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The role of hypoxia inducible factor-1 in cell metabolism – a possible target in cancer therapy

Rachida S Bel Aiba, Elitsa Y Dimova, Agnes Görlach & Thomas Kietzmann†
†*University of Kaiserslautern, Faculty of Chemistry, Department of Biochemistry, Erwin-Schrödinger Strasse 54, D-67663 Kaiserslautern, Germany*

In many cancer types, intratumoural hypoxia is linked to increased expression and activity of the transcription factor hypoxia-inducible factor (HIF-1α), which is associated with poor patient prognosis. This increased the interest in HIF-1α as a cancer drug target. Further, HIF-1α has also a central role in the adaptive cellular programme responding to hypoxia in normal tissues. Many of the HIF-1α-regulated genes encode enzymes of metabolic pathways. Therefore, studying the link and the feedback mechanisms between metabolism and HIF-1α is of major importance to find new and specific therapeutic strategies.

Keywords: hypoxia, hypoxia-inducible factor, von Hippel-Lindau tumour suppressor

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1. Introduction

Over the last decade, many studies focused on the understanding of cancer progression in order to establish new potent therapeutic strategies. This has pointed to two key processes which appear to be closely linked: metabolism and the hypoxia-inducible factor (HIF) system.

Indeed, in various tumours increased metabolic rates and increased levels of HIF, especially HIF-1α, are considered to be associated with a poor prognosis. The link between them is clearly established and is dependent on decreased oxygen supply. Thus, hypoxia is one of the major problems that tumour cells have to deal with. In order to survive and proliferate under extreme conditions, in which nutrients and oxygen are limited, an adaptive process is initiated which promotes the expression of various genes. Several transcription factors, such as activator protein-1 (AP-1), early growth response protein-1 (EGR-1), nuclear factor-κB (NF-κB), CAAT enhancer binding protein-β (C/EBPβ/NF-IL-6) (for review see [1]) and HIF-1 were found to be involved in the modulation of gene expression by hypoxia. Thereby, HIF-1 appears to be of special interest because it activates transcription of glycolytic enzymes and glucose transporters as well as angiogenic growth factors to improve nutrient and blood supply to meet the energy demands of the tumour cell.

2. Oxygen and metabolism

Under physiological conditions, mammalian cells produce energy, which is stored in form of ATP, predominantly by aerobic conversion of glucose, fatty acids and amino acids to CO_2, H_2O and urea, respectively. In addition, small amounts of energy can be produced from the aerobic oxidative metabolism of lactate, glycerol or ethanol and by formation and utilisation of ketone bodies.

Once the major nutrient glucose has entered the cell via glucose transporters, it is converted to glucose-6-phosphate by hexokinases, which exist in several isoforms.

After phosphorylation of glucose to glucose-6-phosphate, glucose is degraded to acetyl-CoA in the glycolytic pathway via the glucose-6-phosphate isomerase reaction, continued by the phosphofructokinase, fructose bisphosphate aldolase plus triose phosphate isomerase reactions, then followed by the glyceraldehydephosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase (PK) and, finally, by the pyruvate dehydrogenase (PDH) reactions (**Figure 1**).

Acetyl-CoA which can also be gained from the metabolism of proteins and fatty acids is then transported to the mitochondria where it is dehydrogenated to CO_2 and reducing equivalents [H] in the citric acid (Krebs) cycle and, thus, these processes constitute the central oxidative energy metabolism (**Figure 1**).

In the citric acid cycle, acetyl-CoA is first combined with the C4-unit oxalacetate to yield the reduced C6-unit citrate in the citrate synthase reaction. This is followed by the aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate thiokinase, succinate dehydrogenase, fumarase and malate dehydrogenase reactions. Of the four dehydrogenase reactions, three yield nicotinamide adenine dinucleotide (NADH), one yields flavin adenine dinucleotide ($FADH_2$) and two produce CO_2 (**Figure 1**).

The NADH formed in substrate dehydrogenations is re-oxidised by an electron transfer chain composed of protein complexes located in the inner mitochondrial membrane. Complex I is an NADH:coenzyme Q oxidoreductase, complex II is identical with the succinate dehydrogenase of the citric acid cycle which reoxidises $FADH_2$ by electron transfer to coenzyme Q, complex III is a coenzyme Q:cytochome c oxidoreductase. Complex IV is a cytochrome c:oxygen oxidoreductase, in which molecular oxygen serves as electron acceptor and becomes reduced to water.

Complexes I, III and IV, but not II, function as proton pumps; they each extrude 3 – 4 protons per transferred electron pair. The export of protons generates a proton-motive force which is used to drive the synthesis of ATP from ADP and Pi catalysed by another multi-protein complex named ATP synthetase (also F_1F_0 ATPase). The vast majority of the ATP formed in the mitochondria is transported to the cytosol by an ATP:ADP exchange translocator in the inner mitochondrial matrix.

Thus, under aerobic conditions, energy production might also be referred to as a redox process that occurs during combustion of food-derived energy substrates serving as electron donors, and molecular oxygen serving as an electron acceptor. The breakdown and complete dehydrogenation of the energy substrates to CO_2 is coupled with the formation of reducing equivalents, mainly NADH, which are required for the formation of a trans-membrane proton-motive force enabling ATP generation. Thereby, the generated electrons react with O_2 which is subsequently reduced to H_2O. In mammals, this reduction from one molecule glucose generates a net amount of 38 molecules ATP.

By contrast, under anaerobic conditions, energy can only be gained by conversion of carbohydrates, which are only partially dehydrogenated to pyruvate under formation of NADH. The electrons from NADH are then transferred to pyruvate, which is converted to lactate, thereby regenerating NAD^+. The net amount of 2 ATP is then formed by conversion of one glucose via substrate level phosphorylation.

Thus, molecular oxygen is an essential component of energy metabolism and its availability can limit the energy balance of the cell. The regulation of oxygen delivery into cells and the control of cellular functions in response to oxygen are essential for all mammals. Under physiological conditions, the average oxygen tension in human arterial blood is between 74 and 104 mm Hg (104 – 146 µmol/l) and in venous blood between 34 and 46 mm Hg (48 – 64 µmol/l) [2,3]. Interestingly, a physiological O_2 gradient exists in liver, the major organ responsible for glucose homeostasis in mammals. Within the smallest functional unit, the acinus, blood flows from the region around the hepatic artery and terminal portal vein (periportal zone) into the sinusoid towards the cells located in the region around the central vein (perivenous zone) [4-6]. Thereby, the oxygen tension reaches 60 – 65 mm Hg in the periportal area and ~ 30 – 35 mm Hg in the perivenous zone [7,8] and it appears that this oxygen gradient functions as a key regulator for the genes encoding carbohydrate-metabolising enzymes [4-6].

In line with this, it was shown that in primary rat hepatocytes net glucose uptake started under hypoxia (2% O_2), whereas CO_2 formation increased in direct proportion to the oxygen concentration. Concomitantly, the gluconeogenic-dependent net glucose output and net lactate uptake increased under higher pO_2 values. By contrast, the net glucose output initiated by glycogen breakdown and the net lactate output started to increase under hypoxia in perivenous-like cells. Thus, the net flow between glucose-6-phosphate and pyruvate in the gluconeogenic direction was enhanced with increasing pO_2 in the periportal-like cells and, conversely, it was increased in the glycolytic direction with decreasing pO_2 in the less aerobic perivenous-like cells [4,9-11].

To operate efficiently under varying O_2 tensions, cells need a reliable O_2 sensing system allowing adequate adaptation of cellular functions to the O_2 available. The first threshold of cellular hypoxia occurs when a decrease in ATP production is measurable; however, sufficient ATP necessary to maintain the vital cellular functions is still produced by metabolic adaptations involving changes in the redox and phosphorylation status of enzymes and increased glycolysis [12,13]. The second threshold occurs when the ATP demand can be met only by anaerobic glycolysis; and the third threshold is reached when glycolysis is not sufficient to produce enough ATP for cell survival [12,13]. Adaptation may be achieved by short-term enzymatic reactions or by long-term regulations via modulating gene expression patterns. Within the latter, HIF-1 appears to be a master regulator for the expression of genes which are activated under low oxygen levels (i.e., hypoxia) [14] (**Figure 1**).

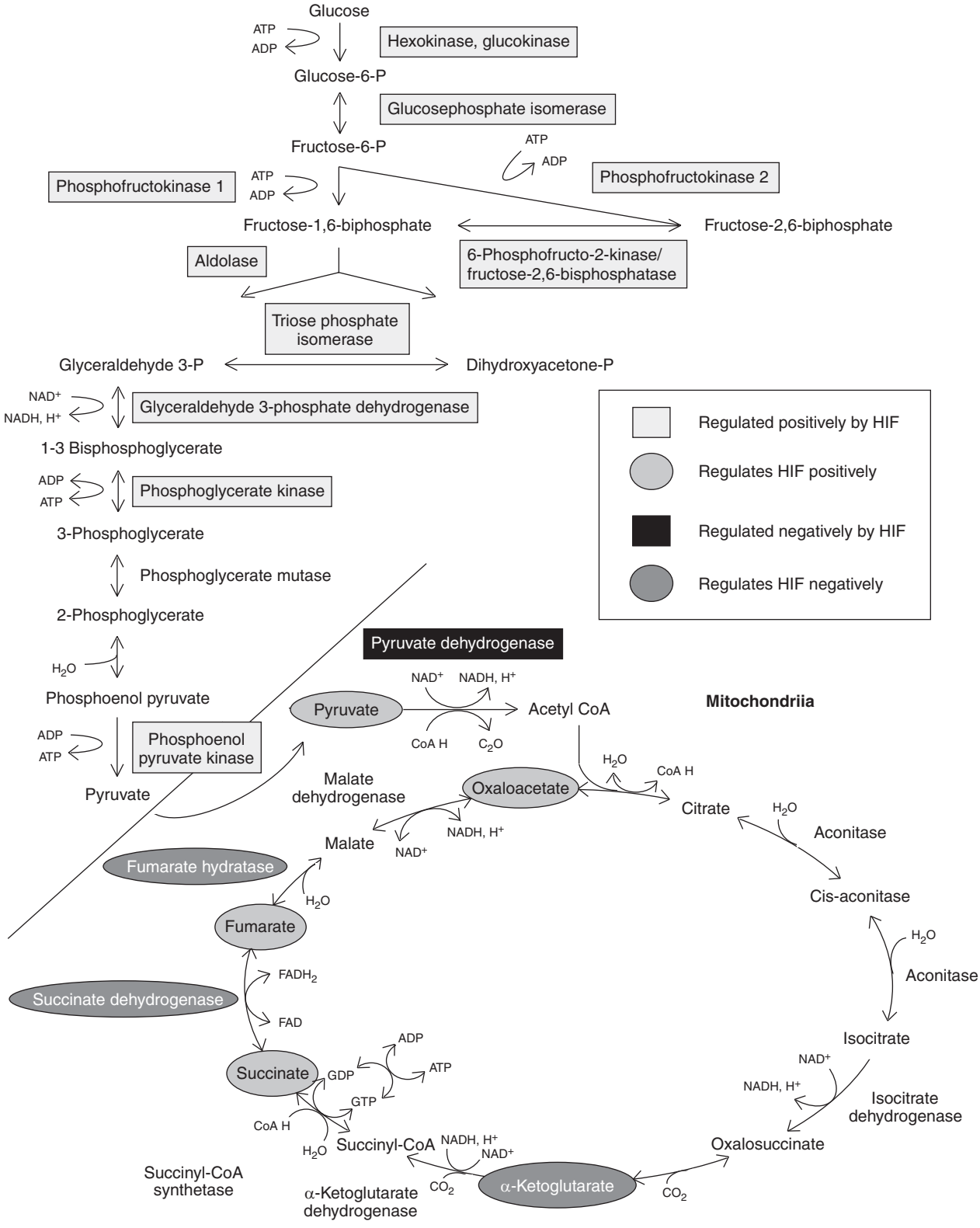

Figure 1. HIF-1 activity and carbohydrate metabolism are closely linked. HIF-1α is involved in the transcriptional regulation of most of the genes encoding enzymes involved in glycolysis. At the same time, it is positively or negatively regulated via several enzymes and metabolites of the citric acid cycle.
HIF: Hypoxia-inducible factor; P: Phosphate.

3. HIF-1 basic biology

3.1 HIF-1α and β subunits

HIF-1 is a heterodimer comprised of HIF-1α and HIF-1β (also termed ARNT). Both subunits belong to the basic-helix-loop-helix (bHLH) Per/Arnt/Sim (PAS) transcription factor family [15]. The dimerisation between both partner proteins is supported by the bHLH domain and a part of the PAS domain, whereas the recognition of hypoxia-responsive elements within promoter DNA is exclusively governed by the bHLH motif. The α-subunit also contains two nuclear localisation sequences, one is N-terminal (amino acid [aa] 17 – 33) and the other is C-terminal (aa 718 – 721) [16]. Transactivation activity is brought about by two transactivation domains (TAD), the N-terminal (TADN, aa 531 – 575) and the C-terminal (TADC, aa 786 – 826) [17-19]. Residues 576 – 785 comprise an inhibitory domain as its deletion was shown to increase transactivation domain function under normoxia [17].

3.2 HIF family

In addition to HIF-1α, which was described to be ubiquitously expressed, new members of the HIF α-subunit family have been identified which appear to be expressed in more restricted areas.

HIF-2α, also known as EPAS1 (endothelial PAS protein), HLF (HIF-like factor) or HRF (HIF-related factor), is predominantly expressed in highly vascularised tissues suggesting that HIF-2α may represent an important regulator of vascularisation and may modulate the regulation of endothelial cell gene expression in response to hypoxia [20-22]. Although HIF-1α and HIF-2α have some overlapping targets, they appear to be needed independently of each other, because knockout of HIF-1α and HIF-2α are lethal *in utero* due to major vascularisation defects [23-25]. In addition to the bHLH-PAS domain, HIF-2α also comprises a TADN and a TADC and is regulated in a similar way as HIF-1α [19].

A third member of the HIF α-family, HIF-3α, was cloned from human, mouse and rat [26-28]. In contrast to the other proteins from that family, it does not contain the TADC domain. Interestingly, several HIF-3α variants have been described: in the mouse an inhibitory PAS protein was identified, which lacks the TADN and the TADC and exerts inhibitory effects possibly by binding to HIF-1α and, likewise, HIF-3α variants have also been described in human cells [29,30]. In addition, two ARNT isoforms, ARNT2 [31] and ARNT3 [32], have been identified. ARNT2 appears to be mainly expressed in the CNS [33]. Recently, ARNT3 was suggested to be involved in the regulation of adipogenesis and lipid metabolism in adult adipocytes [34]. Thus, the existence of different α- and β-subunits suggests the existence of various HIF-isoforms.

3.3 HIF-1 regulation

HIF-1 activity is dependent on the dimerisation of both subunits. Whereas levels of ARNT appear not to be modulated by O_2, HIF-1α protein levels rise dramatically in response to hypoxia. Although transcriptional regulation of HIF-1α by hypoxia cannot be ruled out, the major regulatory mechanisms affect HIF-1α protein stability. Hypoxia-dependent stabilisation of HIF-1α is governed by the oxygen-dependent degradation domain termed ODD (aa 401 – 608) [35,36]. This domain contains prolines (Pro 402 and Pro 564) which under normoxic conditions are targets of O_2-dependent prolyl hydroxylases (PHDs). In order to hydroxylate prolines, PHDs also require iron, α-ketoglutarate and ascorbate as cofactors [37-40].

Four different HIF prolyl hydroxylases have been identified so far: PHD1 (prolyl hydroxylase domain 1; EglN2), PHD2 (EglN1), PHD3 (EglN3) and PHD4 (C-P4H-I) [41-45]. However, the impact of the latter on HIF-1α appears to be not as strong as that of the other types. The extent to which these enzymes are redundant is unclear, although their intracellular localisation is different *in vitro*. Expression of GFP-tagged PHD1 localised this protein into the nucleus, whereas PHD2 was found in the cytoplasm and PHD3 was equally distributed within the cell [46]. However, a recent *in vivo* study showed that PHD1 and PHD2 are found especially in the cytoplasm and not in the nucleus [47].

PHD2 appears to be the primary regulator of HIF-1α, because a decrease in PHD2 activity by RNA interference (knockdown) was sufficient to activate HIF-1α in most cells under standard conditions, whereas depletion of PHD1 was less effective [48,49]. However, all PHDs are contributing to HIF-1α destabilisation as maximal HIF-1α stabilisation can only be observed when all PHDs are knocked-down [49,50]. Once the prolines within the ODD of HIF-1α are hydroxylated, they become recognised by the von Hippel-Lindau tumour suppressor protein (pVHL) that is part of a multiprotein ubiquitin ligase and initiates the ubiquitinylation process [51-53]. Ubiquitinated HIF-1α is thereafter degraded by the 26S proteasome system [54,55]. Interaction between HIF-1α and pVHL, and therefore HIF-1α proteasomal degradation seems to be further enhanced by HIF-1α acetylation by arrest-defective-1 (ARD1) at K532 [56]. Interestingly, recent data showed that histone deacetylase-1 is activated under hypoxia thus decreasing acetylated HIF-1α levels which would allow its activation [57-59].

Recently, the protein small ubiquitin-like modifier-1 (SUMO-1) was associated with HIF-1α stabilisation. Modification of HIF-1α by SUMO-1 occurs at K391 and K477 and, in contrast to ubiquitination which promotes proteasomal degradation, sumoylation acts antagonistically, thus promoting HIF-1α stability and activation [60].

A negative regulator of HIF-1 transactivation, named factor inhibiting HIF-1 (FIH) was identified by using a yeast-two-hybrid system [61]. FIH was then shown to be an asparagine hydroxylase [62,63] which controls, to a major extent, the recruitment of HIF-1α coactivators such as CREB binding protein (CBP)/p300 to the TADC. Under normoxia, N803 is hydroxylated by FIH preventing p300 binding at the TADC. Hypoxic conditions decrease the hydroxylation

process which allows CBP/p300 to contact and to activate HIF-1α. In addition to CBP/p300, other coactivators have been shown to bind HIF-1α and a complete list of those can be found in a recent review [64].

The complexity of HIF-1α regulation is increased by the fact that HIF-1α is also regulated at the translational level under hypoxic conditions [65,66]. Interestingly, hypoxia impairs translation in order to limit energy consumption [65,67,68]. HIF-1α escapes this rule because of an internal ribosome entry site (IRES) in the 5′-UTR which facilitates its translation to be quickly available [65,66].

In addition, the redox state of HIF-1α seems to be important for its activity [69]. Ref-1 (redox factor-1) and its regulator thioredoxin (Trx) have been found to interact with both TADN and TADC and to increase its transactivation [70,71]. In the TADC, Ref-1 reduces C800, thereby potentiating coactivator binding. Moreover, reactive oxygen species appear to mediate the induction of HIF-1α in response to many stimuli including thrombin, growth factors, insulin and angiotensin II under normoxic conditions [69,72-75]. However, H_2O_2 or NO pretreatment prevent hypoxia-dependent induction of HIF-1α [70,76-79], suggesting that the redox balance of the cell is a major regulator of HIF-1α [69].

Furthermore, HIF-1α levels can also be regulated by different signalling cascades. Thereby, the phosphoinositol-3 kinase (PI3K) cascade seems to play an important role as many downstream targets of this pathway have been described to play a preponderant role in HIF-1α activation under hypoxia. The role of the PI3K target protein kinase B (PKB) appears to be dual because downstream components of PKB are described to either activate or inactivate HIF-1α. PKB has also been described to directly influence ARNT and HIF-1α dimerisation under hypoxia by phosphorylating ARNT in cells expressing the Her2 or EGF receptor tyrosine kinase. The PKB target glycogen synthase kinase-3 (GSK3) directly phosphorylates the HIF-1α ODD domain and contributes to HIF-1α destabilisation in response to long-term hypoxia [80]. PTEN (phosphatase and tensin homologue) overexpression results in decreased HIF-1α levels under hypoxia whereas loss of PTEN enhances HIF-1α levels under hypoxic as well as under normoxic conditions [81]. The PKB target HDM2 (mouse double minute homologue) directly interacts with HIF-1α, preventing destabilisation of HIF-1α independently of pVHL [82]. In addition, mTOR (mammalian target of rapamycin) was shown to positively regulate HIF-1α under hypoxia whereas rapamycin decreased hypoxia-induced HIF-1α levels independently of PTEN, involving the ODD domain of HIF-1α [83]. In addition, the mTOR pathway undergoes an interplay with the AMP-activated protein kinase (AMPK) signalling pathway which can be activated allosterically under hypoxia due to an increased AMP:ATP ratio [84].

The MAPK (mitogen-activated protein kinase) pathways have also been shown to contribute to the regulation of HIF-1α. Indeed, inhibitors of these pathways, such as the MEK1 inhibitor PD98059 or the p38MAPK inhibitor SB203580, decreased hypoxia-induced HIF-1α expression [85-88]. Extracellular-regulated kinase-1 (ERK-1) was shown to phoshorylate both the TADN and TADC. These events enhanced not only the stability of HIF-1α, but also its transactivation in response to hypoxia [89,90]. Moreover, overexpression of MKK3 or MKK6 (mitogen-activated kinase kinase), two upstream kinases of p38MAPK, elevated HIF-1α protein levels and activity by hypoxia [85].

4. Role of HIF-1 in cellular metabolism

So far, > 100 targets of HIF-1 have been identified (for review see [64,91]). HIF-1-regulated genes are coding for proteins involved in several processes such as angiogenesis, erythropoiesis, cell proliferation and viability, fibrinolysis, vascular remodeling and vasomotor responses, oxidative stress and inflammation [91,92]. However, one of the most important roles that HIF-1 is playing in the adaptive response to hypoxia is the regulation and the control of energy metabolism (**Table 1**). HIF-1 seems to regulate the expression of almost every single gene whose protein product is involved in glucose uptake and glycolysis (reviewed by [64,93]) (**Figure 1**). However, this appeared to occur in an isoenzyme-specific manner [94] because hypoxia induced the expression of glucose transporters GLUT1 and GLUT3, which are found in all cells (except liver and pancreatic β-cells), whereas expression of GLUT2 was decreased. This isoenzyme-specific regulation by hypoxia was also found in embryonic stem cells from mice containing intact *hif-1α* alleles (HIF-1$\alpha^{+/+}$). By contrast, cells deficient for HIF-1α (HIF-1$\alpha^{-/-}$) did not display hypoxia-dependent induction of mRNAs encoding glycolytic enzymes, such as hexokinase-1, -2, glucosephosphate isomerase, phosphofructokinase L, aldolase A and C, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase 1, enolase 1, pyruvate kinase M and lactate dehydrogenase A [24,95,96]. In addition, these cells showed reduced expression of mRNA for GLUT1, GLUT3, hexokinase-1, -2, phosphofructokinase L, aldolase A and C, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase 1 and lactate dehydrogenase A indicating significant alterations in energy metabolism [24,95,96].

Aside from hexokinase-1 and -2, the key enzyme of glucose utilisation in liver, glucokinase or hexokinase IV, has also been shown to be induced in a HIF-1-dependent manner [97]. Further, liver-type pyruvate kinase L (PK_L) gene expression was hypoxia-inducible whereas under normoxia the predominant mode of activation was glucose-dependent; moreover, the glucose-responsive element within the PK_L promoter seems to be a low-affinity HIF-1 binding site, suggesting a crosstalk between oxygen and glucose signalling pathways [98] (for review see [6]).

The activation of phosphofructokinase 1, which is also a rate-limiting enzyme in glycolysis, is dependent on fructose-2,6-bisphosphate and is allosterically modified by ADP

Table 1. Hypoxia and HIF-1 regulated genes of major metabolic pathways.

		Hypoxia inducible	HIF-1 inducible
Glucose transport	Glucose transporter-1	+	+
	Insulin receptor	+	+
Glycolysis	Glucokinase	+	+
	Phosphofructokinase L	+	+
	PFKFB3 and PFKFB4	+	+
	Aldolase A and C	+	+
	GAPDH	+	+
	Phosphoglycerate kinase 1	+	+
	Phosphoglycerate mutase B	+	+
	Enolase 1	+	+
	Pyruvate kinase M	+	+
	Lactatdehydrogenase A	+	+
Amino acid metabolism	L-Arginine transporter [204]	+	-
	Arginase-1 [204]	+	-
Lipid metabolism	Leptin	+	+
	Leptin receptor	+	+
Xenobiotic metabolism	CYP4B1	+	+
	CYP3A6	+	+
	CYP2C11	+	+
Polyamine metabolism	Ornithine decarboxylase [205]	+	-
	Ornithine aminotransferase [206]	+	-
	Spermidine acetyltransferase [207]	+	-
Iron metabolism	Transferrin	+	+
	Transferrin receptor	+	+
	Heme oxygenase 1	+	+*
Energy phosphate metabolism	Adenylatkinase 3	+	+
	AMP-activated protein kinase family member 5	+	+
pH regulation	CAIX	+	+

CAIX: Carbonic anhydrase IX; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PFKFB: 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase.
For a further detailed list, see [64,96,202,203] and the references therein).

and citrate. Therefore, maintenance of fructose-2,6-bisphosphate levels by a family of bi-functional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) enzymes is important not only for the glycolytic but also for the gluconeogenic pathway [99,100]. Four different genes encode isoforms of the PFKFB (1 – 4) family [100]. Interestingly, all four can be activated by hypoxia in a more or less tissue-specific manner. PFKFB-1, highly expressed in liver, heart and skeletal muscle, showed the strongest response to hypoxia in testis. PFKFB-2, which is mainly expressed in lung, brain and heart, displayed the strongest response to hypoxia in liver and testis. PFKFB-3 is highly expressed in skeletal muscle although all other organs show a variable low basal level of expression; however, hypoxia strongly induced PFKFB-3 expression in lung, liver, kidney, brain, heart and testis. Further, PFKFB-4 was only induced by hypoxia in testis [101]. Despite various hints that the whole family is regulated by HIF-1, this appeared to be the case only for PFKFB-3 and PFKFB-4 as revealed by assays detecting the binding of HIF-1 to their promoters [102-105].

By contrast to the genes encoding glycolytic enzymes, the transcription of the gene encoding phosphoenolpyruvate carboxykinase 1, a rate-limiting enzyme in gluconeogenesis was induced to higher levels by glucagon under aerobic conditions [8,106]. Using transient transfections, a normoxia-responsive element was identified within its promoter, which is a binding target for a yet unknown transcription factor [107].

Under hypoxic conditions, increased glycolysis is paralleled by downregulation of the mitochondrial fatty acid-β-oxidation [108,109] and by decreased expression of genes encoding respiratory chain components; for example, mitochondrial DNA-encoded cytochrome oxidase subunit III, ATP synthase subunit 6, NADH dehydrogenase subunits 1 and 2, and 16S ribosomal RNA, nuclear encoded ATP synthase β-subunit and adenine nucleotide translocase [110]. These adaptative effects might partially be explained by the HIF-1-mediated pyruvate dehydrogenase kinase gene expression. The pyruvate dehydrogenase kinase phosphorylates and inhibits PDH, an enzyme converting pyruvate to acetyl-CoA. The hypoxia-induced inhibition of PDH would reduce formation of acetyl-CoA, its conversion within the citric acid cycle and the subsequent production of the reducing equivalents NADH and FADH. This, in turn, would lower the activity of the mitochondrial respiratory chain and may result in decreased mitochondrial oxygen consumption [111,112].

The importance of HIF-1 in cellular energy metabolism is also pointed out by the fact that HIF-1α stability can be influenced by changes in specific glycolytic and citric acid cycle metabolites. Glucose metabolites, such as pyruvate and oxalacetate, can activate HIF-1α via downregulation of PHDs in human glioblastoma cells [113] and a selective reversal of this response by ascorbate, cysteine, histidine and Fe(II), but not by 2-oxoglutarate was observed [114]. Additionally, two enzymes of the citric acid cycle, succinate dehydrogenase (SDH) and fumarate hydratase (FH) appear to play a role in O_2-dependent energy metabolism. Loss-of-function mutations within those two genes cause accumulation of two intermediate products from the citric acid cycle, succinate and fumarate, respectively. Both can lead to stabilisation of HIF-1α probably via an inhibition of the PHDs. This enhanced HIF-1α stability might be one of the reasons for the inherited cancer syndromes observed in clinical cases characterised with SDH and FH mutations [115-120]. Thus, the potential of the PHDs to be regulated by various metabolic intermediates coming from glycolysis and the citric acid cylce need to be further elucidated.

The upregulation of anaerobic glycolysis under hypoxia also results in increased lactate production and accumulation, and therefore alterations in lactate export and clearance lead to acidosis (review by [13]). Acidosis is also among common metabolic tumour phenotype features and promotes tumour cell invasiveness [121]. In addition, a potential link between metabolism and pH regulation appears to be the carbonic anhydrases (CA), enzymes that catalyse the interconversion of carbon dioxide and water into carbonic acid, protons and bicarbonate ions. Carbon dioxide can freely diffuse in and out of the cell, whereas bicarbonate must be transported. Thus, the conversion of bicarbonate to carbon dioxide facilitates its transport into the cell, whereas the conversion of carbon dioxide to bicarbonate helps to trap the carbon dioxide inside the cell. Additionally, those enzymes have been implicated in ammonia transport, gastric acidity, muscle contraction, gluconeogenesis, renal acidification and normal brain development [122-127]. Several isozymes have been involved in disease states and two isozymes, carbonic anhydrases 9 and 12, are strongly induced by hypoxia in many tumour cells. However, so far, only the CA9 isoform was shown to be a HIF-1-responsive gene [128].

Leptin, an adipocytokine was first described as a hormone produced by adipose tissue [129]. The main function of leptin in the human body is the regulation of energy homeostasis especially under conditions of restricted energy availability, but it also plays a role in immune response, inflammation, haematopoiesis, angiogenesis and reproduction [130-133]. Leptin stimulates growth, migration and invasion of cancer cells *in vitro* and potentiates angiogenesis, thus having the capacity of promoting cancer *in vitro* (reviewed by [134]) Additionally, diabetes, obesity and sterility are associated with leptin administration in ob/ob mice which have a mutation in the leptin gene. Hypoxia markedly increases human leptin gene expression in skin dermal fibroblasts and BeWo cells, a process mediated via a HIF-1-regulated pathway [135,136]. In addition, hypoxia was shown to induce leptin expression in 3T3-F442A adipocyte cells, increasing the possibility that hypoxia, if occurring in adipose tissue, might be a modulator of the angiogenic process through the HIF-1 pathway [137]. Disturbances in leptin metabolism are related to energy imbalance and are associated with coronary heart disease [138], insulin resistance [139], impaired fibrinolysis [140], development of obesity [141], or type 2 diabetes [142], thus suggesting the possible involvement of HIF-1 in these diseases. Additionally, HIF-1 appears to play a role in the regulation of the transglutaminase-2 gene [143]. The primary enzymatic activity of transglutaminase 2 is the Ca^{2+}-dependent transamidation of polypeptide chains through their glutamine and lysine residues (or through polyamines). It also binds GTP (which blocks transamidation) and may act as a G protein. Furthermore, it possesses a protein disulfide isomerase activity and may function as a protein kinase [144-147]. In addition, it can also be secreted by unidentified mechanisms into the cellular environment, where it may participate in cell adhesion processes and extracellular matrix stabilisation [148] and might, thus, have a role in cell proliferation [149,150] or in receptor-mediated endocytosis [151].

Additionally, HIF-1 plays a role in iron metabolism by increasing transferrin [152], transferrin receptor [153] and ceruloplasmin [154] gene expression. Thus, regulation of the iron availability by HIF-1 represents an important feedback mechanism because the activity of PHDs strictly depends on iron (see Section 3.2).

5. HIF-1, cancer and metabolism

Hypoxia and tumour growth are tightly linked to each other. Indeed, tumour progression is characterised by uncontrolled cellular growth which is accompanied by changes in the microenvironment. Below 1 mm^3, tumours are, in general, avascular

and grow slowly. Beyond 1 mm^3 diameter, zones of hypoxia appear within the tumour due to a lack of vascularisation, resulting in a loss of blood supply limiting the access of the growing tumours to oxygen and nutrients. An increase in local hypoxia or anoxia allows the tumour to enter an adaptive process which is initiated by the activation of the HIF system. Newly growing tumours are then developing their own vascularisation by inducing one of the well known targets of HIF-1, the vascular endothelial growth factor (VEGF), but also genes involved in metabolism, such as *Glut1*, in order to supply the high metabolic demand. In fact, the relationship between tumour oxygen tension (pO_2) and survival in advanced head and neck cancer was analysed and evidence was found that suggested tumour hypoxia is associated with a poor prognosis in those patients [155]. Further, it was found that Glut1 expression serves as a marker for an aggressive type of tumour in patients with transitional cell carcinoma of the urinary bladder [156]. In addition, dysregulation of HIF-1α degradation was clearly associated with enhanced risk of cancer or with increased tumour growth. In von Hippel–Lindau (VHL) disease, an autosomal-dominant hereditary disorder caused by mutations in the VHL tumour suppressor gene, HIF-1α is found to be upregulated. This pathology predisposes to the development of various benign and malignant tumours, including angiomas, haemangiomas, pheochromocytomas, pancreatic cysts and renal clear cell carcinomas. In fact, loss of pVHL stabilises HIF-1α, causes activation of HIF-1 thus enhancing tumour growth and angiogenesis. Similarly, HIF-1α was found to be overexpressed in gastric cancer as well as in many oral cancers [157-160]. The evidence that HIF-1α is one of the key players in the regulation of tumour progression is supported by the fact that HIF-1α expression is found in many cancer types and is nearly non-existent in corresponding normal tissue. HIF-1α expression was associated with bad prognosis in several cancers [161,162]. Moreover, inactivation of HIF-1 by knocking down HIF-1α expression dramatically reduces tumour growth *in vivo* and *in vitro* [163-165].

An intriguing observation already made by Otto Warburg about 80 years ago is the ability of tumour cells to maintain high rates of glycolysis even under aerobic conditions [166]. This so called Warburg effect was further confirmed by many publications and a correlation between aggressive tumour phenotype and elevated glycolysis was established [167,168], although the circumstances promoting the switch from oxidative metabolism to increased glycolysis are still poorly understood. However, this switch and the increase in glycolysis appear to be very important because they correlate with decreased tumour sensitivity to chemotherapy and increased tumour resistance to radiotherapy and, therefore, tumour aggressiveness [169-171]. The role of HIF-1 appears fundamental in such a switch because it acts as the major regulator of cell metabolism and at the same time changes in cellular metabolism influence HIF-1α stabilisation and consequently HIF-1 target gene expression. Moreover, tumour cells with dysregulated HIF-1α show not only increased glycolysis even under normoxic conditions, but also a tendency towards more aggressive tumours. It seems then likely that increased levels and activity of HIF-1α might represent a link between enzyme dysregulation and cancer. Furthermore, glycolysis generates intermediates which are used as precursors in different anabolic pathways such as in the synthesis of glycine, serine and purine/pyrimidine; therefore, the flux through glycolysis is critical to maintain carbon skeletons for those synthetic pathways; those pathways, in turn, are essential for cell growth and proliferation [172]. Thus, accelerated glycolysis might facilitate hypoxic tumour growth [173].

6. HIF-1 as an anticancer drug target

It is supposed that suppression of HIF-1 by disrupting the HIF-1 signalling pathway via a wide range of mechanisms, might block the negative effects mediated by HIF-1-responsive genes in cancer. Over the last few years, many reviews focus, summarise and update continuously the therapeutic strategies for HIF-1-targeting in cancer treatment [93,165,174-180] (**Table 2**).

A number of naturally or chemically synthesised compounds which have the potential to inhibit HIF-1α activity have been identified; recent excellent reviews contain a comprehensive list of those [174,178,180]. Often, the exact mechanisms of action for each compound has not been studied up to the last detail and the effects on HIF-1α appear to be rather more indirect than direct. From the compounds with HIF-1α inhibitory potential, only some appeared to act via modulation of 'classical' metabolic enzymes or pathways and they will be summarised below.

Hsp90 is an abundant molecular chaperone participating in the folding and stabilisation of signal-transducing molecules [181], such as steroid hormone receptors, protein kinases and transcription factors (e.g., HIF-1) [182]. The Hsp90 inhibitor geldanamycin was shown to inhibit hypoxia-induced stabilisation of HIF-1α by promoting its degradation in a VHL-independent manner [182-186]. The action of the Hsp90 inhibitors geldanamycin, novobiocin or radicicol might, in part, be explained by their ability to prevent the binding of ATP to Hsp90 which is required for its chaperone function [187]. Interestingly, the effect of geldanamycin is similar to the effect of 2-deoxyglucose (2-DOG) in adult rat ventricular myocytes. Inhibition of glycolysis or mitochondrial respiration in these cells by treatment with 2-DOG or antimycin A resulted in lower ATP concentration and decreased Hsp90 activity; as a result, the Hsp90 client protein ErbB2 is rapidly degraded [188]. These findings suggest that inhibitors of the glycolytic pathway might be involved in HIF-1α degradation via ATP depletion and Hsp90 inactivation.

In addition, flavopiridol, a small molecule that inhibits cyclin-dependent kinases causing an arrest of the cell cycle was shown to downregulate HIF-1α in human glioma U87MG and T98G cells and to decrease VEGF production and tumour cell migration. Intriguingly, this event occurred

Table 2. Reported agents with HIF-1 inhibitory potential.

Mode of action	Agent
Angiogenesis inhibitor	Endostatin
Ca^{2+} blocker	Carboxyamido-triazole
Cyclin-dependent kinase inhibitor	Flavopiridol
Cyclooxygenase-2 inhibitor	Celecoxib, ibuprofen, NS-398
HIF-1α inhibitor	PX-478
HIF-1α/p300 interaction inhibitor	Chetomin
Histone deacetylase inhibitor	FK228, NVP-LAQ824
Hsp-90 inhibitor	Geldanamycin, 17-allyl-amino-geldanamycin, radicol and analogue KF58333
Cytotoxin	TX-402
MAPK pathway inhibitors	PD98095
Microtubule cytoskeleton targeting agent	2-Methoxyestradiol, taxol, vincristine
HER2/neu (erbB2) monoclonal antibody	Trastuzumab
Flavoprotein inhibitor	Diphenylene iodonium
PI3K/PKB/mTOR pathway inhibitor	LY294002, wortmannin, rapamycin, RAD-001, CCl-779
PKC inhibitor	7-Hydroxystaurosporine (UCN-01)
Raf kinase inhibitor	BAY 43-9006 (sorafenib)
RNA synthesis inhibitor	Echinomycin
Thioredoxin inhibitor	1-Methylpropyl-2-imidazolyl-disulfide (PX-12) pleurotin
Soluble guanyl cyclase activator	YC-1
Topoisomerase inhibitor	
Topo I	Topotecan
Topo II	GL331
Tyrosine kinase inhibitor	Genistein, imatinib mesylate, erlotinib, gefitinib
Unknown	103D5, resveratrol

HIF: Hypoxia-inducible factor; Hsp: Heat-shock protein, MAPK: Mitogen-activated protein kinase; PI3K: Phosphoinositol 3-kinase; PKB: Protein kinase B; PKC: Protein kinase C; mTOR: Mammalian target of rapamycin.
For detailed references, see [93,165,174-180].

in the presence of a proteasome inhibitor, a compound normally enhancing HIF-1α stability [189]. Flavopiridol has also been shown to significantly inhibit the rabbit muscle glycogen phosphorylase by binding to its inhibitory site [190]. Moreover, treatment of A549 non-small cell lung carcinoma cells with flavopiridol resulted in an increase in glycogen accumulation [190] pointing out the possible interrelation between glucose homeostasis and flavopiridol [190]. Thus, inhibiting the glycolytic degradation of glucose appears to be an important mechanism for HIF-1α inhibition.

Another natural compound, resveratrol, a polyphenolic phytoalexin with antioxidant and other preventive activities, found in grapes and other food products, [191] was reported to inhibit the uptake of glucose and dehydroascorbic acid in human transformed myelocytic U937 and HL60 cells, most likely by blocking sodium-independent glucose transporters such as GLUT1 and GLUT3 [192]. Additionally, resveratrol was shown to attenuate the stress induced by high glucose levels in human leukaemia K562 cells [193]. Interestingly, resveratrol or oxidative stress have been shown to lead to sirtuin activation which, in turn, renders Foxo1 immobile within the nuclear compartment and promotes Foxo1-dependent transcription of genes [194]. A member of the forkhead transcription factor superfamily, Foxo4, down-regulates the HIF-1α protein levels and results in the suppression of various cellular proteins responsive to hypoxia, such as VEGF, GLUT1 and erythropoietin [195]. Resveratrol also inhibits the hypoxia-induced HIF-1α protein accumulation and the subsequent VEGF expression and transcriptional activation in human A2780/CP70 and OVCAR-3 ovarian cancer cells, in human tongue squamous cell carcinomas (SCC-9) and in hepatoma HepG2 cells [196,197]. Interestingly, similarly to geldanamycin, Foxo4 mediated HIF-1 degradation in a VHL-independent manner, again highlighting the importance of the non-VHL pathways for HIF-1α degradation. Thus, the effects of resveratrol on HIF-1α might be partially explained by activation of the Foxo family which, in turn, suppressed HIF-1 activity.

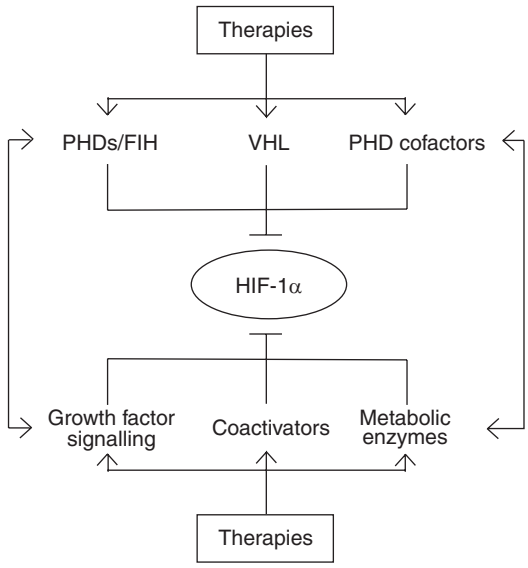

Figure 2. HIF-1α as putative therapeutic target. Potential therapeutic agents may act directly on the HIF-1α protein itself, or in a more indirect manner via the mechanisms degrading it. See Section 7 for explanation.
HIF: Hypoxia-inducible factor; PHD: Prolyl hydroxylase domain; VHL: Von Hippel-Lindau tumour suppressor.

A broad-spectrum tyrosine kinase inhibitor, genistein, an isoflavone found in high concentrations in soybean, was shown to inhibit HIF-1α [198]. It was reported to diminish the blood levels of leptin after intragastric administration in rats and to restrict leptin secretion from isolated adipocytes stimulated with insulin [199]. Indeed, genistein can inhibit glucose transport, cAMP phosphodiesterase and insulin signalling, whereas it stimulates lipolysis [199]. However, the use of alanine instead of glucose in order to overcome the restrictive actions of genistein on glucose transport and glycolysis was not sufficient to restore leptin secretion from isolated adipocytes [199], suggesting that the inhibitory effect of genistein on leptin secretion might arise from its action on glucose metabolism in adipocytes.

Inhibition of HIF-1α stability has also been described for the experimental drug PX-478. Although, PX-478 also decreased VEGF levels [200], its common anti-tumour effect appears to be related to inhibition of glycolysis because PX-478 treatment of mice bearing HT-29 human colon cancer xenografts reduced GLUT1 levels [200]. However, magnetic resonance spectroscopy studies of metabolite changes in HT-29 xenograft tumours after HIF-1α inhibition with PX-478 revealed no significant decreases in lactate and lipid levels *in vivo* and *ex vivo*; however, inhibition of *in vitro* glucose consumption and lactate production by PX-478 was observed [201]. Thus, the potential effects of PX-478 on cellular metabolism with respect to HIF-1α inhibition need further investigations.

7. Expert opinion

HIF-1α appears to be a very attractive target for pharmacological interventions to block tumour progression, and identification of compounds specifically inhibiting HIF-1α is, therefore, an important, although challenging, task. Potential therapeutic agents may act directly on the HIF-1α protein itself, or in a more indirect manner via the mechanisms degrading it. Thus, compounds may specifically activate the HIF-hydroxylases (PHDs and/or FIH) or promote HIF-1α/VHL interaction. In addition, degradation may be promoted by compounds which act as cofactors for the HIF-hydroxylases. The inhibition of HIF-1α could also be achieved in a more indirect manner by interfering with growth factor signalling pathways which enhance HIF-1α levels. Further, preventing association of HIF-1α with its interacting coactivator proteins and regulation of the activities of those metabolic enzymes whose products participate in HIF-1α regulation could contribute to inhibition of HIF-1α (**Figure 2**). This matter is complex due to the presence of feedback loops, as well as other HIF-α subunits, such as HIF-2α and HIF-3α, with activity profiles not completely elucidated so far. Thus, any inhibitor may as well act on HIF-1α, HIF-2α and HIF-3α. Likewise, the existence of several HIF-hydroxylases even more complicates the problem. Thus, large-scale screening attempts might lead to the identification of only a few specific inhibitors. Further, the HIF-system interferes with nearly every signalling pathway in the cell and, thus, the use of these inhibitors, as well as their cross-talk and interference with the major metabolic pathways needs to be carefully determined.

Interference with the HIF-1 system from a metabolic point of view appears to be rationale in terms of either controlling HIF-hydroxylase activities directly or by regulating the production of cofactors, such as 2-oxoglutarate, Fe^{2+} or factors modulating the activity of enzymes controlling the PDH activity. In addition, patients with defects in the SDH, FH or VHL gene may also benefit from those inhibitors, especially from those which can induce HIF-1α degradation in a VHL-independent manner (as geldanamycin).

Together, the use of HIF-1α inhibitors in combination with the classical chemotherapy and radiotherapy might represent a new strategy in anticancer therapy for those patients with tumours containing high HIF-1 levels, especially when side-effects of the new drugs can be minimised.

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Affiliation

Rachida S Bel Aiba[2], Elitsa Y Dimova[1], Agnes Görlach[2] & Thomas Kietzmann[†1]
[†]Author for correspondence
[1]University of Kaiserslautern, Faculty of Chemistry, Department of Biochemistry, Erwin-Schrödinger Strasse 54, D-67663 Kaiserslautern, Germany
Tel: +49 631 205 3419; Fax: +49 631 205 4953; E-mail: tkietzm@gwdg.de
[2]Technical University Munich, Experimental Pediatric Cardiology, Department of Pediatric Cardiology and Congenital Heart Disease, German Heart Center Munich, Germany