

# Hypoxia-inducible factor in cancer: from pathway regulation to therapeutic opportunity

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## ABSTRACT

Cancer remains one of the most formidable challenges in modern medicine, due to its complex and dynamic nature, which demands innovative therapeutic approaches. One major challenge to cancer treatment is the tumour microenvironment and in particular tumour hypoxia (low oxygen levels), which contributes to tumour progression and immune evasion. At the cellular level, this is primarily governed by hypoxia-inducible factor (HIF). HIF is a transcription factor that orchestrates cellular responses to low oxygen levels, driving angiogenesis, metabolic adaptation and immune regulation. HIF's dysregulation is frequently observed in various cancer types and correlates with increased aggressiveness, metastasis, resistance to therapy and poor patient prognosis. Consequently, understanding the cellular mechanisms underlying HIF activation and its downstream effects has become crucial to developing targeted cancer therapies for improving cancer patient outcomes and represents a key step towards precision medicine.

Recent advancements in drug development have led to the emergence of HIF inhibitors, which aim to disrupt HIF-driven processes in cancer providing therapeutic benefit. Here, we provide a review of the molecular mechanisms through which HIF promotes tumour growth and resistance, emphasising the potential clinical benefits of HIF-targeted therapies. This review will discuss the challenges and opportunities associated with translating HIF inhibition into clinical practice, including ongoing clinical trials and future directions in the development of HIF-based cancer treatments.

## INTRODUCTION

Oxygen, an essential molecule for sustaining life, is vital for energy production and the survival of all multicellular organisms.<sup>1</sup> In humans, the maintenance of oxygen homeostasis is a finely tuned process that ensures adequate supply of oxygen to match the demand of every cell and tissue within the body.<sup>2</sup> Adding to the complexity of this task is the fact that cells exist in different microenvironments where oxygen levels may fluctuate significantly, impacting their function. Hypoxia is a condition characterised by an insufficient supply of oxygen to cells.<sup>3</sup> This poses

a significant stress which cells must adapt to survive and function. Hypoxia also plays a significant role in the development and progression of cancer. Tumour hypoxia is a common feature of many solid tumours such as breast, cervix and head and neck cancers.<sup>4–6</sup> This occurs when tumour cell expansion outstrips the blood supply, leading to inadequate oxygen delivery to tumour cells resulting in hypoxic regions within the tumour. From a clinical perspective, tumour hypoxia is associated with poor patient prognosis and therapy resistance, therefore, understanding the underlying processes governing this response has been a major area of research.<sup>7–9</sup>

Critical to the transcriptional response to hypoxia is the oxygen-regulated family of transcription factors known as hypoxia inducible factors (HIFs), which when activated, trigger a diverse range of transcriptional targets that contribute to every aspect of cancer progression including, metabolic reprogramming, cell motility, metastasis, resistance to therapy and vascularisation.<sup>10–11</sup> HIF activation has been observed in numerous solid tumours including colon, breast, lung and kidney, making targeting HIF activity in cancer a prime therapeutic target.<sup>12–15</sup> It is worth noting that the HIF pathway has not evolved to cause cancer, but instead is a rescue mechanism that allows cells to adapt to their oxygen environment. As tumours are typically hypoxic, cancer cells use this pathway to promote various processes, which contribute to tumour progression. Although targeting transcription factor activity has proved to be therapeutically challenging, several advances have been made in targeting HIF activity in cancer. This review will summarise the current understanding of HIF regulation and its role in cancer and summarise the progress



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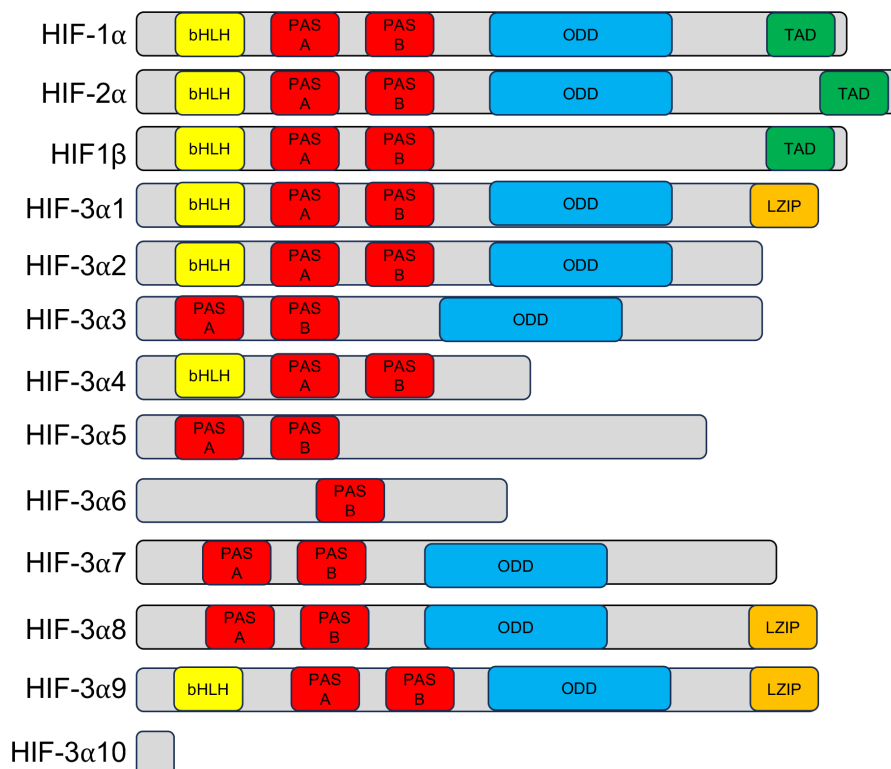


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**Figure 1** HIF subunit composition. The domain structure of different HIF isoforms is depicted as follows. The basic Helix-Loop-Helix (bHLH) and Per-ARNT-Sim (PAS) domains are essential for DNA binding and dimerisation. Within HIF- $\alpha$  subunits, the oxygen-dependent degradation domain (ODD) imparts sensitivity to oxygen levels. Notably, the ODD region contains conserved proline residues, subject to hydroxylation in an oxygen-dependent manner by prolyl hydroxylase (PHD) enzymes. Additionally, the transactivation domain (TAD) is crucial for achieving full transcriptional activity. HIF-3 $\alpha$  presents a greater complexity, featuring multiple splice variants that exert diverse biological effects. Of particular significance, HIF-3 is unique among the subunits in possessing an LZIP motif, which also confers DNA binding capabilities.

into clinical targeting the HIF pathway in cancer and potential alternative strategies.

### HIF pathway in cancer

#### HIF subunits and composition

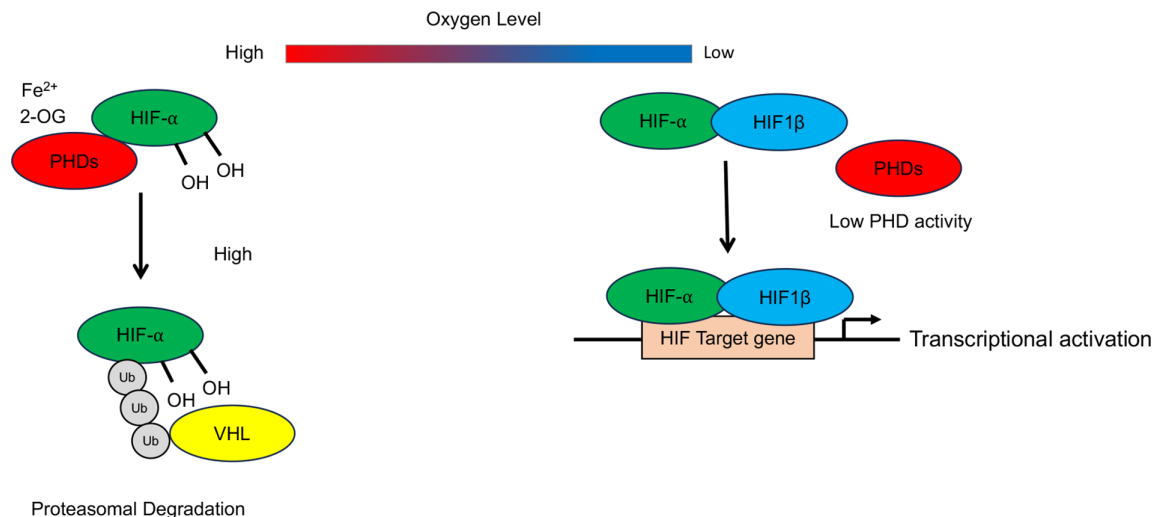
HIFs are a family of transcription factors stabilised in response to diminished oxygen availability. They act as key coordinators of the transcriptional response to lower oxygen levels. HIF functions as a heterodimeric transcription factor, composed of an oxygen-sensitive HIF- $\alpha$  subunit and a constitutively expressed but oxygen-insensitive HIF1 $\beta$  subunit (also known as ARNT).<sup>16</sup> HIF-1 $\alpha$  was initially identified through analysis of the expression of the erythropoietin gene,<sup>17</sup> and currently, there are three known isoforms of HIF- $\alpha$  (HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$ )<sup>18</sup> (figure 1).

Structurally, all HIF- $\alpha$  subunits share common functional domains. At the N-terminus HIF possesses a basic helix-loop-helix domain, which is responsible for DNA binding.<sup>16</sup> This domain is followed by a Per/ARNT/Sim (PAS) domain, consisting of two vital regions termed PAS-A and PAS-B, crucial for heterodimerisation with HIF1 $\beta$ .<sup>19</sup> The oxygen-dependent degradation domain (ODD) is what renders HIF- $\alpha$  sensitive to oxygen levels.<sup>20</sup> Additionally, HIF- $\alpha$  possesses a C-terminal transactivation

domains (C-TAD), necessary for full transcriptional activation.<sup>21</sup>

HIF-1 and HIF-2 have been the subject of extensive research and share a significant degree of sequence similarity. While they are known to share common target genes, they have also been found to modulate distinct gene targets and exhibit different DNA binding patterns.<sup>22</sup> Specifically, HIF-1 primarily associates with gene target promoters, whereas HIF-2 is frequently situated at gene promoters and enhancer sequences.<sup>23</sup> Emerging evidence indicates that HIF-1 and HIF-2 possess unique functions and may even have opposing roles.<sup>22</sup> The mechanisms underlying how HIF-1 and HIF-2 target specificity are regulated continue to be a topic of ongoing investigation. In stark contrast to HIF-1 $\alpha$ 's ubiquitous expression, HIF-2 $\alpha$  is more tissue-specific<sup>24–27</sup> and differs in its activation kinetics.<sup>28</sup> Both isoforms are often found to be overexpressed in many cancer types, which is associated with unfavourable disease prognoses, resistance to chemotherapy and radiation therapy, and the emergence of highly aggressive tumours.<sup>29</sup>

HIF-3 $\alpha$  remains the least explored of the three isoforms and is the most tissue specific. Its regulation is notably more complex when compared with HIF-1 and HIF-2 due to the generation of multiple splice variants, some



**Figure 2** Regulation of HIF by oxygen. Under normal oxygen conditions, HIF- $\alpha$  subunits are hydroxylated by prolyl hydroxylase (PHD) enzymes in an oxygen-dependent manner. This hydroxylation marks them for proteasomal degradation by creating a binding affinity for the E3 Ligase VHL. In contrast, under hypoxic conditions, PHD activity is inhibited, leading to the stabilisation and translocation of HIF- $\alpha$  subunits to the nucleus. There, they heterodimerise with HIF-1 $\beta$ , and activate the transcription of target genes involved in adaptation to low oxygen environments. HIF, hypoxia-inducible factor; VHL, von Hippel-Lindau.

of which lack DNA binding capabilities and sensitivity to oxygen levels.<sup>30</sup> This complexity has posed significant challenges in understanding the biological role of HIF-3 $\alpha$ . While initially thought to function mainly as a transcriptional regulator by competitively inhibiting the interaction between HIF- $\alpha$  and HIF1 $\beta$ , several more recent studies have revealed that HIF-3 $\alpha$  possesses transcriptional activity.<sup>31–33</sup> Consequently, the precise role of HIF-3 $\alpha$  in cancer progression remains poorly characterised. However, recent research suggests that it may contribute to the promotion of metastasis in pancreatic cancer.<sup>34</sup>

#### Oxygen-dependent regulation of HIF

The primary regulation of HIF- $\alpha$  subunits occurs at the post-transcriptional level, through hydroxylation-dependent proteasomal degradation (figure 2). When oxygen is plentiful, HIF- $\alpha$  undergoes rapid hydroxylation at two critical proline residues within the ODD.<sup>35</sup> Specifically, in HIF-1 $\alpha$ , these proline residues are Pro 402 and 564, while in HIF-2 $\alpha$ , they are Pro 405 and Pro 531.<sup>20</sup>

Pioneering studies conducted in worms and flies revealed that prolyl hydroxylase enzymes (PHDs) are responsible for this hydroxylation process.<sup>35</sup> The PHDs, consisting of three known isoforms (PHD1, PHD2 and PHD3), belong to the larger class of enzymes known as 2-oxoglutarate-dependent dioxygenases (2-OG DD). These enzymes rely on oxygen, iron ( $\text{Fe}^{2+}$ ) and 2-oxoglutarate as essential cofactors for their catalytic activity. Consequently, these enzymes can act as sensors of oxygen levels, metabolic states and iron availability within the cellular environment.<sup>36</sup> PHD-dependent hydroxylation of HIF- $\alpha$  creates a binding affinity for von Hippel-Lindau (VHL) ubiquitin ligase complex which binds to HIF- $\alpha$  and catalyses its polyubiquitination, targeting HIF- $\alpha$  for proteasomal-mediated degradation.<sup>37–39</sup>

As oxygen levels decrease, the activity of the PHDs is inhibited, resulting in the stabilisation of HIF- $\alpha$  subunits. HIF- $\alpha$ , then translocates to the nucleus, where it forms a heterodimer with HIF1 $\beta$ . The HIF complex then interacts with consensus hypoxia-responsive element (HRE) sequences, (5'-RCGTG-3') within the promoters and enhancers of HIF target genes, initiating their transcription and activating the cellular adaptation to hypoxia.<sup>40</sup> The HIF response encompasses the activation of genes involved in a myriad of adaptive mechanisms that help cells cope with the challenges posed by reduced oxygen availability. To add an additional level of complexity, HIF's transcriptional activity is also regulated by factor inhibiting HIF (FIH), an asparagine hydroxylase. FIH hydroxylates a crucial asparagine residue located within the C-TAD of HIF- $\alpha$ , thereby obstructing its interaction with transcriptional coactivators, CBP and p300.<sup>41</sup> The interaction with p300/CBP is required for full activation of a subset of HIF target genes.

It is noteworthy that other members within the 2-OGDD enzyme family, such as the DNA demethylase TET enzymes and the histone demethylases within the KDM family, also play significant roles in orchestrating the HIF transcriptional response.<sup>42–44</sup> These enzymes have emerged as crucial factors in influencing the gene expression patterns and epigenetic landscape in hypoxic conditions and in cancer development.

In addition to the posttranscriptional control, the expression of HIF is also intricately modulated at the translational level through a complex interplay with the mammalian target of rapamycin (mTOR) complex. mTOR is a serine/threonine kinase that functions as a sensor and integrator of signals from the extracellular environment.<sup>45</sup> It comprises two functionally distinct complexes, namely, mTOR complex 1 (mTORC1) and

mTOR complex 2 (mTORC2). While mTORC1 primarily governs cell growth and metabolism by influencing protein translation, mTORC2 regulates cell survival and proliferation.<sup>46</sup> In numerous cancer types, mTOR is frequently overactivated due to the loss of tumour suppressors such as phosphatase and tensin homologue (PTEN), activation of oncogenes such as phosphoinositide 3-kinase (PI3K) and metabolic reprogramming.<sup>47</sup> Numerous studies have underscored the vital involvement of the mTORC1 complex in regulating HIF-1 $\alpha$  protein expression at both the translational and transcriptional level.<sup>48,49</sup> In contrast, hypoxia and the expression of HIF-1 $\alpha$  exert a negative regulatory influence on mTOR activity through transcriptional control of the mTOR inhibitor REDD1, resulting in translational shutdown.<sup>50</sup> Intriguingly, the activation of HIF-2 has an opposing effect, where the stabilisation of HIF-2 promotes mTORC activity through the transcriptional regulation of the amino acid transporter SLC7A5. This leads to an augmented uptake of amino acids, activation of mTORC1 and enhanced in tumour growth of xenograft models.<sup>51</sup>

#### Metabolic activation of HIF in cancer

In addition to the regulation of HIF by oxygen levels, HIF activity can also be influenced by the metabolic environment of the cell. This is because the proper functioning of PHD enzymes, relies on the availability of 2-oxoglutarate. Several in vitro studies have demonstrated that PHDs, along with other oxygen and 2-OGDD enzymes, such as DNA and histone demethylases, can be inhibited by various metabolic intermediates, including succinate and fumarate.<sup>52</sup>

The validity of these findings was further confirmed by the identification of patients with rare hereditary cancer syndromes carrying familial mutations in the tricarboxylic acid (TCA) cycle enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH). Patients harbouring mutations in SDH and FH are predisposed to cancer syndromes that manifest as paragangliomas, pheochromocytomas and renal cancer.<sup>53,54</sup> These tumours are highly vascular, have elevated concentrations of succinate and fumarate which inhibit PHD activity and result in HIF activation. However, it is important to note that while HIF activation is observed in these tumour types, it should not be assumed that HIF activity is the sole driver behind tumour progression. Several other studies have suggested that a combination of metabolic reprogramming, changes in the epigenetic landscape and upregulation of other transcription factors such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) also contribute to this process.<sup>55–57</sup>

More recently, individuals with germline mutations in the 2-oxoglutarate dehydrogenase complex (OGDH) have been observed to develop recurring pheochromocytomas and paragangliomas.<sup>58</sup> Initially, the underlying mechanisms were unclear, but using forward genetic screens, it was identified that the loss of OGDH leads to the accumulation of 2-oxoglutarate (2-OG) and its

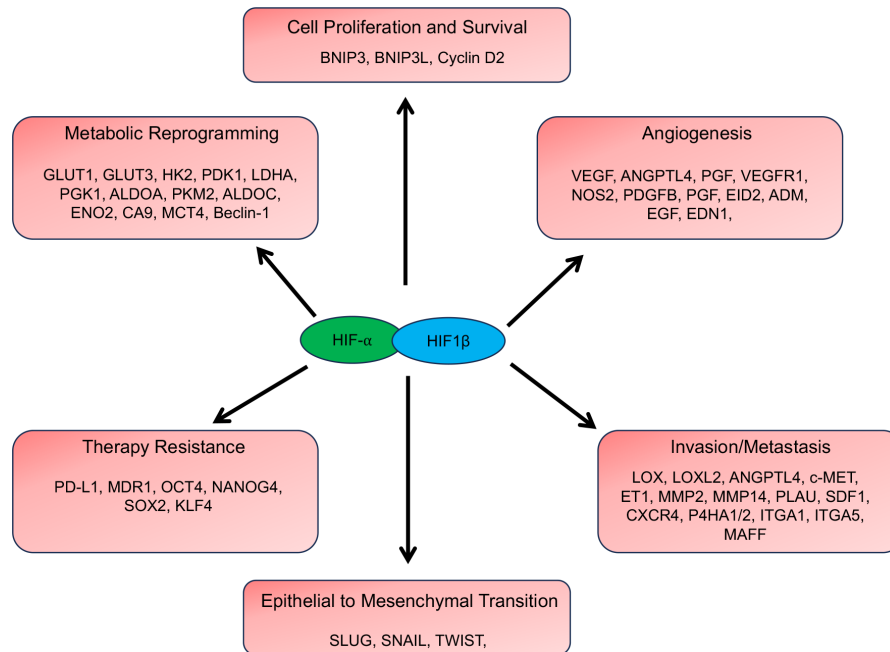
subsequent conversion into the L-enantiomer of 2OG, 2-hydroxyglutarate (2-HG). Notably, 2-HG is a potent inhibitor of PHD activity, as well as other 2-ODD enzymes, suggesting L-2HG accumulation may contribute more broadly to cancer progression outside of HIF activation.<sup>59</sup> This is an active area of research.

#### HIF target genes in cancer

##### *Metabolic reprogramming*

HIF target genes play a pivotal role in governing various cellular processes that contribute to critical aspects of cancer progression, identified in Hanahan and Weinberg's Hallmarks of Cancer<sup>60</sup> (figure 3). Among the notable factors driving cancer advancement is the Warburg Effect, a phenomenon whereby cancer cells undergo metabolic reprogramming. This rewiring involves a switch from oxidative metabolism towards glycolysis. This adaptation fulfils the biosynthetic demands arising from uncontrolled cancer cell proliferation, enabling heightened glucose consumption, providing carbon sources for anabolic processes such protein synthesis and cell growth.<sup>61</sup>

HIF-1 $\alpha$  serves as a critical regulator of metabolic reprogramming, orchestrating the cellular transition from oxidative phosphorylation to glycolytic metabolism in hypoxic tumours.<sup>62</sup> In response to hypoxia, the activation of HIF triggers an upregulation in the expression of many glycolytic enzymes, including glucose transporters GLUT1 and GLUT3, pyruvate dehydrogenase kinase (PDK1) and hexokinase II (HK2). These enzymes facilitate increased glucose influx into cells, redirecting glucose away from the TCA cycle towards anaerobic glycolysis.<sup>63</sup> Moreover, HIF activation within tumours induces the expression of lactate dehydrogenase (LDHA), which catalyses the conversion of pyruvate into lactate and H<sup>+</sup> ions.<sup>64</sup> The surplus H<sup>+</sup> ions are actively expelled from the cells, in an HIF-dependent manner, through upregulation of transporters such as NHE1, CA9 and the monocarboxylate transporters MCT4.<sup>65,66</sup> The removal of excess H<sup>+</sup> ions from cancer cells acidifies the extracellular environment, the consequences include diminished infiltration and activity of T cells coupled with an elevated expression of PD-1, leading to T-cell exhaustion<sup>67,68</sup> and a highly immunosuppressive environment. To tackle this problem, numerous ongoing studies are actively working on the development of specific inhibitors targeting LDHA, in combination with immunotherapy for potential therapeutic advantages.<sup>69</sup> However, more substantial progress has been achieved in the creation of specific inhibitors of CA9. CA9 is highly expressed in breast cancer and pancreatic ductal adenocarcinoma (PDAC), where its elevated expression is correlated with unfavourable prognoses and metastasis.<sup>70,71</sup> Beyond its role in regulating the cellular microenvironment, CA9 also is important maintaining cancer stem cells (CSCs),<sup>72</sup> which are highly resistant to therapeutics. Initial investigations into targeting CA9 as an anticancer therapy involved the use of the compound acetazolamide (AZM). Treating cells with



**Figure 3** HIF regulates multiple processes involved in tumour progression. In the tumour microenvironment, hypoxia or low oxygen levels, leads to the stabilisation of HIF-1 $\alpha$  and HIF-2 $\alpha$  subunits. HIF- $\alpha$  subunits translocate to the nucleus, where they heterodimerise with HIF- $\beta$  and bind to hypoxia response elements (HREs) in the promoter regions of target genes. HIF activation results in the transcription of genes that promote various aspects of cancer progression, including angiogenesis, metastasis, metabolic reprogramming and resistance to therapy. These HIF-driven processes collectively contribute to the aggressiveness and therapeutic challenges associated with cancer. Figure highlights some key gene targets that can influence cancer progression. HIF, hypoxia-inducible factor.

AZM led to an increase in intracellular pH, improved absorption of the chemotherapeutic drug doxorubicin and enhanced cytotoxicity. These results suggested that modulating CA9 activity could be a promising strategy to alleviate the acidity of the tumour microenvironment and boost the efficacy of other anticancer drugs.<sup>73</sup> Currently, there are two CA9 inhibitors that have entered clinical trials: SLC-0111 and DTP348. These small molecules have demonstrated their ability to selectively inhibit CA9 and penetrate hypoxic niches.<sup>74</sup> SLC-0111 is presently undergoing evaluation in a phase I clinical trial (<https://clinicaltrials.gov/ct2/show/NCT02215850>), focusing on assessing its safety and effectiveness in cancer treatment. Concurrently, DTP348 is under investigation in a phase I clinical trial (<https://clinicaltrials.gov/ct2/show/NCT02216669>), where it is being studied in conjunction with radiotherapy as a potential treatment option for patients with solid tumours.

In addition to its role in metabolic adaptation, recent studies have demonstrated a crucial role for HIF-2 $\alpha$  in maintaining endoplasmic reticulum (ER) homeostasis in clear cell renal cell carcinoma (ccRCC).<sup>75</sup> ccRCC is the most prevalent form of kidney cancer, and in approximately 90% of cases is characterised by the loss of the VHL gene and constitutive activation of HIF activity. Numerous studies have underscored the significance of HIF-2 activity in driving the progression of ccRCC through its ability to promote angiogenesis and metastasis.<sup>76</sup> One prominent hallmark of ccRCC is the

presence of intracellular lipid droplets, which serve as storage sites for triglycerides and cholesterol esters.<sup>77</sup> Through the analysis of patient samples and cell line models, it was revealed that HIF-2 regulates this process by controlling the transcription of PLIN2, a critical factor required for lipid droplet formation and lipid storage. Functionally, this regulation is essential to prevent a cytotoxic ER stress response, which can occur due to the increased pressure on the ER caused by elevated protein synthesis.

In a recent investigation, it was also revealed that HIF-2 can promote the uptake of cholesterol by regulating the transcription of the cholesterol scavenger receptor known as SCARB1 in ccRCC.<sup>78</sup> Cholesterol uptake is crucial for cancer progression, as it provides fuel for tumour growth, is an important component of cellular membranes and impairs immune cell function.<sup>79</sup> Current research has unveiled that, under hypoxic conditions, de novo synthesis of cholesterol, which is highly dependent on oxygen availability is inhibited in hypoxic cancer cells, through HIF-dependent and independent pathways due to its high dependence on oxygen.<sup>80 81</sup> Consequently, hypoxic tumours such as ccRCC are believed to develop a dependency on taking cholesterol from the outside environment, potentially creating an opportunity for therapeutic intervention by targeting HIF-2 activity and the process of cholesterol scavenging. This approach holds promise as a potential treatment strategy for ccRCC and other hypoxic tumour types.

### Angiogenesis

The HIF-dependent control of angiogenesis in cancer represents a pivotal mechanism propelling the progression and proliferation of tumours. Angiogenesis is the formation of fresh blood vessels from pre-existing ones, which enables cancer cells to access vital nutrients and oxygen necessary for their continued growth.<sup>82</sup> When HIF activation occurs in oxygen-deprived tumour environments, it stimulates the expression of a variety of proangiogenic genes, including VEGF, ANGPTL4, MMP-2, MMP-9 and LOX. These genes promote the expansion of endothelial cells, thereby promoting the development of new blood vessels<sup>83</sup> into the tumour.

In addition to nutrient supply, these newly formed blood vessels can also act as conduits for cancer cells, enabling them to enter the bloodstream and metastasise to distant organs.<sup>84</sup> In essence, HIF-driven angiogenesis not only sustains tumour growth but also facilitates the spread of cancer cells throughout the body.<sup>85</sup> Antiangiogenic therapies are an effective approach to cancer treatment. Various anti-VEGF/VEGFR inhibitors, such as sunitinib and sorafenib, are currently employed to treat advanced renal cancer, pancreatic cancer and other solid tumours.<sup>86</sup> Antiangiogenic therapy can reduce tumour angiogenesis and starve the tumour of growth factors and nutrients resulting in reduced cell growth and cell death.<sup>87</sup> In contrast to normal vasculature, vessels formed through tumour angiogenesis exhibit significant abnormalities, including a dilated lumen and irregular architecture.<sup>88</sup> Paradoxically, antiangiogenic therapy can also result in blood vessel 'normalisation' by restoring tumour perfusion and reducing tumour hypoxia to tumours which can increase the efficacy of chemo, radio and immunotherapy.<sup>89</sup> Despite the success of anti-VEGF therapy, it has proven ineffective in numerous patients. This may be attributed to the extensive array of proangiogenic genes regulated by HIF in hypoxic tumours, leading to therapy escape. Furthermore, a reduction in angiogenesis may exacerbate hypoxia within the tumour, potentially promoting metastasis and therapy resistance due to insufficient therapeutic delivery.<sup>90</sup>

### Cell invasion and migration

Cancer metastasis represents the foremost cause of mortality in patients with cancer.<sup>91</sup> This intricate process involves multiple stages including tumour cell infiltration, intravasation into blood or lymphatic vessels, exit from these vessels (extravasation) and unchecked proliferation. A key event in the initiation of cancer metastasis is the epithelial-mesenchymal transition (EMT), where cells transition from a rigid, structured state to a highly mobile phenotype. This transformation involves extensive alterations in cellular interactions and substantial remodelling of the extracellular matrix.<sup>92</sup> At the heart of these interactions is E-cadherin, an adhesion molecule that governs

cell-cell interactions, preserving an epithelial cellular identity through the regulation of  $\beta$ -catenin activity. Notably, earlier studies have demonstrated that the loss of E-cadherin amplifies cellular metastasis in cancer models, whereas its overexpression inhibits this process. HIF is known to orchestrate EMT by transcriptionally regulating key repressors of E-cadherin, TWIST, SNAIL and SLUG that downregulate E-cadherin expression, promoting a mesenchymal cell phenotype<sup>93–95</sup> which supports cancer metastasis.

For cancer cells to metastasise, they must also extensively modify the extracellular matrix (ECM). This entails the creation of a robust network, primarily composed of collagen that facilitates cell migration and invasion. HIF is a key mediator in this process by governing the expression of enzymes such as lysyl oxidases (LOX, LOXL2), procollagen hydroxylases (P4HA-1, P4HA-2) and integrins (ITGA1, ITGA5) and MAFF.<sup>96–98</sup> Substantial evidence underscores the indispensability of these proteins in driving metastasis in models of breast head and neck cancer.

### Therapy resistance

For cancer to progress, it must devise strategies to evade therapeutic interventions and remain undetected by the immune system. Hypoxic tumours, exhibit potent immunosuppressive properties, and show heightened resistance to chemotherapy and radiotherapy.<sup>99</sup> HIF activity coordinates various mechanisms that undermine the immune system's ability to combat tumour growth. To enable cancer cells to flourish and acquire metastatic potential, they must evade both the adaptive and innate components of the immune system such as, cytotoxic T cells and natural killer (NK) cells.<sup>100–101</sup> Extensive research has uncovered that tumour hypoxia creates a highly immunosuppressive environment, facilitating the unchecked proliferation of cancer cells.<sup>102–105</sup> As mentioned previously, HIF-mediated increases in tumour microenvironment acidity due to the Warburg effect, exacerbates immunosuppression.<sup>106</sup> Additionally, exposure to chemotherapeutic drugs such as doxorubicin and paclitaxel and hypoxia has been shown to induce the upregulation of programmed death ligand 1 (PD-L1) through direct binding of HIF-1 to the PD-L1 promoter, resulting in immunosuppression and, on occasion, apoptosis.<sup>107</sup> PD-L1 interacts with the programmed death 1 (PD-1) receptor found on T-cells and NK cells, contributing to immune evasion by causing T-cell exhaustion.<sup>108</sup>

A significant hurdle in the effectiveness of cancer therapy is the recurrence of cancer in patients who initially responded well to treatment. The development of therapy resistance is closely linked to the presence of CSCs within the tumour.<sup>109</sup> These cells possess unique capabilities, including self-renewal and the ability to differentiate into various cell types allowing them to adapt and to environmental conditions, ultimately acquiring resistance to therapeutic interventions. Key transcription factors involved in CSC formation include OCT4, SOX2,

KLF4 and NANOG.<sup>110–112</sup> Exposure of cancer cells to hypoxia and chemotherapeutic agents such as paclitaxel increase the proportion of CSCs and promote resistance to chemotherapy in cell culture, in an HIF-dependent manner.<sup>113</sup> Mechanistically, a substantial body of evidence supports that this is mediated by both HIF-1 and HIF-2,<sup>111</sup> which can directly and indirectly control the expression of OCT4, SOX2, KLF4 and NANOG.<sup>114 115</sup>

HIF activation can also contribute to therapy resistance by upregulating the expression of multidrug resistance 1 (MDR1), a cellular transporter responsible for efflux of chemotherapeutic agents from tumour cells. In lung adenocarcinoma and triple-negative breast cancer (TNBC), both MDR1 and HIF1 are markedly overexpressed, and this correlates with increased resistance to chemotherapy and unfavourable patient outcomes.<sup>116</sup>

#### *Cell proliferation and cell survival*

The progression of tumour growth heavily relies on the proliferation and survival of tumour cells.<sup>60</sup> During hypoxia, cells must adapt their functionality to ensure survival. The transition to glycolysis during hypoxia is particularly noteworthy, given the diminished energy supply resulting from reduced oxidative phosphorylation.<sup>117</sup> Tumour cells address this energy deficit by activating autophagy, a cellular mechanism that selectively breaks down and recycles cellular components to conserve energy.<sup>118</sup>

Autophagy activation during hypoxia contributes to energy provision by breaking down cellular components such as damaged organelles and proteins, generating smaller molecules for both protein synthesis and ATP production.<sup>119 120</sup> Simultaneously, research suggests that hypoxia can induce mitochondrial dysfunction and the production of reactive oxygen species (ROS), causing cellular damage.<sup>121</sup> In response to hypoxia, selective autophagy targeting mitochondria (mitophagy) is initiated, facilitating the elimination of impaired mitochondria and mitigating the detrimental effects of ROS.<sup>122</sup> Central to the regulation of autophagy and mitophagy in hypoxia is HIF-1, which directly binds to and triggers the transcription of BNIP3 and BNIP3L.<sup>123</sup> The induction of autophagy by HIF in hypoxic conditions has been shown to confer resistance to radiotherapy in lung and colon cancer cells.<sup>124 125</sup> While BNIP3 and BNIP3L can trigger autophagy, their activation represents a double-edged sword, as in specific scenarios, it can lead to the apoptosis and necrosis of cells.<sup>126</sup>

Tumour growth is significantly reliant on cell proliferation. Early investigations into the impact of hypoxia on cell progression, using cancer cell line models, revealed that hypoxia induces G1 cycle arrest due to the induction of the cyclin-dependent kinase (CDK) inhibitors p21 and p27.<sup>127 128</sup> The regulation of p27 during hypoxia remains a subject of ongoing discussion, with various studies proposing both HIF-dependent and independent mechanisms.<sup>129 130</sup> However, the induction of p21 is dependent

on HIF-1. In contrast to other HIF-dependent regulators of tumour progression that involve direct transcriptional regulation, the regulation of p21 involves a complex interplay with the c-Myc transcriptional network.<sup>127</sup>

The activation of c-Myc within tumours is a pivotal driver of cell cycle progression, functioning as a transcriptional activator of cyclins such as cyclin D2 and as a transcriptional repressor of p21.<sup>131</sup> Transcriptional regulation by c-Myc relies on binding to its transcriptional partner Max.<sup>132</sup> In a study from the Simon group, the induction of HIF-1 during hypoxia disrupted the interaction between c-Myc and Max, resulting in reduced expression of cyclin D2 and relief of repression of p21, leading to cell cycle arrest. Conversely, HIF-2 has been demonstrated to play an opposing role to HIF-1 concerning c-Myc activity. Induction of HIF-2 enhances the interaction between c-Myc and Max, resulting in increased cyclin D2 expression and decreased p21 expression, facilitating cell cycle entry.<sup>133</sup>

In this specific context, it could be hypothesised that HIF-1 is acting as a tumour suppressor; however, it is essential to recognise the distinction in activation kinetics between HIF-1 and HIF-2. HIF-1 activation is rapid and associated with acute hypoxia, whereas HIF-2 is associated with chronic hypoxia due to its slower and more sustained activation.<sup>28</sup> This underscores that the initial cell cycle arrest triggered by HIF-1 could function as a mechanism to facilitate the reprogramming of cellular metabolism and energy production. This adaptation could lay the foundation for subsequent cell cycle entry, a process facilitated by the later activation of HIF-2. Consequently, this presents a potential mechanism through which HIF-1 and HIF-2 collaboratively propel tumour progression in hypoxic tumours.

#### **Clinical progress in targeting the HIF pathway**

Radiotherapy, chemotherapy, targeted therapy and immunotherapy stand as the pillars of cancer treatment. However, a formidable obstacle of therapy resistance remains a prominent challenge in the realm of cancer therapy.<sup>134</sup> It is well established that hypoxia plays a pivotal role in most solid tumours, where cancer cells thrive in oxygen-depleted conditions markedly distinct from the oxygen levels in healthy tissues, therefore, current work is focused on developing therapeutic strategies capable of modulating the hypoxic microenvironment to confer therapeutic advantages.<sup>135</sup>

Numerous approaches are currently under investigation, including the development of bioresponsive prodrugs that selectively activate within hypoxic environments.<sup>136</sup> Additionally, the use of nanotechnology has emerged as a novel area of research to tackle hypoxic tumours. Researchers have engineered nanoparticles designed not only for precise drug delivery to tumour sites but also for the meticulous control of drug dosage. In early preclinical studies, the use of nanoparticles to release oxygen at tumour sites in conjunction with doxorubicin

has demonstrated highly promising outcomes in cell and mouse models.<sup>137</sup>

Another promising avenue for targeting tumour hypoxia involves direct inhibition of the HIF pathway itself. Given that HIF is an intracellular transcription factor, which lacks easily accessible active sites, the pursuit of inhibiting HIF activity has posed significant challenges.<sup>138</sup> Initial studies identified compounds capable of indirectly impeding HIF activity by reducing mRNA expression, translation, degradation and transcriptional activity. Examples include compounds such as PX-478 and digoxin, which inhibit HIF-1 $\alpha$  protein expression by inhibiting Hsp90 activity.<sup>139 140</sup> Although these compounds exhibited immense promise in preclinical models involving cancer cell lines and mice, they lacked specificity and as such, proved to be excessively toxic for human use and did not reach clinical trial.

More recently, a collection of new HIF-1 $\alpha$  inhibitors have surfaced. For example, chetomin disrupts the DNA binding capacity of the HIF complex by interfering with the interaction between HIF-1 and its chaperone heat shock protein 90 (Hsp90). Chetomin demonstrated antitumour efficacy in human myeloma cell lines and in patient samples, and similar positive results have emerged in studies involving prostate cancer.<sup>141 142</sup> Acriflavine, a small molecule, interferes with the interaction between HIF-1 $\alpha$  and its binding partner p300. This reduces the activation of specific subsets of HIF target genes, leading to enhanced cell death in glioma cells and prolonged survival in in vitro and in vivo studies.<sup>143 144</sup> Recent studies have highlighted the potential application of natural plant compounds, known as phytochemicals, for modulating HIF activity. Compounds such as baicalein and oroxylin have demonstrated the ability to diminish HIF

activity and enhance cytotoxicity through diverse mechanisms.<sup>145 146</sup> While these compounds show promise, it remains challenging to determine whether the observed cytotoxic effects are solely linked to the reduction in HIF activity. Further research is needed to gain a deeper understanding of their mechanisms of action. Presently, the primary challenge associated with HIF-1 inhibitors lies in their limited specificity (table 1). All existing inhibitors function as indirect inhibitors, leading to numerous off-target effects. These effects stem from issues related to DNA intercalation, causing complications in DNA replication and the cell cycle.<sup>147</sup>

Targeting of HIF-2-specific activity has been more successful. Unlike HIF-1 inhibitors which were identified using compound screening, HIF-2 compounds that specifically inhibit HIF-2 activity were identified using a structural biology approach.<sup>148–150</sup> Currently, there are two compounds, PT2385 (second generation PT2977) and PT2399 (belzutifan), that directly inhibit HIF-2 activity (table 1). These compounds function by selectively binding to HIF-2 $\alpha$ , preventing its dimerisation with HIF1 $\beta$ . These compounds were rapidly investigated for their potential in treating ccRCC, where HIF-2 has been identified as a significant oncogenic driver.<sup>151</sup> Both in vitro and in vivo animal studies using the HIF-2 inhibitors have demonstrated remarkably potent antitumour effects.<sup>76 150</sup> During phase I clinical trials involving patients with advanced ccRCC, PT2385 and PT2399 exhibited good antitumour activity and had good safety profiles.<sup>152</sup> In a phase II clinical trial, PT2399 demonstrated substantial efficacy against RCC as well as other tumours associated with VHL disease, leading to its FDA approval in this circumstance.<sup>153</sup>

**Table 1** HIF-1 and HIF-2 inhibitors

Inhibitor	Effect
<b>HIF-1 Inhibitors</b>	
EZN-2968 (synthetic oligonucleotide)	Targets HIF-1 mRNA leading to decreased HIF-1 protein levels.
PX-478	Inhibits HIF translation by blocking its interaction with the ribosome. Also increases p53 and reduces c-Myc translation.
Acriflavine	Inhibits the interaction with HIF and p300 (Hsp90 inhibitor), Topoisomerase inhibitor.
32-134D	Inhibits HIF-1 transcription. Based on Acriflavine. Off targets need further investigation.
Chetomin	Blocks interaction of HIF-1 with Hsp90. Hsp90 inhibitor.
Digoxin	Inhibits HIF-1 activity by blocking its interaction with Hsp90. Cardiac glycoside that inhibits the Na <sup>+</sup> K <sup>+</sup> ATPase.
<b>HIF-2 Inhibitors</b>	
PT2385	Direct inhibitor of HIF-2-dependent activity. Blocks heterodimer formation between HIF-2 $\alpha$ and HIF1 $\beta$ .
PT2977 (second generation)	Direct inhibitor of HIF-2-dependent activity. Blocks heterodimer formation between HIF-2 $\alpha$ and HIF1 $\beta$ .
PT2399 (belzutifan)	Direct inhibitor of HIF-2-dependent activity. Blocks heterodimer formation between HIF-2 $\alpha$ and HIF1 $\beta$ .
Summary of current HIF-1 and HIF-2 inhibitors and their effects. HIF, hypoxia-inducible factor.	



Undoubtedly, the discovery of HIF-2 inhibitors marks a significant advancement in ccRCC treatment. Nevertheless, it is important to note that employing HIF-2 inhibition as a treatment for ccRCC is not a universal solution. Several studies have reported mechanisms of resistance to HIF-2 inhibitors, including mutations in HIF-2 that prevent inhibitor binding and the utilisation of escape mechanisms involving p53.<sup>154</sup> Furthermore, preclinical studies using patient-derived RCC cell lines and xenograft studies have revealed that not all patients responded equally to HIF-2 inhibition.<sup>76</sup> The authors suggest that further research is necessary to identify markers that can stratify patients into those who will and will not respond favourably to HIF-2 inhibition.

The discovery of HIF inhibitors capable of counteracting cellular adaptations to hypoxia in cancer presents significant potential for enhancing the effectiveness of combination therapies. Although various HIF-1 inhibitors are currently undergoing clinical trials alongside chemotherapy, radiotherapy and immunotherapy agents, the indirect nature of these inhibitors makes it challenging to attribute the clinical benefits of combination therapy to the inhibition of HIF-1 activity. Identifying more specific HIF-1 inhibitors could enable their combination with anti-PD-1 immunotherapy agents, potentially enhancing efficacy by reducing PD-L1 expression in tumour cells. Simultaneously, there is a specific focus on exploring HIF-2 inhibition to improve outcomes in immunotherapy. Several studies have demonstrated that, in contrast to other cancer types, the regulation of PD-L1 expression in ccRCC is primarily governed by HIF-2 $\alpha$ .<sup>155 156</sup> Moreover, mouse studies of PDAC have shown that stromal HIF-2 activity reduces the efficacy anti-CTLA-4 and anti-PD1 therapy.<sup>157</sup> This underscores the therapeutic opportunity to combine immunotherapeutic agents with HIF-2 inhibition. Currently, this hypothesis is under investigation in a clinical trial assessing the combination of PT2385 with nivolumab (an anti-PD-L1 antibody) in advanced ccRCC.<sup>152</sup>

#### Alternative strategies to targeting the HIF pathway in cancer: focus on chromatin regulators

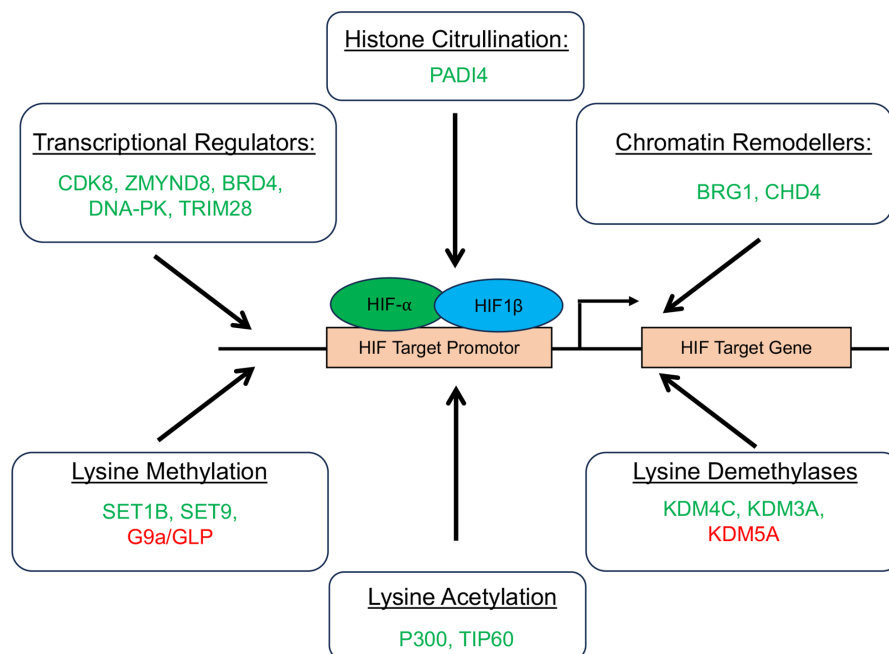
Therapeutically targeting HIF activity is an attractive strategy in cancer treatment. However, it is essential to recognise that besides its role in regulating tumourigenesis, HIF also plays crucial functions in various tissues throughout the body, including immune cells, neurons and the carotid body.<sup>24 27 158</sup> Additionally, a one-size-fits-all approach of inhibiting both HIF-1 and HIF-2 may not be advantageous, particularly in certain tumour types such as ccRCC, where HIF-1 is believed to act as a tumour suppressor.<sup>159</sup> Moreover, as HIF plays multiple roles in the body, blanket inhibition of HIF activity may result in unintended toxicity. Furthermore, clinical studies using HIF-2 inhibitors have revealed the emergence of therapy

resistance mechanisms in patients.<sup>154</sup> Consequently, alternative strategies for targeting HIF activity in cancer are needed.

An ongoing conundrum in the field of hypoxia research is understanding how HIF activates a transcriptional response at the chromatin level. While both HIF-1 and HIF-2 bind to the same HRE consensus sequence, chromatin immunoprecipitation sequencing studies have unveiled distinct genome binding patterns for both isoforms.<sup>23 160</sup> This suggests the existence of unique mechanisms that orchestrate HIF isoform binding and transcriptional control. One potential mechanism is through the interaction with distinct transcriptional and chromatin complexes that act as regulatory hubs that facilitate HIF transcriptional specificity. By understanding how these complexes provide specificity to HIF transcriptional activity it is possible they could be targeted therapeutically to provide therapeutic benefit in cancers where HIF is driving tumour progression (figure 4). Transcriptional regulation ultimately hinges on the accessibility of chromatin. Chromatin accessibility can be modulated through various mechanisms, with one of the most extensively studied mechanisms being the posttranslational modification of histone tails by enzymes known as chromatin writers.<sup>161</sup> Early research in this area highlighted the significance of the acetyltransferase complex CBP/p300 in orchestrating the expression of specific subsets of HIF-dependent genes.<sup>162</sup>

#### Histone acetylation

While p300/CBP was traditionally considered the primary acetyltransferase governing HIF's transcriptional activity for many years, depletion of p300 in cell line models only resulted in reduced expression of a subset of HIF target genes.<sup>41</sup> This observation suggested that other transcriptional regulators may coordinate HIF's broader transcriptional repertoire. Recent studies have used genetic screening techniques to identify novel HIF coactivators. Through an siRNA screen conducted in *Drosophila*, scientists identified Pontin and Reptin, which are components of the TIP60 complex, as coactivators of the *Drosophila* equivalent of HIF, Sima.<sup>163</sup> The TIP60 complex is a large, multisubunit assembly responsible for catalysing the acetylation of histone 3 (H3) lysine 9 and histone 4, modifications associated with transcriptional activation.<sup>163</sup> Subsequent investigations in human cancer cells demonstrated that Pontin, Reptin and TIP60 itself can physically interact with HIF1 $\alpha$ . Mechanistically, the HIF complex recruits the TIP60 complex to HIF target genes, where it facilitates the acetylation of H3K9 and H4. This acetylation, in turn, leads to the phosphorylation and activation of RNA polymerase II.<sup>164</sup> The therapeutic potential of targeting TIP60 activity has been proposed for the treatment of non-small cell lung carcinoma and malignant pleural mesothelioma.<sup>165 166</sup> However, it remains unclear whether the impact of inhibiting TIP60 on cancer growth is contingent on a reduction in HIF activity.



**Figure 4** Regulators of HIF transcriptional activity. Diagram illustrating the categorised regulators of HIF transcriptional activity during hypoxia, segmented based on their functional roles. Proteins highlighted in green serve as positive regulators, enhancing HIF activity, while those in red function as negative regulators, suppressing HIF activity. HIF, hypoxia-inducible factor.

#### Histone lysine methylation and demethylation

Histone methylation of lysine and arginine residues is a crucial regulator of gene expression, with lysine methylation being the most studied in the regulation of the hypoxia response. Lysine methylation involves the addition of methyl groups to specific lysine residues on histones. Lysine residues can undergo mono, di or trimethylated and the precise lysine modified can exert profound effects on gene expression. Lysine methylation is catalysed by families of enzymes known as histone lysine methyltransferases (KMTs), while removal of these methyl groups relies on histone demethylases (KDMs).<sup>167</sup>

A notable subset within the KDM family consists of enzymes featuring the Jumonji C (JmjC) domain. Like the PHD enzymes, the KDMs depend on molecular oxygen, iron and 2-oxoglutarate for their enzymatic activity.<sup>168</sup> Intriguingly, a body of research has revealed that many of these enzymes are direct transcriptional targets of HIF, underscoring their essential role in orchestrating the hypoxia response.<sup>169–173</sup> One such example is KDM4C, which can interact with and enhance the transcriptional activity of HIF-1 $\alpha$ , with no discernible impact on HIF-2 $\alpha$ . Mechanistically, KDM4C demethylates H3K9me<sub>3</sub>, an epigenetic mark associated with transcriptional repression, thereby promoting increased HIF-1 $\alpha$  binding and increased transcriptional activity.<sup>173</sup>

HIF-1 $\alpha$  similarly directly regulates the transcription of KDM3A. As observed with KDM4C, KDM3A can associate with HIF-1 and is indispensable for the upregulation of glycolytic genes in cancer cells.<sup>174–175</sup> Notably, both KDM4C and KDM3A have been identified as key players in various cancer types, including breast and ovarian

cancers.<sup>176</sup> Therapeutically, targeting these enzymes may provide a means of selectively addressing the metabolic reprogramming mediated by HIF-1 in these cancer types.

Recent research has shed light on the pivotal role of KDMs in governing the epigenetic landscape during hypoxia. In two concurrent investigations conducted by the Kaelin and Rocha groups, KDM5A and KDM6A emerged as crucial oxygen sensors orchestrating the epigenetic landscape and cell fate in hypoxia.<sup>177–178</sup> Using cell culture models, these studies revealed that hypoxic exposure reduces the activity of these enzymes, resulting in epigenetic alterations and changes in gene transcription. Interestingly, the loss of KDM5A resulted in elevated histone 3 lysine 4 trimethylation (H3K4me<sub>3</sub>), which is typically linked to the activation of gene transcription even in normoxic conditions. This led to the expression of HIF-dependent genes, mirroring the cellular response to hypoxia.<sup>178</sup> These findings underscored the significance of chromatin as an oxygen sensor. Given that various KDM family members have been implicated in cancer progression, ongoing research is exploring their therapeutic potential.<sup>179–180</sup> However, further investigations are required to unravel the mechanisms governing KDM specificity, particularly in regulating the expression of oncogenes and tumour suppressors and to determine if they also methylate non-histone methylation substrates, which may contribute to the HIF response in cancer.

The increase in histone methylation observed at HIF target genes during hypoxia cannot be solely attributed to histone demethylase activity, as active methylation processes are also in play. In a recent investigation, a CRISPR-based forward genetic screen identified the H3K4 histone methyltransferase SET1B as a coactivator

for HIF transcriptional activity. This research revealed that, in response to hypoxia, SET1B interacts with the HIF complex and is recruited to specific HIF target sites in an HIF-dependent manner, where it influences the levels of H3K4me3 and HIF transcriptional activity. SET1B can interact with both HIF-1 $\alpha$  and HIF-2 $\alpha$  and predominantly regulates genes associated with angiogenesis. Mouse xenograft studies illustrated that the loss of SET1B resulted in reduced tumour growth and establishment, with tumours that were more hypoxic and more apoptotic.<sup>181</sup> This suggests that SET1B could be a potential therapeutic target to treat cancers which are driven by HIF activity. Although not fully explored, SET1B mRNA expression is elevated in ccRCC and increases with the metastatic potential of the tumour.<sup>182</sup> This is intriguing as ccRCC is driven by HIF-2 activity, suggesting SET1B may play a potential role in ccRCC progression by sustaining HIF-2 activity and promoting metastasis. Although the recruitment of SET1B is crucial for augmenting HIF activity at specific target genes, it remains unclear whether this effect is solely attributed to SET1B's H3K4me3 activity or if an alternative mechanism is at play. SET1B is a member of a broader family of histone methyltransferases, including SET1A and MLL1-4.<sup>183</sup> Their roles in the hypoxia response have not been thoroughly explored, and there is also a suggestion that they might methylate non-histone substrates.<sup>184 185</sup> H3K4me3 has also been shown to be important in modulating the release of paused RNA polymerase II.<sup>186</sup>

Early studies into the roles non-histone methyltransferases in the hypoxia response identified SET9 as a specific regulator of HIF-1-dependent activity. Notably, SET9 stabilises HIF1 $\alpha$  and facilitates its binding to chromatin, thereby activating specific subsets of HIF-1 target genes, particularly those associated with glycolysis. This is achieved through its ability to prevent HIF degradation.<sup>187</sup> Consequently, inhibiting SET9 activity presents a potential strategy to selectively block the activation of HIF target gene subsets in tumours. This approach may hold promise, especially in cases such as breast cancer, where both HIF1 and SET9 are associated with disease progression.<sup>188 189</sup>

#### Histone citrullination

In addition to well-established roles of histone methylation and acetylation, histone citrullination was recently identified as a new regulator of HIF activity in breast and liver cancer.<sup>190</sup> Histone citrullination involves the conversion of arginine residues to citrulline, which reduces the positive charge of histones, thereby facilitating transcription. This modification is catalysed by the enzyme's peptidyl arginine deiminases, PADI2 and PADI4.<sup>191</sup> Through a variety of biochemical approaches, it was determined that PADI4 is a direct target of HIF and is essential for the expression of nearly all HIF target genes in hypoxic conditions. Mechanistically, this is attributed to PADI4's capability to influence the epigenetic landscape surrounding HIF target genes.<sup>190</sup> Elevated levels of

PADI4 have been observed in TNBC and hepatocellular carcinoma.<sup>192 193</sup> Analysis of human breast cancer patient samples revealed a significant correlation between PADI4 expression and HIF expression, as well as angiogenesis within the tumour. It remains to be studied whether inhibition of PADI4 activity would be of clinical benefit in dealing with tumour hypoxia.

#### Chromatin remodelling

In addition to modifications made to histones after translation, the accessibility of chromatin can also be shaped by ATP-dependent chromatin remodelling complexes. These complexes use DNA-dependent ATPase activity to reposition and reorient histones, thereby altering the overall chromatin accessibility.<sup>194</sup> At the level of histones, chromatin remodelling is primarily regulated by the Switch/Sucrose-Nonfermentable (SWI/SNF) complex. The SWI/SNF complex uses its ATPase activity to impact chromatin accessibility by removing and shifting nucleosomes.<sup>195</sup> The composition of this complex can vary, with some complexes containing the BRG1 protein while others contain BRM1. SWI/SNF complexes containing BRG1 can interact with HIF-1 $\alpha$  and HIF-2 $\alpha$ , enhancing their recruitment to a subset of HIF target genes.<sup>196</sup> In a separate study, the CHD4 subunit of the nucleosome remodelling and deacetylase complex (NuRD) has been associated with the coactivation of HIF-1-dependent and HIF-2-dependent activities. Mechanistically, it is thought that CHD4 regulates the loading and release of paused RNA polymerase II upon HIF binding.<sup>197</sup> The precise mechanism by which CHD4 identifies specific target genes remains unknown. Interestingly, this regulation of HIF activity seems to be independent of CHD4's helicase activity or other NuRD complex subunits. This suggests that hypoxia may influence the composition of various chromatin-associated protein complexes, potentially leading to novel functions. Further research is necessary to determine if this is the case, but it could offer therapeutic opportunities for targeting protein complexes that exist in hypoxic tumours.

#### Transcriptional regulators

Although chromatin remodelling is key to promoting HIF binding to its transcriptional targets this is not ultimately enough to fully activate transcription. RNA polymerase II (RNAP II) is a pivotal enzyme responsible for the transcription of eukaryotic genes. Transcription begins with the initiation of RNAP II activity at specific promoter regions, where the enzyme assembles with various transcription factors to form a preinitiation complex. This initial step, known as transcriptional initiation, sets the stage for the subsequent elongation phase. During transcriptional elongation, RNAP II travels along the DNA template, synthesising an RNA molecule that is complementary to the coding strand of the gene.<sup>198</sup> These two fundamental processes, initiation and elongation are essential for the accurate and regulated expression of genes and are highly dependent on phosphorylation of

specific residues within the C-terminal domain (CTAD) of RNAP II.<sup>199</sup>

Numerous investigations have demonstrated that the HIF complex primarily associates with regions of open chromatin, where it stimulates the release and elongation activity of paused RNA polymerase II.<sup>200</sup> However, this process is not solely reliant on HIF binding; it is also dependent on the recruitment of other transcriptional complexes. One such complex is the CDK8 mediator complex, a multisubunit assembly capable of enhancing RNA polymerase II phosphorylation and transcriptional elongation. In a study conducted by Galbraith *et al*, it was revealed that the CDK8 mediator complex interacts with and is recruited to HIF1 $\alpha$ -dependent target genes. Through genetic depletion experiments, it was CDK8 was found to be indispensable for the expression of the majority of HIF-1 $\alpha$  dependent target genes.<sup>201</sup> In a clinical context, cancer cells are known to exploit CDK8 to promote tumorigenesis, making it an appealing target for therapeutic intervention.<sup>202</sup> Notably, one CDK8 inhibitor, RVU120 (SEL120), is currently under investigation in a phase I/II clinical trial involving patients with relapsed metastatic solid tumours.<sup>203</sup>

Methylation and acetylation modifications on histone residues are detected by specific chromatin reader proteins, that couple alterations in histones to transcriptional regulation. One such chromatin reader is Zinc Finger MYND-type containing 8 (ZMYND8), which recognises methylation and acetylation of histone 3 and 4.<sup>204</sup> Notably, in the context of breast cancer cells, ZMYND8 was identified as a coactivator for HIF-1 $\alpha$  and HIF-2 $\alpha$ . When exposed to hypoxic conditions, ZMYND8 interacts with HIF-1 $\alpha$  and HIF-2 $\alpha$ , enhancing the expression of the majority of HIF target genes.<sup>205</sup> This was also found to be dependent on acetylation, facilitated by p300 and its association with BRD4. Intriguingly, ZMYND8 is subject to direct transcriptional regulation by both HIF-1 and HIF-2, resulting in an amplified HIF response, which was found to be integral to the promotion of breast cancer progression and metastasis.

More recently, a study conducted by Yang *et al* employed rapid immunoprecipitation mass spectrometry of endogenous protein (RIME), a proteomic method aimed at identifying chromatin-associated complexes, to elucidate the requirements for an HIF-1 $\alpha$ -dependent transcriptional response in breast cancer cells. This investigation identified DNA-dependent protein kinase (DNA-PK) and tripartite motif-containing protein 28 (TRIM28) as pivotal regulators of HIF-1 $\alpha$ -dependent activity. In response to hypoxia, HIF-1 $\alpha$  recruits DNA-PK and TRIM28 to chromatin, initiating DNA-PK activation and the subsequent phosphorylation of TRIM28. Phosphorylation of TRIM28 triggers the recruitment of CDK9, the release of negative elongation factors from RNA polymerase II, and the activation of transcriptional elongation.<sup>206</sup> Importantly, DNA-PK and TRIM28 as well as HIF-1 expression have been observed to increase in various cancer types and are associated with poor patient prognosis.<sup>207–209</sup> Several

DNA-PK inhibitors are currently undergoing clinical trials for cancer treatment.<sup>209</sup> Given that inhibition of HIF activity leads to reduced tumour angiogenesis, metastasis and immune evasion, it is plausible that some of the therapeutic benefits of DNA-PK inhibition stem from its impact on HIF activity.

## CONCLUSION AND FUTURE PERSPECTIVES

The cellular mechanisms driving cancer growth are incredibly intricate, involving a web of interconnected processes. These range from genetic mutations that initiate tumorigenesis to complex signalling pathways that sustain the proliferation and survival of tumour cells. Notably, the challenge of tumour hypoxia significantly impacts the efficacy of cancer therapies. The contribution of HIF to cancer progression has been extensively studied since its discovery, providing a deep understanding of how HIF governs various aspects of tumour biology. This knowledge has paved the way for the development of several therapeutics, now making their way into clinical practice for cancer treatment, either as stand-alone agents or in combination with other treatments such as immunotherapy and radiotherapy.

However, targeting HIF in cancer remains challenging, as complete inhibition of HIF activity may lead to unintended toxicity in tissues reliant on HIF for normal functioning. Moreover, while a specific inhibitor for HIF-2 has been developed, the current HIF-1 inhibitors primarily work through indirect mechanisms, necessitating further research to develop more precise HIF-1 inhibitors.

A promising strategy to address specific aspects of HIF activity is to unravel how HIF transcriptional specificity is coordinated. Although numerous HIF coactivators have been identified, understanding how these factors collaborate to facilitate the HIF response remains unclear. Future studies should focus on delineating how HIF and its identified coactivators collaborate to initiate HIF transcriptional activity, ideally at the individual gene level. Furthermore, while many HIF activators have been shown to interact with HIF-1 and HIF-2 isoforms, detailed structural studies are warranted to comprehend the physical interactions between these regulators. This will not only gain deeper mechanistic insights into how HIF functions with these proteins but also offer potential for designing drugs to target these interactions. When contemplating the therapeutic targeting of chromatin regulators, a comprehensive understanding of the broader role of these enzymes in cell biology is crucial to mitigate possible off-target and toxicity issues. If the chromatin regulators identified are selectively recruited by the HIF complex to HIF target loci, structural studies could be leveraged to pinpoint small molecules capable of disrupting the interaction of these regulators with the HIF complex.

This approach would minimise the potential off-target effects associated with targeting chromatin regulators.

In addition to its role as a transcription factor, recent studies have highlighted non-transcriptional functions of HIF.<sup>210–211</sup> For instance, in a study by Kierans *et al*, it was revealed that HIF promotes glycolysis by facilitating the formation of large metabolic complexes in intestinal epithelial cells.<sup>212</sup> Understanding if this HIF-driven mechanism operates in tumour cells and exploring its potential as a target to reduce glycolysis within tumours holds great promise.

Beyond targeting HIF subunits directly, there is a growing interest in the potential of targeting PHD activity in cancer. PHDs not only control the levels of HIF in cells but can also hydroxylate non-HIF targets.<sup>213–216</sup> For example, a recent study by Liu *et al* demonstrated that PHD1 hydroxylates histone H3 at proline 16, regulating gene transcription and promoting TNBC.<sup>217</sup> However, the question of whether PHDs possess additional targets remains a subject of ongoing debate, as *in vitro* studies have struggled to confirm *in vivo* PHD targets.<sup>218</sup> This discrepancy could be attributed to the limitations of the *in vitro* methodology, potentially omitting crucial components necessary for hydroxylation that are present within cells.<sup>219–220</sup> Further research is needed to fully comprehend the roles of PHDs outside of HIF signalling. This is particularly encouraging from a clinical standpoint as the PHD inhibitor Daprodustat has been FDA-approved for treating anaemia in patients with chronic kidney disease.<sup>221</sup>

Another promising avenue in cancer research is the identification of genes that exhibit synthetic lethality in combination with VHL loss. This is particularly relevant in ccRCC, where 90% of cases involve VHL mutations resulting in VHL inactivation.<sup>76</sup> This approach holds significant promise, as its effectiveness has been convincingly demonstrated, as exemplified by the combination of PARP inhibition in tumours carrying BRCA1/2 mutations.<sup>222</sup> Numerous studies using genetic, compound and computational screening have identified potential candidates, and their clinical efficacy remains to be explored.<sup>223–226</sup>

### Questions for future research

- ⇒ Can targeted therapies towards the hypoxia-inducible factor (HIF) pathway enhance the potency of current cancer treatments?
- ⇒ How does HIF work with its coactivators to trigger an HIF response, and can this be exploited pharmacologically?
- ⇒ Are there unconventional roles for HIF in driving cancer progression beyond its canonical role in transcription?
- ⇒ Is it both pertinent and a potent strategy to direct our focus towards other elements within the HIF pathway, aside from the HIF subunits themselves to treat cancer?

In summary, the HIF pathway plays a pivotal role in shaping tumourigenesis. Understanding how this pathway is regulated is of paramount importance for identifying novel therapeutic strategies in cancer treatment and improved cancer management.

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