to occur. However, given the flexibility of CRISPR-Cas nuclease systems to cut DNA in distinct manners and even target RNA [82], we will continue to see rapid advances in these screening strategies.

VHL and synthetic lethality

One of the first areas to take advantage of genetic screening approaches was the search for genes that would be synthetically lethal in combination with *VHL* mutations (**Figure 2**). 90% of renal cell carcinomas have *VHL* mutations [83], and identifying genes or molecules that are synthetically lethal with VHL could offer major therapeutic advantages, as recently demonstrated with the combination of PARP inhibition in tumours defective in BRCA1/2 [84-86].

Screening approaches for synthetic lethality with VHL loss have typically used chemical inhibition and RNAi in renal cell carcinoma cells (e.g. RCC4 or 786-O cells). A number of potential targets and compounds have been identified, but there is generally low concordance between the studies. For instance, Turcotte et al screened VHL-deficient and VHL-reconstituted RCC4 cells with a 64,000 drug compound library, and identified that STF-62247 selectively killed the VHL-deficient cells [87]. Cell death was HIF independent and thought to be mediated by STF-62247 accumulation in lysosomes, and inhibition of late stage autophagy [88]. Thompson et al also used RCC4 cells, and screened with a library of pharmacologically active compounds [89]. They identified Rho-associated coiled-coil containing protein kinase (ROCK) as a potential synthetic lethal target [89]. Treatment with ROCK inhibitors reduced cell proliferation, migration, and induced cell death in VHL-deficient cells, presumably in a Rho-dependent manner. ROCK inhibition can alter also autophagy [90], and it is possible that autophagy is involved in this synthetic-lethal interaction, as observed with STF-62247. However, this has not yet been experimentally addressed.

Rather than using a chemical library, Bommi-Reddy et al used a kinase focused short-hairpin RNA (shRNA) library in 786-O and RCC4 cells, identifying a dependence on CDK6, MER and MAP2K1 activity [91]. Loss of viability following CDK6 and MAPK2K1 depletion was independent of HIF, consistent with VHL having additional roles outside of the hypoxia response pathway [92, 93]. CDK4/6 small molecule inhibition also decreased the viability of VHL null cells, and these studies have been replicated using an siRNA screen in *Drosophila* that was coupled with a targeted inhibitor library screen in renal cancer cell lines [94]. CDK4/6 inhibition may be a therapeutically tractable strategy in renal cancer, as in combination therapy with the HIF-2 α inhibitor, PT2399, CDK4/6 inhibition produced a synergistic suppression of cell viability [95].

Targeted shRNA libraries have also been used to examine depletion of histone and chromatin modifiers in a range of VHL-deficient renal cancer cell lines [96]. VHL loss was associated with increased H3K27 acetylation and monomethylation, and synthetic lethality was observed with depletion of the H3K27 methyltransferase EZH1 [96]. Interestingly, H3K27me1 can be removed by

the demethylases KDM6A and KDM6B [11, 97] which are both transcriptionally regulated by hypoxia, and function as ccRCC repressors [98]. It is thought that KDM6A is already downregulated in renal cancer cells, contributing to their hypersensitive EZH1 H3K27 methyltransferase activity [96]. The screen also identified another demethylase KDM4A, which demethylates the H3K36 mark, as a potential synthetic lethal partner of VHL loss [99, 100]. Although this has not been experimentally validated, it is speculated that this may counteract the loss of the H3K36 SETD2 methyltransferase, which is frequently lost in renal cancers [101].

Renal cancer cell lines have proved valuable in studying VHL genetic interactions, but may not be truly representative of tumours *in vivo*, where growth is also influenced by the microenvironment and immune surveillance. Wolff et al sought to create a more representative *in vivo* setting by co-culturing VHL-deficient and -proficient cells and using a ~12,800 small molecules library [102]. Their high throughput imaging screening method identified homoharringtonine (HHT), an FDA-approved drug used in the treatment of chronic myeloid leukaemia, as a synthetic lethal partner of VHL loss, although mechanistically it is not understood what mediates this interaction [102]. Moreover, this co-culturing approach does not fully recapitulate tumorigenesis, and there is still a need to establish studies that more faithfully capture the growth of renal tumours in a clinical context. One such approach may be to apply *in vivo* CRISPR mutagenesis [103, 104] to renal cell tumours, and it will be of interest to understand how these approaches inform on the role of recapitulating the tumour microenvironment.

Lastly, complementary computational methods have been used to screen for synthetic lethality with VHL loss [105-107]. One computational approach, termed DIASY (data mining synthetic lethality identification pipeline), based on the assumption that synthetically lethal interactions can be identified by pairs of genes whose co-inactivation in tumours occurs much less than expected by chance alone, identified several candidate genes that may be synthetically lethal with VHL loss, including EZH1 [106]. They did not identify CDK4/6 or other targets identified in experimental screens. Overall, it remains unclear why there is considerable variation in the targets/compounds identified in the different screening approaches, but it likely depends on the methodologies used and differences in the cell lines. CDK4/6 and EZH1 are the most promising targets, but there may still be other synthetically lethal interactions to be uncovered.

Regulation of HIF stability

Genetic screens have been helpful in delineating pathways that can influence HIF stability. Prior to the advent of KBM7 near-haploid human cells, Vaux et al took advantage of the near-haploid nature of CHO cells, to mutagenise cells expressing HRE-linked cell surface markers, and screen for increased HRE activity [108]. Clones that demonstrated increased cell surface expression also showed increased HIF-1 α levels. However, this was not due to mutations in HIF-1 α itself, but predominantly arose due to *VHL* mutagenesis and impaired ubiquitination. Two clones showed increased HIF-1 α protein levels that were not due to *VHL* mutations, indicating that other pathways that influence HIF- α stability were still to be found.

Bemis et al took a complementary approach, and used a retroviral cDNA library in U-251 glioblastoma cells expressing an HRE driving GFP to find genes involved in aerobic activation of HIF- α [109]. They found a COP9 signalosome (CSN) component, CSN5, activated HIF-1 α in aerobic conditions. CSN5 bound the C-terminal ODD in HIF-1 α , reducing prolyl hydroxylation and stabilising HIF-1 α . This study and those undertaken in CHO cells [108] highlight the dominance of the PHD-VHL axis, but still raise the question that other signals may be involved in HIF- α stability.

Rather than expressing a cDNA library, we applied genome-wide mutagenesis screens to near-haploid human cells expressing a dynamic HIF- α reporter [68, 110, 111]. This approach was initially applied to KBM7 cells using the retroviral gene-trapping mutagenesis strategy developed by Brummelkamp and others [75, 77]. We generated KBM7 cells stably expressing a HIF GFP reporter, consisting of an HRE(3x) driving GFP fused to the ODD of HIF-1 α , and following mutagenesis with the gene-trapping retrovirus, used flow cytometry to sort and enrich for cells that showed high GFP levels in aerobic conditions. By mapping the insertion sites of the gene-trapping retrovirus, we identified several pathways and genes involved in HIF-1 α stability, independent of oxygen availability. Four main pathways were identified: 1) The canonical PHD-VHL axis, 2) intracellular iron metabolism, 3) mTOR, and 4) 2-OG metabolism [68, 111] (**Figure 2**). An unexpected finding was that mutations in the OGDHc, a rate limiting step in the TCA that converts 2-OG to succinyl CoA, was critical in regulating HIF levels in aerobic conditions. Loss of OGDHc activity led to stabilisation of HIF in a non-prolyl hydroxylated form, which is counterintuitive, as 2-OG levels were increased. However, we uncovered that 2-OG accumulation and reductive metabolism drives the formation of L enantiomer of 2-HG (L-2-HG), which inhibited PHD catalytic function [68]. In addition to the control of HIF-1 α levels, L-2-HG also had a significant effect on the activity of the TET enzymes in control of DNA methylation. We hypothesised that the consequences of impaired PHD and TET activity following OGDHc mutations may be analogous to other TCA enzyme mutations, and predispose to

tumour formation. This indeed proved to be the case, and subsequent to our studies Remacha et al identified that human germline mutations in one of the OGDHc subunits, *DLST*, led to recurrent pheochromocytomas and paragangliomas [67], placing the OGDHc alongside SDH and FH in hereditary cancer syndromes that involved aerobic activation of HIFs (**Table 1**).

In addition to uncovering a role for OGDHc, the near-haploid genetic approach identified a role for mitochondrial lipoylation in the HIF response. Lipoylation is an essential post-translational fatty acid modification for several mitochondrial enzymes, including the OGDHc, where it undergoes cyclical oxidation and reduction on DLST to form an intermediate with the succinyl moiety. Mutations in lipoic acid synthase (*LIAS*) were highly enriched in the mutagenesis screen. We found that *LIAS* loss decreased OGDHc activity, and thereby led to L-2HG formation and PHD inhibition [68]. Interestingly, we also observed HIF-1 α stabilisation in fibroblasts from patients with germline mutations in lipoic acid synthesis genes. These individuals present with severe neurological disease in early childhood (a form of Leigh syndrome), and it is possible that HIF- α activation here may be beneficial in relation to this form of mitochondrial disease, but this requires further investigation.

A further area in which our genetic approach has been helpful is in understanding the role of the vacuolar H⁺ATPase (V-ATPase) in HIF- α stability. Prior studies had identified that V-ATPase inhibition resulted in increased HIF-1 α levels, suggesting that HIFs could be a substrate for lysosomal degradation [112]. However, it was unclear under what conditions this would occur, as the degradation of HIF through VHL-mediated ubiquitination is dominant. The mutagenesis screen confirmed that V-ATPase loss can lead to activation of the HIF response, as a number of V-ATPase subunits were enriched for gene-trap insertions [111]. In addition, two uncharacterised genes were identified, (*CCDC115* and *TMEM199*), and we subsequently showed these to be required for V-ATPase activity [111]. However, we did not observe lysosomal degradation of HIF-1 α . Instead, we found that PHD activity was inhibited when the V-ATPase was not active, and this was due to intracellular iron depletion, highlighting the involvement of the endosomal-lysosomal pathway for cellular iron metabolism, and how this links with the HIF pathway.

More recently, we have used genome-wide CRISPR-Cas9 mutagenesis to complement the near-haploid screens [110]. These genome-wide pooled libraries are equivalent to the gene-trapping method in uncovering the main pathways involved in HIF stability, and have also provided additional insights by delineating the function of previously uncharacterised genes. The putative mitochondrial gene *ABHD11* was highly enriched for sgRNAs following genome-wide CRISPR-Cas9 mutagenesis. Given the propensity of the screens to identify components of 2-OG metabolism, we hypothesised that *ABHD11* may regulate 2-OG abundance. We observed that *ABHD11* associates with the OGDH

complex and is required for basal OGDHc activity. Interestingly, ABHD11 was required to maintain functional lipoylation and redox cycling of the OGDHc [110]. The physiological role of ABHD11 is not yet known, but it is intriguing that an enzyme involved in redox regulation of the TCA cycle interfaces with the HIF oxygen-sensitive pathway.

A remaining question that has not been fully answered is whether there is a requirement for other ubiquitin ligases or DUBs in HIF- α protein stability. Bett et al used a 'ubiquitome' siRNA library to identify ubiquitin enzymes involved in HIF stability, conducting the screen in 1% oxygen rather than aerobic conditions [113]. They identified that USP52 (ubiquitin-specific protease 52)/PAN2 (poly(A) nuclease 2) depletion decreased HIF-luciferase reporter levels. Interestingly, USP52 is likely to be a 'pseudo-DUB' without isopeptidase activity, and they went on to show an involvement of USP52 in maintaining HIF-1 α mRNA stability in cytoplasmic P-bodies. While these findings did not show a direct involvement of deubiquitination, other studies suggest that DUBs are involved in HIF regulation. Bremm et al took a focused approach to explore the role of Otubain DUBs in HIF-1 α regulation, and used siRNA-mediated depletion to screen for their effect on a HRE-luciferase reporter in hypoxia [114]. Cezanne (OTUD7B) was found to be involved in HIF-1 α degradation, but in a VHL and PHD independent manner. Subsequent studies identified that Cezanne altered *HIF-2 α* expression, which was dependent on E2F1 [115]. The detailed mechanisms of how Cezanne controls HIFs remains to be fully understood, but these studies highlight that DUBs involved in HIF regulation may occur at both the translation and post-translational level.

Activation of the HIF response

Genetic screens to find determinants of HIF- α stability have informed on the involvement of metabolic and other cellular pathways in the HIF response. A distinct query relates to how HIFs activate a transcription response once stabilised. Both HIF-1 α and HIF-2 α can bind to the same HRE, but chromatin immunoprecipitation sequencing (ChIP-seq) experiments demonstrate that these isoforms can occupy distinct loci within the genome. This raises the question of how HIF isoforms target gene selectively?

We and others have applied genetic approaches to understand activation of the HIF response and the interaction of HIFs with chromatin (**Figure 2**). The Wappner group performed an unbiased genome wide screen using an RNAi library using *D. Melanogaster* cells expressing a HIF specific reporter[73]. Cells were treated with the iron chelator (DFO) to inhibit PHD function and activate HIF activity, and cells that failed to activate HIF activity were selected. Around 30 genes

were identified, which included previously identified components of the CSN [109], mTOR and the phosphoinositide 3-kinase (PI3K) pathway. They also identified an essential role for miRNA system in the regulation of *Sima* mRNA, dependent on *Agonaute 1 (Ago1)* [73]. Subsequent studies have identified around 40 miRNAs that can regulate *HIF- α /HIF1 β* expression directly or indirectly, reflecting the complex nature of RNA silencing [116]. A number of chromatin remodellers were also identified in the *D. Melanogaster* siRNA screen, including components of the SWI/SNF complex [73]. Kenneth et al have consequently shown that the SWI/SNF complex can alter *HIF-1 α* mRNA expression [117]. The involvement of SWI/SNF is likely to be more complicated though, as several components of the complex are frequently mutated in renal cell tumours (e.g. *PBRM1*) [118], where HIF levels are increased.

Transcription factor binding and gene transcription is ultimately controlled by chromatin accessibility and epigenetic modifications. One of the primary mechanisms through which chromatin accessibility is controlled is through N-terminal post-translational modification of histone tails, where lysine acetylation and methylation can help regulate gene activation and repression. Early work in the hypoxia field demonstrated the role of CBP/p300, an acetyltransferase complex, in transcriptional activation of certain HIF target genes [119]. HIF- α binds p300/CBP through its N-terminal transactivation domain, which is counteracted by FIH-1 [8, 9]. More recent studies have used genetic screens to further understand how HIFs interact with epigenetic regulators. Pontin and Reptin, *Drosophila* orthologues of the human TIP60 complex, were identified as potential regulators of HIF activation by the Wappner group in their siRNA screen [73]. Perez-Perri et al went on to explore the functional requirement for TIP60 (KAT5 acetyltransferase) in *Drosophila* and human cells, and observed that TIP60 depletion altered the expression of a subset of HIF-1 α target genes [120]. Interestingly, this overlapped to some extent with prior findings whereby the CDK8-Mediator complex interacted with HIF-1 α to regulate distinct target gene expression [121]. These studies suggest that HIFs can interact with several acetyltransferases to promote gene transcription, and show the involvement of the CDK8-Mediator to initiate recruit RNA PolII activation through recruitment of the Super Elongation Complex.

Recently, we applied CRISPR-Cas9 mutagenesis to further understand how a HIF transcriptional response is achieved. Using our dynamic fluorescent HIF reporter system in human cells, we screened for mutagenised cells that failed to accumulate a fluorescent signal in hypoxia, identifying several genes required for transcriptional activation at HIF target loci [122]. Among the most enriched genes for sgRNA mutagenesis was the histone methyltransferase *SET1B (SETD1B)*, which catalyses the histone 3 lysine 4 trimethylation mark (H3K4me3) associated with open regions

of chromatin and transcriptional activation. We found that H3K4me3 deposition at specific HIF target loci is dependent on both HIF and SET1B binding, with HIF-1 α recruiting SET1B to chromatin when stabilised [122]. Interestingly, HIF-dependent SET1B target genes are for the most part distinct from those utilising CDK8-Mediator/TIP60 [120], and typically involved genes which were not expressed or expressed at low levels in 21% oxygen. While it is clear that SET1B recruitment by HIF enhances gene transcription at these sites, whether this is solely dependent on H3K4me3 deposition or an alternative function of SET1B remains to be determined.

Essentiality and viability screens in oxygen limiting conditions

Essentiality or 'cell fitness' screens have recently attempted to shed light on how other cellular processes promote survival when oxygen is scarce. Jain et al performed genome-wide CRISPR growth screens at 21%, 5%, and 1% oxygen, to determine essential genes dependent on oxygen availability [123]. They identified ~200 essential genes in high oxygen and ~100 genes in low oxygen conditions. Canonical HIF pathway genes were not detected as essential, with VHL loss only showing a growth defect in 21% oxygen. The reasons for these findings are not entirely clear, but may relate to differences in growth of the cancer cell line used (K562, lymphoblast cells), and the requirement for HIFs in an *in vivo* setting, where consequences of HIF activation, such as promoting angiogenesis, will be more apparent. Cells in low oxygen instead relied on lipid metabolism, with genes involved in peroxisomes being essential. Supplementation with unsaturated fatty acids (oleic acid) restored cell growth, but this essentiality phenotype was only observed in HEK293T and K562 cells. Therefore, it is likely that hypoxia cell fitness genes differ between cell types and tissues, and the relative importance of such variations remains to be determined.

In contrast to the reliance on lipid metabolism in hypoxia, most fitness genes in a high oxygen setting were mitochondrial, with Complex I components particularly evident as essential in 21% oxygen but not 1% oxygen [123]. These findings complement prior studies by the Mootha group whereby they used a genome-wide CRISPR Cas9 genetic screen to identify genes that could suppress a model of mitochondrial disease (Complex III inhibition) with VHL being the top hit [124]. Using a variety of genetic and chemical tools, Jain et al showed that hypoxia was protective in preventing cell death in a zebrafish and a mouse model of mitochondrial disease (*Ndufs4* KO, Complex I deficiency) [124]. This survival benefit is not due to HIF activation, which failed to improve brain disease in the *Ndufs4* null mice, but is thought to arise from neuronal oxygen toxicity that occurs when the respiratory chain is disrupted [125].

These cell fitness screens highlight the relationship between oxygen availability and mitochondrial function, but several questions remain. Why cell types differ in genes that are essential are not yet clear, and how non-cancer cells behave when oxygen is limiting is not known. It is not straightforward to distinguish between genes that alter cellular oxygen levels (e.g. oxygen consumption) in these fitness screens versus those that are due to changes in oxygen-sensing. Combining essentiality screens with mitochondrial or HIF reporters may overcome some of these challenges.

Conclusion and future directions

Over the past 20 years genetic studies, advances in gene editing and phenotypic screening approaches have contributed to our understanding of how cells respond to oxygen. Most studies have been focused on the HIF pathway, and despite the central involvement of VHL-mediated ubiquitination of HIF- α , other components of the ubiquitin machinery remain to be fully determined. This may relate to certain pathways and genes being preferentially selected in HIF phenotypic screens, with loss of VHL or PHD activity always being identified across multiple cell types, or redundancy within the ubiquitin system. Whilst the detection of VHL and PHDs serve as useful controls in screens, their preferential selection may conceal other genes or signals that have intermediate effects on HIF stability/activity. Designing focused sgRNA libraries excluding genes already known to regulate HIF may help overcome the preferential selection of VHL and PHD loss. Alternatively, supplementation with PHD co-factors (e.g. iron or 2-oxoglutarate) may help avoid bias towards particular cell pathways.

The ability to now undertake gene editing in any cell type should allow genetic screens to move away from cancer cell lines to primary cells, and to explore oxygen-sensing in specialist cell types. For example, it is now theoretically possible to interrogate the rapid oxygen-sensing in the glomus cells of the carotid body using CRISPR-Cas systems, and potentially determine the relative contribution of the respiratory electron transport chain and ROS generation to the membrane depolarisation.

Lastly, advances in CRISPR-Cas technology are likely to overcome some of the existing limitations: (1) Lethal phenotypes in mutagenesis screens can be potentially avoided with CRISPRi and CRISPRa approaches, (2) *in vivo* CRISPR-Cas screens may allow a more detailed understanding of cell autonomous and non-autonomous phenotypes, (3) genetic redundancy is still an obstacle, but combinatorial CRISPR screens have been developed, where co-disruption of gene function leads to

decreased cell fitness [126], and (4) CRISPR-Cas9 knock-ins will help inform on endogenous gene regulation, without the need for overexpressed or surrogate reporter systems. Combining these technological advances with complementary genetic data from population human studies [127] will provide further insights into the complexity of oxygen-sensing pathways and open up new therapeutic avenues to explore.

Author contributions

J.A.N. and B. M.O. conceptualized the review, wrote and edited the manuscript. J.A.N. was responsible for funding acquisition.

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Table 1: Genetic mutations associated with the HIF pathway and tumour formation

Gene	Mutations	Associated tumours	HIF Activation	Epigenetic Changes
VHL	VHL deletion (Gene inactivation), (Promotor hypermethylation)[22]	ccRCC, pheochromocytomas, haemangioblastomas	Yes, (predominantly HIF-2 α)	Histone and DNA methylation
SDHB	C213T STOP, 221insCAG,C270G, G436A, T708C, G721A, 847delTCTC (frameshift), C859A, C881A, P131R, M71fsX80 [49-51]	Paragangliomas, pheochromocytomas, renal cell carcinomas (rare)	Yes	DNA methylation
SDHC	R133T [46-47, 49]	Paragangliomas, pheochromocytomas	Yes	DNA methylation
SDHD	H102L, P81L, N92Y, G14A STOP, C33A STOP, C112T STOP, G274T, C361T STOP, R38X, Q109X, L128fsC134 [45-49]	Paragangliomas, pheochromocytomas,	Yes	DNA methylation
FH	A117P, T381I, A194T N329S [48, 57-58]	Paragangliomas, pheochromocytomas leiomyomas, renal cell carcinomas.	Yes	Histone and DNA methylation
IDH	R132H, (IDH1) R132C (IDH1) R140Q (IDH2) R172K (IDH2) [61-65]	Glioblastoma, acute myeloid leukaemia	No (accumulation of D-2-HG)	Histone and DNA methylation
OGDHC (DLST)	G374E [67]	Paragangliomas, pheochromocytomas	Yes, (2-HG accumulation)	DNA methylation

Summary of known mutations in genes associated with HIF activation and cancer syndromes. Associated epigenetic mutations that may arise from impaired 2-OG DD activity by KDMs and TETs also indicated.

Figure Legends

Figure 1: 2-oxoglutarate dependent dioxygenases regulate diverse cellular functions.

2-OG DDs can sense and adapt to cellular availability of oxygen, iron, and 2-OG. These enzymes have diverse roles, including 1) DNA/RNA demethylation (e.g AlkBH enzymes and FTO), 2) DNA hydroxymethylation (TETs), 3) Histone demethylation (KDMs), 4) HIF- α stability (PHDs) and transcriptional activation of gene targets (FIH-1), and 5) collagen prolyl hydroxylation (CPH enzymes).

Figure 2: Insights into the HIF pathway and VHL biology using genetic screening approaches.

Summary of pathway components identified using genetic screening regulating: 1) HIF- α levels and stability of the protein, 2) transcriptional activation of the HIF target genes, and 3) genes and compounds that are potentially synthetically lethal with VHL in ccRCCs. Highlighted in light grey are other genes which had previously been validated using alternative methods, or have been identified through screening but not yet validated.

Figure 1: 2-oxoglutarate dependent dioxygenases regulate diverse cellular functions

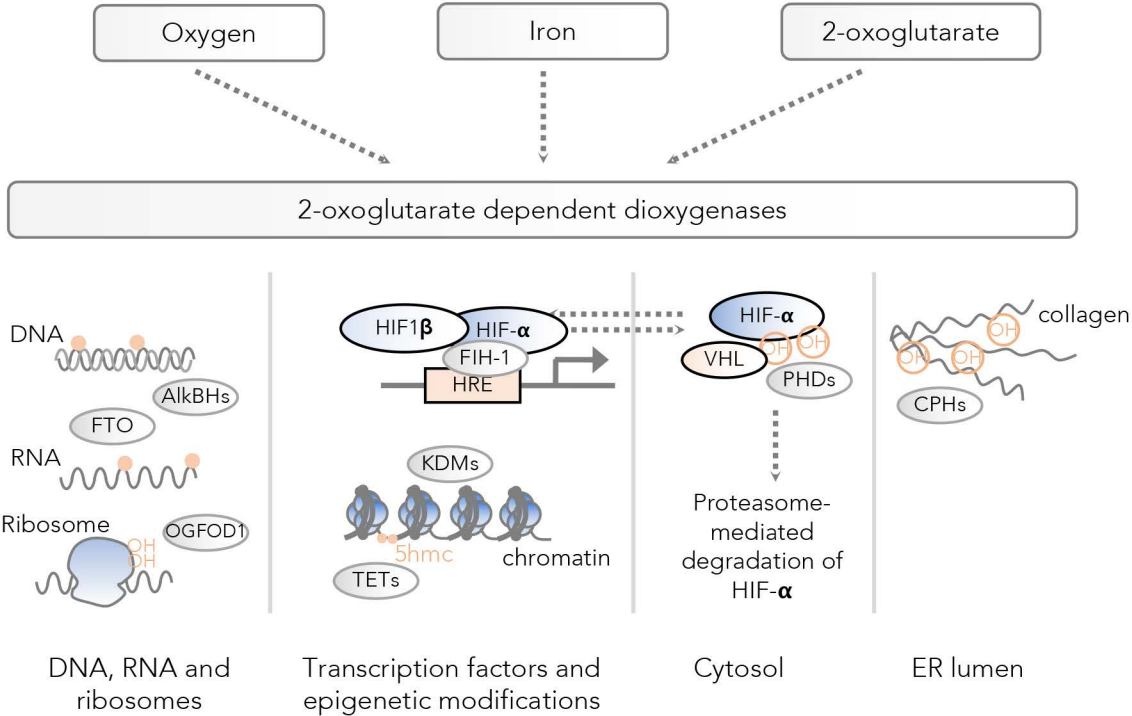


Figure 2: Insights into the HIF pathway and VHL biology using genetic screening approaches

