Interplay between hypoxia-inducible factor 1α and SIRT1 protein deacetylase in hypoxic cancer cells

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PhD Thesis

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Abstract

Hypoxia is a common feature in solid tumors. Activation of the transcription factor hypoxia-inducible factor 1α (HIF-1α) initiates key cellular adaptations to promote cell survival in the hypoxic tumor microenvironment. Several experimental and clinical studies validate HIF-1 as a target for anti-cancer therapy. SIRT1 is a protein deacetylase, which is induced in response to various cellular stresses such as calorie restriction, DNA damage, oxidative and oncogenic stress. By deacetylating and regulating key transcription factors, SIRT1 induces cellular adaptations and thereby promotes cell survival.

My thesis focuses on the complex interplay between HIF-1α and SIRT1 in hypoxic cancer cells. Given the role of SIRT1 to promote survival in response to conditions of stress by deacetylating key transcription factors, Chapter 2 investigates the effect of SIRT1-mediated deacetylation on HIF-1α stability and transcriptional activity. The data demonstrate a physical interaction between SIRT1 and HIF-1α and identify HIF-1α as a new target for SIRT1-mediated deacetylation. In addition, the results demonstrate that SIRT1 is required for the activation of HIF-1α, thus revealing a posttranslational modification that controls the expression and function of HIF-1α. Chapter 3 investigates the role and regulation of SIRT1 in response to hypoxia. SIRT1 protein expression remains constant under hypoxic stress, however, hypoxic cancer cells depend on SIRT1, as its inhibition leads to a growth arrest in these cells. Taken together, the inhibition of SIRT1, which results in hyperacetylation and consecutive degradation of HIF-1α, potentially provides a new therapeutic strategy to combat HIF-1-expressing tumors.

Abbreviations

ARD1	Arrest-defective protein 1
ATP	Adenosintriphosphat
bHLH	Basic helix-loop-helix
CA IX	Carbonic anhydrase IX
COX	Cytochrome c oxidase
C-TAD	C-terminal transactivation domain
CTGF	Connective tissue growth factor
DBC1	Deleted in breast cancer 1
EGFR	Epidermal growth factor receptor
eIF-4E	Eukaryotic translation initiation factor 4E
EPO	Erythropoietin
ERK	Extracellular-signal-regulated kinase
FIH	Factor inhibiting HIF
Glut-1	Glucose transporter 1
Glut-3	Glucose transporter 3
GSK-3	Glycogen synthase kinase 3
HADC	Histone deycetylase
HAT	Histone acetyl-transferase
HCC	Hepatocellular carcinoma
HDACi	HDAC inhibitors
HIC1	Hypermethylated in cancer 1
HIF-1	Hypoxia-inducible factor 1
HIF-1α	Hypoxia-inducible factor 1alpha
HSP90	Heat-shock protein 90
IGF1R	Insulin-like growth factor-1 receptor
IGF2	Insulin-like growth factor-2
JNK	c-Jun N-terminal kinase
LDHA	Lacate dehydrogenase A
MAPK	Mitogen-activated protein kinase

MCT4	Monocarboxylate transporter 4
MEK	MAP/ERK kinase
MTA1	Metastasis-associated protein 1
mTOR	mammalian target of rapamycin
NAD^+	Nicotinamide adenine dinucleotide
NDRG1	N-myc downregulated protein 1
NES	Nuclear export signal
NHE1	Sodium-hydrogen exchanger
NOS	Nitric oxide synthase
N-TAD	N-terminal transactivation domain
ODD	Oxigen-dependent degradation domain
p300/CBP	p300/ CREB binding protein
PAS	Per/ARNT/SIM
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1α
PHD 1-3	Prolyl hydroxylase domain (1-3) enzymes
PI3K	Phosphoinositide-3-kinase
PKB	Protein kinase B
PTEN	Phosphatase and tensin homolog
RBX1	Ring box protein 1
ROS	Reactive oxygen species
RSUME	RWD-containing sumoylation enhancer
SENP1	SUMO1/sentrin specific peptidase 1
Sir2	silent mating type information regulation 2
sirtuin	sirtuin (silent mating type information regulation 2 homolog)
SIRT1	sirtuin (silent mating type information regulation 2 homolog) 1
SUMO	Small ubiquitin-like modifier
TCA	Tricarboxylic acid
TGF-α	Transforming growth factor-α
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	VEGF receptor 1
VHL	Von Hippel-Lindau tumor suppressor protein

Chapter 1

General Introduction

1. Tumor hypoxia and HIF-1

In rapidly growing solid tumors, significant areas lose access to the supporting blood vessels due to an inefficient formation of the tumor vasculature (1). In these regions, O_2 delivery is insufficient to meet O_2 demand and the tumor suffers from hypoxic stress (insufficient O_2 supply). The most well characterized mechanism by which tumor cells adapt to a hypoxic environment is the activation of the hypoxia-inducible transcription factor, HIF-1. Activation of HIF-1 has evolved as the central and key molecular response mechanism used by cells to adapt to hypoxic conditions. HIF-1 exerts its function by regulating genes that encode proteins involved in glucose metabolism, angiogenesis, erythropoiesis, cell survival and metastasis. In addition to its hypoxia-mediated induction, altered oncogenic signaling pathways contribute to overall activation of HIF-1 in tumors (Figure 1, reviewed in (2)). In the first part of my general introduction, I will focus on two key molecular adaptations regulated by HIF-1 in a hypoxic tumor microenvironment, namely tumor cell metabolism and tumor angiogenesis.

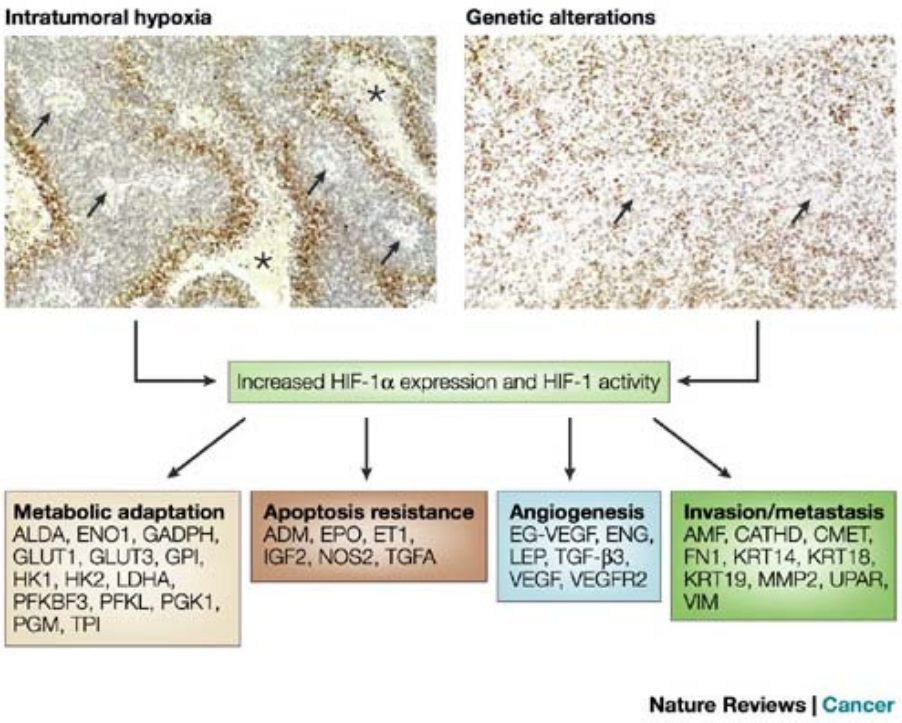


Figure 1| Mechanisms and consequences of HIF-1 activity in cancer cells.
Semenza, Nat Rev Cancer. 2003 Oct;3(10):721-32.

Immunohistochemical analysis of HIF-1α levels in two separate oropharyngeal cancers. The biopsy section on the left shows HIF-1α protein (brown staining) in viable cancer cells surrounding areas of necrosis (indicated by asterisk). The cancer cells that express the highest levels of HIF-1α are at the greatest distance from a blood vessel (indicated by arrows) and are therefore the most hypoxic. In the biopsy section on the right, there are no areas of necrosis and HIF-1α is detected in cancer cells throughout the field, including cells that are immediately adjacent to a blood vessel (arrows), indicating that increased HIF-1α levels are being driven by an O_2-independent mechanism, such as through genetic alteration. These two mechanisms are not mutually exclusive- genetic alterations can amplify the response to hypoxia. In either case, increased HIF-1 activity leads to upregulation of genes that are involved in many aspects of cancer progression, including metabolic adaptation, apoptosis resistance, angiogenesis and metastasis. See Appendix for gene names

1.1. HIF-1 regulates cancer cell metabolism

More than 70 years ago, Otto Warburg described a significant difference in the metabolism of solid tumors compared to normal tissue. Whereby normal tissues generate ATP predominantly by oxidative phosphorylation in the mitochondria, in tumors, cellular energy is mainly produced by glycolysis (3).

Oxidative phosphorylation is the more efficient way of utilizing glucose for energy production. In the presence of O_2, glucose is completely oxidized to CO_2 and H_2O. In a first step of this process, glucose is metabolized to pyruvate through the activity of glycolytic enzymes in the cytoplasm. Pyruvate is then shuttled into the mitochondria, where in the presence of molecular O_2 it is further metabolized by pyruvate dehydrogenase (PDH), tricarboxylic acid (TCA) cycle enzymes, and the electron transport chain (4). By the complete oxidation of one molecule of glucose, approximately 36 molecules of ATP are generated (5). If there is a lack of O_2, cells generate ATP by anaerobic glycolysis. In this process, glucose is similarly broken down to pyruvate, however, instead of shuttling pyruvate into the O_2-dependent mitochondria, pyruvate is converted to lactate through the activity of lactate dehydrogenase A (LDHA) in the cytoplasm. The breakdown of glucose by anaerobic glycolysis has a low efficiency in generating ATP, as one molecule of glucose produces only two molecules of ATP. Interestingly, in tumor cells the switch in energy production from oxidative phosphorylation to glycolysis occurs even when O_2 levels would be sufficient to support mitochondrial function (3). Therefore, glycolysis in the presence of O_2 is termed aerobic glycolysis (Warburg effect). Because of the low efficiency of glycolysis in generating ATP, it causes tumor cells to consume more glucose for adequate energy production.

The molecular mechanisms that mediate the switch from oxidative to glycolytic metabolism get more and more unraveled and have recently been demonstrated to be largely dependent on HIF-1 (6-8). HIF-1 activation leads to key metabolic adaptations such as an increase in glycolysis and a decrease in oxidative phosphorylation (Figure 2, reviewed in (9)). HIF-1 actively reprograms metabolism in tumor cells by influencing various enzymes involved in the process of energy production and through its inhibition of mitochondrial metabolism and biogenesis.

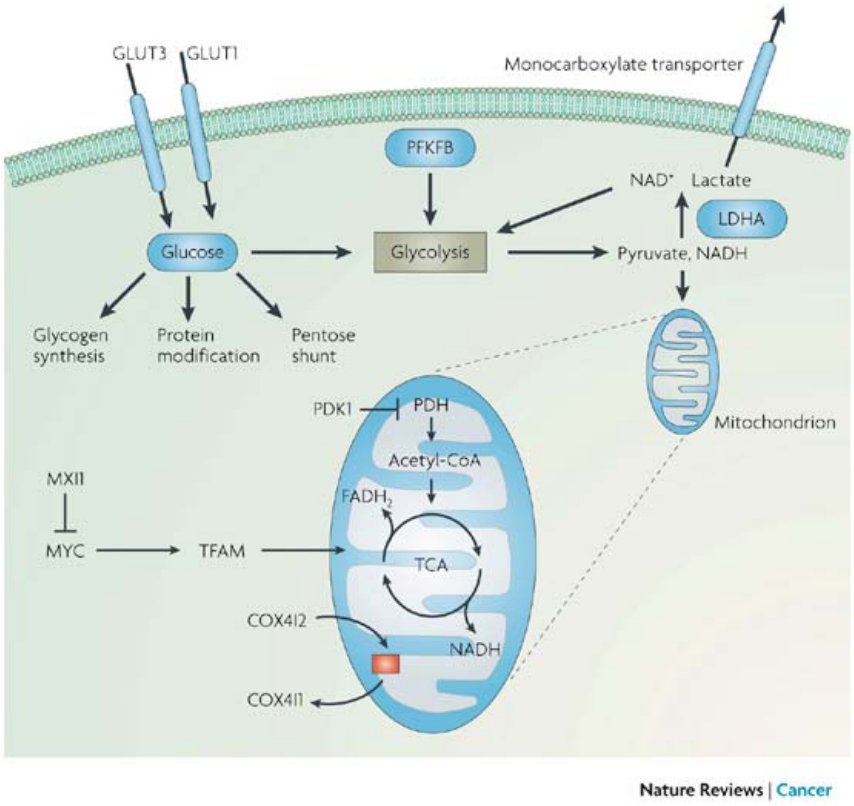


Figure 2| Overview of HIF-1-mediated regulation of tumor cell metabolism.
Denko, Nat Rev Cancer. 2008 Sep;8(9):705-13.

HIF-1 activation leads to an increase in several metabolic pathways (such as glycolysis) and a decrease in others (such as oxidative phosphorylation). First, there is increased uptake of glucose into the cell through the upregulation in expression of the glucose transporters GLUT1 and GLUT3. The intracellular glucose is then metabolized by the increased levels of the glycolytic enzymes. Increased glycolysis generates increased pyruvate, which is largely converted to lactate by HIF-inducible lactate dehydrogenase A (LDHA) and removed from the cell by the monocarboxylate transporter. In the mitochondria, decreased pyruvate flow into the tricarboxylic acid (TCA) cycle owing to HIF1-dependent pyruvate dehydrogenase kinase 1 (PDK1) induction, decreased mitochondrial biogenesis through MAX interactor 1 (MXI1) induction (which antagonizes MYC activity) and switched cytochrome oxidase subunit 4 isoform 1 (COX4I1) to the high-efficiency COX4I2 subunit. PDH, pyruvate dehydrogenase; PFKFB, 6-phosphofructo-2-kinase/ fructose 2,6-bisphosphatase.

HIF regulation of glucose metabolism

HIF-1 transcriptionally regulates the expression of numerous proteins which are required for glucose metabolism. HIF-1 induces the expression of glucose transporters (Glut-1 and Glut-3), which facilitate the uptake of glucose into cancer cells (10-13). Intracellular glucose is utilized by several pathways, of which, its enzymatic breakdown to pyruvate by glycolysis is the predominant one (Figure 2, reviewed in (9)). HIF-1 increases the amount of all the glycolytic enzymes required for glycolysis. Pyruvate is the end product of glycolysis and its increased cellular levels need to be removed (14-18). Pyruvate can either be converted to lactate by lactate dehydrogenase (LDHA) in the cytoplasm or it can be shuttled into the mitochondria where it is metabolized by pyruvate dehydrogenase (PDH). HIF-1 plays a key role in determining the fate of pyruvate. LDHA is a HIF-1 target gene and its induction by hypoxia (or HIF-1 activation) leads to increased conversion of pyruvate to lactate (19, 20). Moreover, HIF-1 induces pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and thereby inactivates PDH (6, 8, 21). Taken together, the HIF-1-mediated induction of LDHA and PDK1 promotes the glycolytic pathway and inhibits the oxidative phosphorylation in the mitochondria. Increased levels of lactate are removed from the cell by the HIF-1-induced monocarboxylate transporter 4 (MCT4) (22, 23). In addition, the intracellular and extracellular pH is also largely regulated through the induction of the HIF-1 target genes such as carbonic anhydrase IX (CA IX) and sodium-hydrogen exchanger (NHE1) (24, 25). The maintenance of an alkaline intracellular pH and an acidic extracellular pH is critical for tumor cell proliferation and invasion.

HIF-1 regulation of mitochondria

One main mechanisms of how HIF-1 inhibits mitochondrial metabolism is through the induction of PDK1, which as mentioned above inactivates PDH. Inactivation of PDH blocks the flow of pyruvate into the mitochondria, decreases oxidative phophorylation and ultimately leads to reduced O_2 consumption and reduced reactive oxygen species (ROS) generation (6, 8). Another mechanism reported to reduce the O_2 demand is the HIF-1-mediated inhibition of mitochondrial biogenesis (26). The reduction of mitochondrial mass is the effect of a complex interplay between HIF-1 and MYC and other transcription factors (27, 28). One recent finding demonstrated a role of HIF-1 upon hypoxia in fine-tuning mitochondrial function by increasing the efficiency of

cytochrome c oxidase (COX). COX is the O_2 consuming enzyme in the electron transport chain. Under hypoxia, HIF-1 induces an isoforn switch of a subunit of this enzyme (switch of COX4-1 to COX4-2) (29). This isoform switch provides an adaptation mechanism upon reduced O_2 availability, which leads to a more efficient use of O_2 and to decreased ROS production while ATP is still generated by oxidative phosphorylation.

The question of how changes in metabolism give tumor cells a survival and growth advantage has raised a lot of speculation (recently reviewed in (9)). One big field of controversy is the role of ROS. ROS are produced in the mitochondria and in large amounts can be toxic to the cells (30, 31). One possible scenario is that the HIF-1-mediated reduction of mitochondrial function leads to decreased ROS levels, which has been correlated with increased survival in some studies (6, 26, 29), however, not in others (32-34). Another possible mechanism, by which HIF-1-mediated reduction of mitochondrial function is likely to provide a growth advantage is an increase of anabolic substrates. Tumor cells have a marked increase of glucose uptake and require more substrates for cell division such as ribose for nucleic acid synthesis. By HIF-1-mediated inhibition of mitochondrial function more of these substrates are made available to the rapidly proliferating cancer cells (35).

In conclusion, HIF is the key regulator of the switch from oxidative to glycolytic metabolism. This metabolic reprogramming does not occur solely due to a limitation in O_2 concentration. HIF-1 actively promotes the metabolic switch at O_2 concentrations which are still far above the critical concentration required for oxidative phosphorylation (33, 36, 37). It is well recognized that HIF-1 is not solely activated by (tumor) hypoxia. HIF-1 can be stimulated by oncogene activation or loss of tumor suppressors (discussed in detail in section/ 2.3. Oncogenic activation of HIF-1). It is now appreciated that the activation of HIF-1 (independent of any specific mechanism) represents a general mechanism underlying the Warburg effect in cancers.

1.2. HIF-1 regulates angiogenesis

Angiogenesis is an important feature of tumor progression. As mentioned above, in expanding tumors the diffusion distances from the existing vascular supply increase and result in hypoxia. Therefore, expansion of a solid tumor requires new blood vessel formation to adequately provide rapidly proliferating tumor cells with oxygen and nutrients. The key regulator of hypoxia-induced angiogenesis is HIF-1. In hypoxic tumors, HIF-1 induces multiple target genes, which modulate angiogenesis by activating endothelial cells. Although HIF-1 is the main regulator of hypoxia-induced angiogenesis, it is important to note that there are alternative, HIF-1-independent pathways, which can promote angiogenesis in response to hypoxia. Here, I will only briefly discuss the role of HIF-1 in promoting angiogenesis (reviewed in (38, 39)).

Initially, tumors remain quiescent in an avascular phase and there progression is limited by an inadequate vascular supply (40). The process in which tumors progress from a non-angiogenic to an angiogenic phenotype, has been termed the angiogenic switch (41). HIF-1 plays a key role in promoting this angiogenic switch by inducing HIF-1 target genes such as VEGF and VEGFR-1 (VEGF receptor) (42, 43). VEGF has a strong mitogenic effect on endothelial cells and is critically required for tumor growth (44). Implantation of VEGF-/- transformed mouse embryonic fibroblasts into immunocompromised mice resulted in dramatically reduced tumor growth compared to wild type cells (45). The important role of HIF-1 in inducing VEGF upon hypoxic conditions was assessed in xenograft tumors using hepatoma (Hepa-1) cells (10). Hepa-1 cells that were deficient for HIF-1β and therefore unable to form HIF-1, resulted in a reduction of VEGF mRNA expression and more importantly in reduced tumor vascularization and growth. Moreover, it was demonstrated by different studies that HIF-1 is involved in an autocrine signaling pathway between VEGF and its receptors and regulates tumor cell survival and angiogenesis (46, 47).

HIF-1 can induce the transcription of other targets, which are involved in angiogenesis such as nitric oxide synthases (NOS) (48-51). NOS produce NO, which acts as an endothelial cell survival factor by promoting cell proliferation (52, 53). Different in vivo studies revealed a role of NOS in promoting tumor growth and angiogenesis (54, 55).

Finally, it is important to note that several growth factor signaling pathways concomitantly to promote cell growth simultaneously increase HIF-1α levels. Activation of various receptor tyrosine kinases is associated with HIF-1-mediated angiogenesis (discussed in 2.3. Oncogenic activation of HIF-1α).

2. HIF-1 regulation

HIF-1 is a heterodimeric transcription factor that consists of an alpha and a beta subunit. HIF-1β is constitutively expressed, whereas the expression of HIF-1α protein is highly regulated. As for any protein, the expression of HIF-1α protein is determined by its rate of protein synthesis and protein degradation (Figure 3). The rate of HIF-1α protein synthesis is (mainly) regulated by O_2-independent mechanisms. HIF-1α protein synthesis generally remains constant under various physiological conditions, however, certain genetic alterations can increase HIF-1α protein synthesis (discussed in 2.3. Oncogenic activation of HIF-1α protein). It is important to point out that HIF-1α protein levels are mainly dependent on the rate of its degradation. HIF-1α protein degradation is mainly regulated via an O_2-dependent mechanism. Briefly, in the presence of O_2, HIF-1α protein is hydroxylated by specific O_2-dependent enzymes. This posttranslational modification (hydroxylation) of HIF-1α protein leads to its ubiquitination and proteasomal degradation. Under hypoxic conditions (low O_2 concentrations) these specific O_2-dependent enzymes are inactive, which leads to stabilization of HIF-1α protein by avoiding its proteasomal degradation. During the last years, numerous studies revealed the importance of other posttranslational modifications which can influence HIF-1α protein stability and transcriptional activity. Posttranslational modifications such as acetylation/deacetylation, phosphorylation, S-nitrosylation and SUMOylation were shown to critically influence HIF-1α protein stability and can either inhibit or promote HIF-1α protein degradation (Figure 4).

In this section of the introduction, I will first briefly describe the structure of HIF-1 (2.1. Structure of HIF-1). Since one of the central aspects of my thesis work is a specific posttranslational modification and its impact on HIF-1α protein stability and activity (see Chapter 2), the main focus of this section is on the different posttranslational modifications, which have been described to influence HIF-1α protein stability (2.2. Posttranslational modifications of HIF-1α). Finally, I will close up this section with a short summary of some genetic alterations, which influence HIF-1α protein synthesis (2.3 Oncogenic activation of HIF-1α).

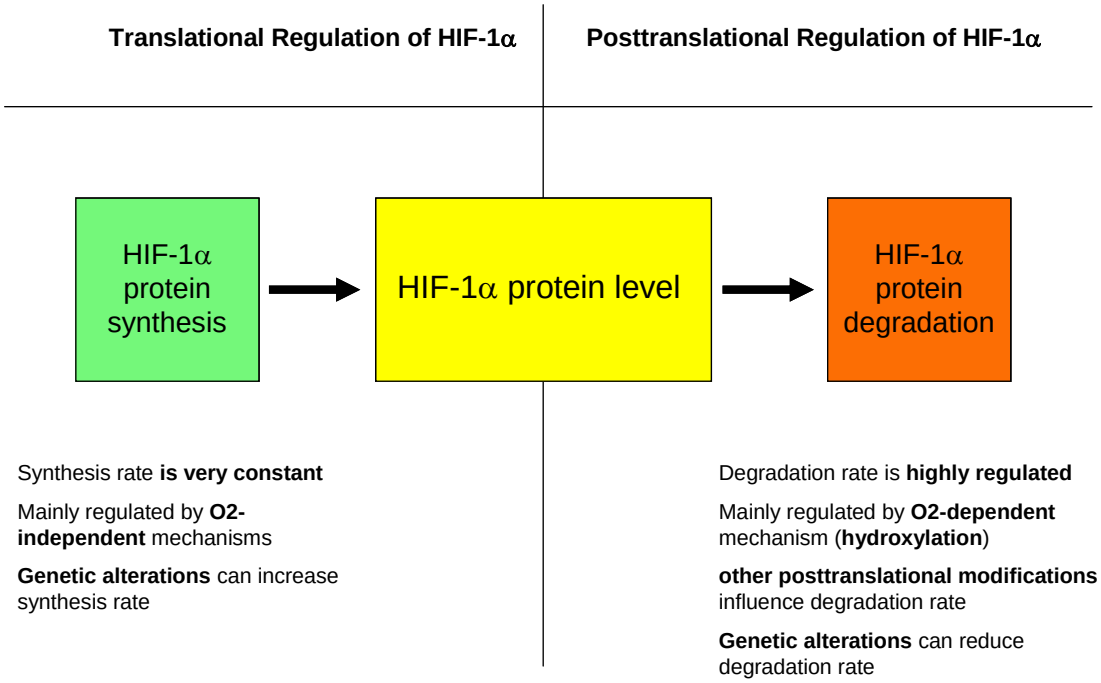
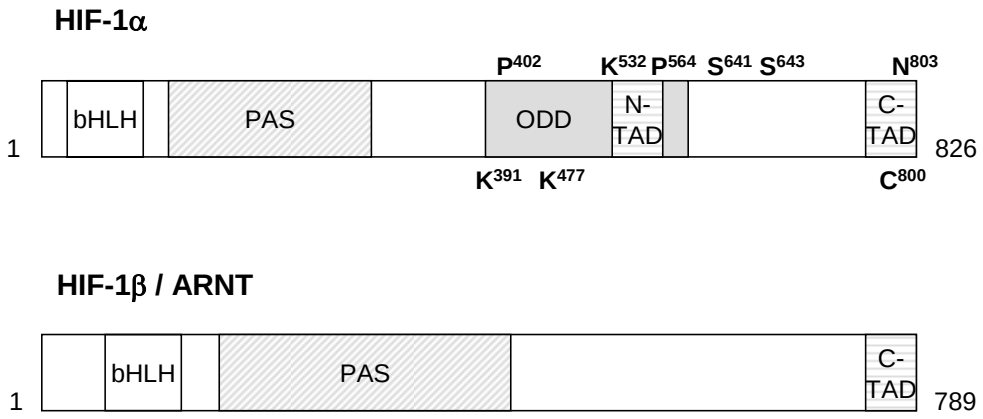


Figure 3| Regulation of HIF-1α protein levels

HIF-1α protein synthesis is (mainly) regulated by O_2-independent mechanisms. The rate of HIF-1α protein synthesis is very constant under various physiological conditions. However, certain genetic alterations can lead to an increased rate of HIF-1α protein synthesis (discussed in 2.3. Oncogenic activation of HIF-1α protein). HIF-1α protein levels are mainly determined by the degradation rate of HIF-1α protein. The degradation rate is highly regulated due to its dependency on O_2. In the presence of O_2, HIF-1α is hydroxylated, ubiquitinated and degraded. When O_2 is limited, HIF-1α is stabilized. However, recently other posttranslational modifications such as acetylation and phosphorylation of HIF-1α (O_2-independent) have been demonstrated to influence its degradation rate (discussed in 2.2. Posttranslational modifications of HIF-1α). In addition to these posttranslational modifications, genetic alterations such as VHL mutations can influence the degradation rate of HIF-1α.

2.1. Structure of HIF-1

HIF-1 is a heterodimeric transcription factor consisting of an α and β subunit. The bHLH (basic helix-loop-helix) and PAS (Per/ARNT/SIM) domains of HIF-1α are mainly required for the dimerization with the HIF-1β subunit. Moreover, bHLH is needed for the binding of transcription factor HIF-1 to DNA (56). Two transactivation domains, N-TAD (N-terminal transactivation domain) and C-TAD (C-terminal transactivation domain) activate transcription of target genes (57). The C-TAD interacts with p300/CBP, transcriptional co-activators. The ODD (oxygen-dependent degradation) domain is target of several posttranslational modifications, which influence HIF-1α stability and transcriptional activity. The oxygen-dependent enzymatic hydroxylation of two specific prolyl residues within the ODD domain of HIF-1α leads to its subsequent ubiquitination and proteasomal degradation. More recently, other posttranslational modifications of HIF-1α, most of which target and modify the ODD domain of HIF-1α have been identified and were shown to critically influence its stability as discussed below.



HIF-1α	**P^{402} , P^{564}**	**PHD Hydroxylation**
	N^{803}	**FIH-1 Hydroxylation**
	K^{532}	**ARD1 Acetylation**
	S^{641}, S^{643}	**MAPK Phosphorylation**
	K^{391}, K^{477}	**SUMOylation**
	C^{800}	**S-nitrosation**

Figure 4| Structure and posttranslational modification sites of HIF-1

2.2. Posttranslational modifications of HIF-1α

Hydroxylation and polyubiquitination

Under normoxic conditions HIF-1α is hydroxylated by Prolyl Hydroxylase Domain (PHD) enzymes at two specific prolyl residues (P 402 and P 564) located in the ODD domain of HIF-1α (58-60). For the hydroxylation reaction of the prolyl residues the PHDs require O_2 and α-ketoglutarate as substrates. The hydroxylated prolyl residues of HIF-1α serve as a recognition site for the tumor suppressor protein VHL (58-61). VHL is the recognition component of an E3 ubiquitin-protein ligase complex that contains further subunits: Elongin C, Elongin B, Cullin 2, Ring Box Protein 1 (RBX1) and an E2 ubiquitin conjugating enzyme (E2). Ubiquitination of HIF-1α by this multiprotein complex leads to HIF-1α degradation by the 26S proteasome (62-66).

In humans three PHDs have been described: PHD1, PHD2 and PHD3 (67-70). PHD1 and PHD2 hydroxylate both prolyl sites, whereas PHD3 only modifies the C-terminal prolyl residue (P 564). It was demonstrated that specific gene silencing of PHD2 by siRNA was sufficient to stabilize HIF-1α under normoxic conditions. However, silencing of PHD1 and PHD3 had no effect on HIF-1α stability (71). Consistent with these findings, it was shown that PHD2 has the highest specific activity for HIF-1α hydroxylation (72). PHD2 silencing not only led to HIF-1α stabilization, moreover it mediated HIF-1α translocation to the nucleus, where HIF-1 was found to be transcriptionally active (71). Interestingly, it was demonstrated that PHD2 and PHD3 mRNAs as well as PHD2 protein were induced by hypoxia (68, 73). These findings provided evidence of an autoregulatory mechanism that helps to explain the previous observation, that the more severe the hypoxic stress (leading to a strong induction of PHD2 protein) the faster the degradation of HIF-1α upon reoxygenation (74). Recently, OS-9, the protein product of a widely expressed gene, was demonstrated to interact with both PHD2 and HIF-1α. This interaction was shown to promote the hydroxylation reaction and led to an increased proteasomal degradation of HIF-1α (75).

As described above, HIF-1α protein stability is mainly regulated by the oxygen-dependent PHD2 enzyme. HIF-1 transcriptional activity is regulated by another O_2-dependent enzyme, termed Factor Inhibiting HIF-1 (FIH-1). For the transcriptional activity of HIF-1 two domains of HIF-1α are required: the N-terminal and C-terminal

transactivation domains. The C-TAD interacts with the transcriptional co-activators p300/CBP to enhance transcription of HIF-1 target genes. Hydroxylation of a specific asparagines residue (N 803) in the C-TAD by FIH interrupts the interaction of C-TAD and p300/CBP, thereby inhibiting HIF-1 transcriptional activity (76).

In summary, the mammalian O_2-sensing pathway involves prolyl and asparaginyl hydroxylation of the HIF-1α subunit, which in the presence of O_2 inactivates HIF-1 by proteasomal degradation and inhibition of transcriptional co-activator recruitment. Under hypoxic conditions, these mechanisms are blocked by the lack of molecular O_2, allowing HIF-1α stabilization, nuclear translocation, binding to target genes and enhanced transcriptional activation through the recruitment of co-activators.

Acetylation and deacetylation

More recently, several studies demonstrated an important role of two opposing groups of enzymes, histone acetyl-transferases (HATs) and histone deacetylases (HDACs), in the stabilization of HIF-1α. These enzymes are known to posttranslationally modify the acetylation level of histone proteins. In yeast, in which these enzymes initially were discovered, HDACs generally are associated with gene silencing. HDACs remove acetyl groups from specific lysine residues on histone tails, thereby enhancing the electric charge of histones and the concomitant attraction between positively charged histone proteins and negatively charged DNA. HATs generally have the opposite effect, promoting gene transcription by acetylating and thereby neutralizing the positive charge of specific lysines on histone tails. HDACs can be classified into four groups : class I consists of HDAC1, 2, 3 and 8 (nuclear localization), class II consists of HDAC4, 5, 6, 7, 9 and 10 (cytoplasm and nuclear localization), class III consists of sirtuins (SIRT1-7) and class IV consists of HDAC11, which shares features of both classes I and II HDACs. Whereas classes I, II and IV HDACs share structural and sequential homologies and require a Zinc (Zn^+) ion for their enzymatic activity, the class III HDACs (sirtuins) are a completely distinct group of HDACs and share no similarities in their structure or sequence with class I, II or IV HDACs. Moreover, sirtuins have the unique property among all classes of HDACs to depend on nicotinamide adenine dinucleotide (NAD^+) for their enzymatic reaction (77).

During the last years an increasing amount of non-histone proteins, among them several key transcription factors (e.g. p53, E2F1, NF-kB, MyoD) or transcriptional co-activators (e.g. p300, PGC-1α), have been shown to be modified by HATs and HDACs. The acetylation level of transcription factors influences their stability, transcriptional activity and interaction with transcriptional co-activators as well as their DNA binding affinity. In the case of HIF-1α conflicting data concerning its acetylation has been published. It was demonstrated in a yeast two-hybrid assay that an acetyl-transferase termed mouse ARD1 (mARD1) acetylates a specific lysine residue (K 532) located in the ODD domain of HIF-1α under normoxic conditions. Acetylated HIF-1α showed enhanced binding to VHL, thus promoting HIF-1α degradation (78). As reported by different groups, a mutated HIF-1α protein, in which the lysine (K 532, the site of lysine acetylation) was changed to an arginine, was more stable than wild-type HIF-1α in human cells under normoxic conditions (66, 78, 79). Unlike the PHDs, enzymatic activity of HATs is independent of oxygen. However, under hypoxic conditions mARD1 mRNA expression decreased, leading to a reduction of HIF-1α acetylation and to increased HIF-1α stability (78). Controversial to that, several other studies in various human cell lines revealed similar human ARD1 (hARD1) mRNA and protein levels upon normoxia and hypoxia (80, 81). It was further demonstrated that hARD1 associates with the ODD domain of human HIF-1α, however does not acetylate and destabilize HIF-1α (82, 83). Although several above mentioned publications provide evidence that hARD1 is unable to acetylate HIF-1α, one report suggested an anti-angiogenic role of CTGF (Connective Tissue Growth Factor) by accelerating HIF-1α degradation through ARD1-dependent acetylation (84).

So far only one acetyl-transferase (mARD1), however several class I and II HDACs have been implicated in the regulation of HIF-1α acetylation levels. In the past years, an increasing amount of small-molecule inhibitors of class I and II HDACs (HDACi) have been described to exhibit an anti-angiogenic activity. Different mechanisms of the HDACi-mediated antitumoral effects have been proposed, some of them are suggested to directly influence HIF-1α stability or transcriptional activity. HIF-1α has been shown to interact with HDAC1 and 3 (class I), and HDAC4, HDAC6 and HDAC7 (class II) (85-87). Thus far, nothing is known about the interaction of class III HDACs (sirtuins) and HIF-1α (see Chapter 2). Hepatitis B virus X protein (HBx) was

shown to induce the expression of both metastasis-associated protein 1 (MTA1) and HDAC1 in hepatocellular carcinoma (HCC) (88). MTA1, HDAC1, HDAC2 and HIF-1α physically interact and MTA1/HDAC1 complex stabilizes HIF-1α by deacetylation, thus potentially playing a critical role in angiogenesis and metastasis of HCC (79). Silencing of HDAC4 by specific shRNA revealed increased levels of HIF-1α acetylation, associated with decreased HIF-1α stability. These results indicate an important function of HDAC4 deacetylase activity on HIF-1α stability (85). The same group demonstrated, that HDAC6 was required for HIF-1α stabilization. Knockdown of HDAC6 led to a decrease of HIF-1α protein, however, unlike knockdown of HDAC4, it had no influence on the acetylation level of HIF-1α (85). In contrast to the mARD1-mediated VHL-dependent HIF-1α degradation (78), inhibition of HDAC4 and HDAC6 was shown to mediate HIF-1α degradation in a VHL-independent, but proteasome-mediated pathway (85). Inhibition of HDAC6 by different HDACi was suggested to decrease HIF-1α stability by interfering with HSP90 chaperone function. HSP90 is a chaperone, responsible for the correct folding and maturation of several proteins such as HIF-1α. HSP90 antagonists were demonstrated to decrease HIF-1α in a VHL-independent pathway (89). Consistent with this study, inhibition of HDAC6 has been shown to increase acetylation levels of HSP90, thereby impairing its chaperone function and concomitantly degrading HIF-1α levels in a VHL-independent, proteasome-mediated pathway (90). The same group postulated another mechanism, involved in HDACi-mediated HIF-1α inhibition. HDACi enhances the acetylation level of p300, a transcriptional co-activator of HIF-1α. An increase of p300 acetylation leads to a decrease in p300 interaction with C-TAD of HIF-1α, thus repressing the transcriptional activation of HIF-1 target genes (91). Finally, it was reported that HDAC7 co-translocates to the nucleus together with HIF-1α among hypoxic conditions. Nuclear HDAC7 and HIF-1α interact with p300. Hereby HDAC7 increases transcriptional activity of HIF-1α (87).

Different HDACs have distinct functions in regard to HIF-1α regulation. Various HDACs are required for the stabilization and/or transcriptional activation of HIF-1α. Considering that HDACs are generally involved in transcriptional silencing, it is interesting to see, that as in the case of HIF-1α, HDACs can have important roles in

inducing gene expression by the stabilization and transcriptional activation of the transcription factor HIF-1.

Phosphorylation

Phosphorylation of various transcription factors has been shown to influence their activity. This is a well-known regulatory mechanism. In the case of HIF-1α, several studies reported a role of phosphorylation in enhancing HIF-1 transcriptional activity. HIF-1α phosphorylation by p42/p44 mitogen-activated protein kinases (MAPK) leads to enhanced HIF-1 transcriptional activity. In the same study the authors demonstrated, that other family members of MAPK such as p38 MAPK or c-Jun N-terminal kinase (JNK) do not phosphorylate HIF-1α (92). However, it was shown by an other group, that p38 MAPK was able to phosphorylate and activate HIF-1 transcription upon p38 MAPK activation by a specific viral oncogene (93).

The phosphorylation status of HIF-1α was propagated to be critical to whether cells undergo apoptosis or survive. Whereas phosphorylated HIF-1α binds to HIF-1β, dephosphorylated HIF-1α preferentially interacts with p53, thus promoting apoptosis (94). Recent data suggests two specific serine residues (Ser 641 and Ser 643) of HIF-1α as MAPK phosphorylation targets (95). Phosphorylated HIF-1 shows enhanced transcriptional activity. Inhibition of phophorylation by site-directed mutagenesis of the two serine residues or by MAPK pathway inhibitors showed much lower transcriptional activity than wild-type HIF-1 (95). Furthermore the same group demonstrated that inhibition of HIF-1α phosphorylation impairs its nuclear accumulation. They identified a nuclear export signal (NES) in HIF-1α that interacts with CRM1, a protein involved in the nuclear export of HIF-1α (96). It was suggested that phosphorylation of the two described serine residues by MAPK promotes nuclear accumulation and transcriptional activity of HIF-1α by inhibition of the interaction between the NES of HIF-1α and the CRM1 (96). A recent study described phosphorylation of 3 specific phosphorylation sites (S 551, T 555 and Ser 589) in the ODD of HIF-1α by GSK-3 (glycogen synthase kinase 3) (97) Interestingly, it was additionally shown in this study, that GSK-3 inhibition or mutations of the GSK-3 phosphorylation sites within HIF-1α, enhanced HIF-1α protein levels. Moreover, GSK-3-mediated HIF-1α degradation is VHL-independent and involves

ubiquitylation and the proteasome (97). These findings are somewhat contradictory to several above described studies, which consistently revealed transcriptional activation of HIF-1α through phosphorylation, rather than HIF-1α protein degradation.

S-nitrosylation

Controversial data in regard to the impact of NO in the control of HIF-1α stability and transcriptional activity have been published in the last years. On one hand it was suggested that under normoxic conditions, NO inhibits the enzymatic activity of PHDs by replacing oxygen, thus leading to normoxic stabilization of HIF-1α protein (98). On the other hand, it was suggested, that under hypoxic conditions, NO-mediated redistribution of intracellular oxygen resulted in increased availability of oxygen for PHDs, thereby consecutively degrading HIF-1α (99). Another report suggested direct activation of PHDs by NO (100). In addition, it was suggested that HIF-1α could be posttranslationally modified by S-nitrosylation (101). The same group reported in another study, that S-nitrosylation of a cysteine residue (C 800) within the C-TAD activates the interaction between HIF-1α and its transcriptional co-activator p300, thereby promoting HIF-1 transcriptional activity. This effect was not observed when the cysteine residue was substituted by alanine (C 800 A) (102).

A recent study reported, that NO-mediated S-nitrosylation of HIF-1α increased its stabilization and activity in normoxia. In murine tumors, ionizing radiation stimulated the production of NO, thereby promoting S-nitrosylation of the only cystein residue (C 533) within the ODD of murine HIF-1α (103). The mechanism by which NO-mediated S-nitrosylation was suggested to enhance HIF-1α stability, is interruption of VHL binding to the S-nitrosylated ODD of HIF-1α. These findings were further confirmed by demonstrating, that mutation of the cystein residue in the ODD (C 533 S) did not decrease binding with VHL in the presence of NO, thereby leading to its continuous degradation (103).

SUMOylation

SUMOylation has been discovered to be an important and dynamic posttranslational modification of proteins in the last decade. The pivotal role of protein SUMOylation is reflected in the genetic loss of SUMO, leading to death in S. cerevisiae, C. elegans, Arabidopsis and mice (104). Humans express 3 functional SUMO isoforms (SUMO-1, SUMO-2 and SUMO-3) and an additional isoform (SUMO-4), which`s role is not clear. As in the case of ubiquitination, the covalent SUMOylation of proteins requires E1 (activating), E2 (conjugating), E3 (ligating) enzymes. SENPs (SUMO-isopeptidases) have different functions, one of them being the deSUMOylation. The regulation and extent of protein SUMOylation may be dependent on other posttranslational modifications of the target protein. Under certain stress conditions such as hypoxia, a general increase of protein SUMOylation has been described (105, 106). It was shown that HIF-1α was targeted for SUMOylation by several studies. However, which influence SUMOylation has in the stabilization and transcriptional activation of HIF-1α is discussed controversial. On one hand, a study demonstrated increased HIF-1α stability and transcriptional activity by SUMOylation of two lysine residues (K 391 and K 477) within its ODD (107). Consistent with these results, another group described a role for RSUME (RWD-containing sumoylation enhancer), a protein that is induced by hypoxia and enhances HIF-1α SUMOylation, thus promoting its stabilization and transcriptional activity (108). On the other hand, there is data, demonstrating reduction of HIF-1 transcriptional activity by SUMOylation (109). A recent study revealed a critical role for SUMO-specific protease 1 (SENP1) in the regulation of HIF-1α stability in hypoxia. SENP1-/- mice embryos show severe fetal anemia due to deficient erythropoietin (EPO) production (110). SENP1 controls EPO by the regulation of HIF-1α stability during hypoxia. Hypoxia induces HIF-1α SUMOylation and promotes VHL binding and consecutive ubiquitination and degradation. SENP1 reverses HIF-1α SUMOylation.

Several studies demonstrated reversible SUMOylation of HIF-1α. The role of this posttranslational modification remains controversial and requires future investigations.

2.3. Oncogenic activation of HIF-1α

Humans are obligate consumers of O_2. The more cells are present in a tissue, the more O_2 is consumed. When cells divide and proliferate O_2 consumption increases. Therefore, it seems logical that the main pathways, which promote cell proliferation and survival also induce HIF-1α (Figure 5, reviewed in (2)). The strategy of proliferating cells to induce HIF-1α ensures the maintenance of O_2 homeostasis. For example, proliferating cells express VEGF, which induces angiogenesis to provide the increasing number of cells with sufficient O_2 and nutrients. As discussed previously (1.1. HIF-1 Regulates Cancer Cell Metabolism), rapidly proliferating cells switch from oxidative to glycolytic metabolism. Both of these processes (angiogenesis and metabolic reprogramming) are partly mediated by HIF-1 (6-8, 111). The growth-factor-stimulated increase in HIF-1α levels has two important differences compared to the hypoxia-mediated increase in HIF-1α levels. The first difference is that growth factors induce HIF-1α in a cell-type-specific manner, whereas hypoxia induces HIF-1α in virtually all cell types. The second difference is the mechanism, which leads to an increase in HIF-1α protein levels. Whereas hypoxia decreases HIF-1α degradation, growth factors and other signaling molecules increase HIF-1α protein synthesis. Activation of MAPK and PI3K pathways and its downstream target mTOR (mammalian target of rapamycin) increases HIF-1α protein synthesis by a complex mechanism (Figure 4, reviewed in (2)). The 5`-untranslated regions of HIF-1α mRNA regulate translation in response to S6 kinase activation. The increase of mRNA translation is dependent on mTOR because mTOR regulates S6 kinase, which is required for efficient translation (112-114).

Several growth factors such as insulin-like growth factor-2 (IGF2) and transforming growth factor-α (TGF-α) are known HIF-1 target genes. Binding of these growth factors to their receptors (IGF2 binds to insulin-like growth factor 1 receptor (IGF1R) and TGF-α binds to epidermal growth-factor receptor (EGFR)) activate signal-transduction pathways, which lead to increased HIF-1α protein synthesis. Increased HIF-1α levels lead to increased HIF-1 transcription of target genes, including IGF2 and TGF-α. Therefore, HIF-1 contributes to autocrine signaling pathways, which are critically involved in cancer progression.

Finally, besides the above discussed activation of oncogenes, loss-of-function mutations in tumor suppressor proteins such as PTEN can lead to increased HIF-1α protein

synthesis. As depicted in Figure 3, loss of function mutations of other tumor suppressors such as VHL and p53 lead to increased HIF-1α protein levels through a decrease of its ubiquitination and degradation. Therefore, these mutations influence HIF-1α protein at the posttranslational level and do not (directly) influence HIF-1α protein synthesis.

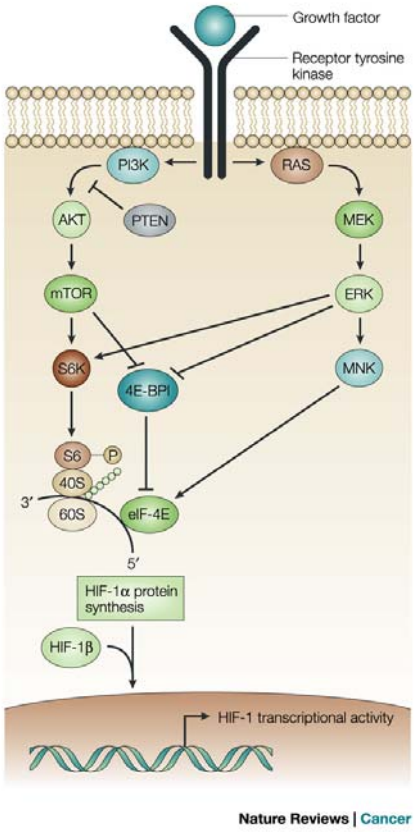


Figure 5| Regulation of HIF-1α protein synthesis.

Semenza, Nat Rev Cancer. 2003 Oct;3(10):721-32.

Growth-factor binding to a cognate receptor tyrosine kinase activates the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. PI3K activates the downstream serine/threonine kinases AKT (also known as protein kinase B (PKB)) and mammalian target of rapamycin (mTOR). In the MAPK pathway, the extracellular-signal-regulated kinase (ERK) is activated by the upstream MAP/ERK kinase (MEK). ERK, in turn, activates MNK. ERK and mTOR phosphorylate p70 S6 kinase (S6K) — which, in turn, phosphorylates the ribosomal S6 protein — and the eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1). Binding of 4E-BP1 to eIF-4E inactivates the latter, inhibiting cap-dependent mRNA translation. Phosphorylation of 4E-BP1 prevents its binding to eIF-4E. MNK phosphorylates eIF-4E and stimulates its activity directly. The effect of growth-factor signalling is an increase in the rate at which a subset of mRNAs within the cell (including HIF-1α mRNA) are translated into protein.

3. HIF-1 as a therapeutic target in cancer

3.1. Targeting HIF-1 for cancer therapy

HIF-1 transcriptionally regulates many genes which are critically involved in cancer pathogenesis such as metabolic reprogramming, angiogenesis, metastasis and cancer cell survival. Independent of any specific mechanism, HIF-1α overexpression is associated with increased patient mortality in several cancers (reviewed in (2, 115)). In experimental animal models it was demonstrated that human cancer cell lines that were genetically manipulated to increase HIF-1α expression, showed increased tumor growth, angiogenesis and metastasis, whereas genetic manipulations that decrease HIF-1α expression resulted in decreased tumor growth, angiogenesis and metastasis (116-120). These studies clearly validate HIF-1 as therapeutic target in cancer. An increasing number of anti-cancer agents have been demonstrated to inhibit HIF-1 activity. The aim of this section is to briefly describe the different mechanisms or strategies, which are used to target HIF-1α for cancer therapy. This section neither provides a complete list of known HIF-1 targeting agents, nor will it specifically address the clinical relevance of the different agents. HIF-1 targeting anti-cancer agents can be divided into different groups according to their mechanisms of action (Figure 6, adapted from (39)).

1. inhibition of HIF-1α protein translation
2. inhibition of HIF-1α protein by promoting its proteasomal degradation
3. inhibition of HIF-1 DNA binding capacity
4. inhibition of HIF-1 transcriptional activity

1. Inhibition of HIF-1α protein translation

The rate of HIF-1α protein synthesis in cancers is largely determined by mTOR activity. The constitutive activation of receptor tyrosine kinases and the downstream PI3K/ AKT and RAS/ MAPK signal transduction pathways in cancer cells leads to increased mTOR activity and increased HIF-1α protein synthesis (2, 114) Thus, various inhibitors of these oncogenic signaling pathways lead to reduced HIF-1α protein levels and reveal biological consequences such as impaired angiogenesis. Different studies demonstrated that mTOR inhibition was associated with a decrease of HIF-1 target genes through the

inhibition of HIF-1α mRNA translation (121, 122). Inhibitors of topoisomerases (topotecan) are another class of HIF-1α targeting agents, which inhibit HIF-1α protein translation (123, 124). However, their exact mechanism of action remains to be established.

2. Inhibition of HIF-1α protein by promoting its proteasomal degradation

HSP90 inhibitors generally decrease HIF-1α protein levels by promoting its proteasomal degradation. However, different mechanisms by which HSP90 inhibitors lead to proteasomal degradation of HIF-1α protein have been described (90, 125-128). Histone deacetylase inhibitors (HDACi) are another group of agents, which inhibit HIF-1α protein by inducing its proteasomal degradation. The mechanism by which HDACi lead to the proteasomal degradation of HIF-1α protein, is particularly relevant to my thesis work. Therefore, I will briefly discuss the role of HDACi on HIF-1α protein degradation in a separate section (3.2. HDAC Inhibitors target HIF-1α protein).

3. Inhibition of HIF-1 DNA binding capacity

HIF-1 activates gene expression by binding to a specific DNA sequence, which is present in all HIF-1 target genes. One group of agents (echinomycin and polyamides) inhibits HIF-1 binding to these specific DNA sequences within HIF-1 target genes (129).

4. Inhibition of HIF-1 transcriptional activity

HIF-1 transcriptional activity is largely mediated by two functional domains of HIF-1α, N-TAD and C-TAD, which interacts with the transcriptional coactivator p300/CBP to enhance transcription. One class of HIF-1 targeting agents inhibits HIF-1 transcriptional activity by disrupting the interaction of HIF-1α with p300 and by other, yet to be defined mechanisms (130, 131).

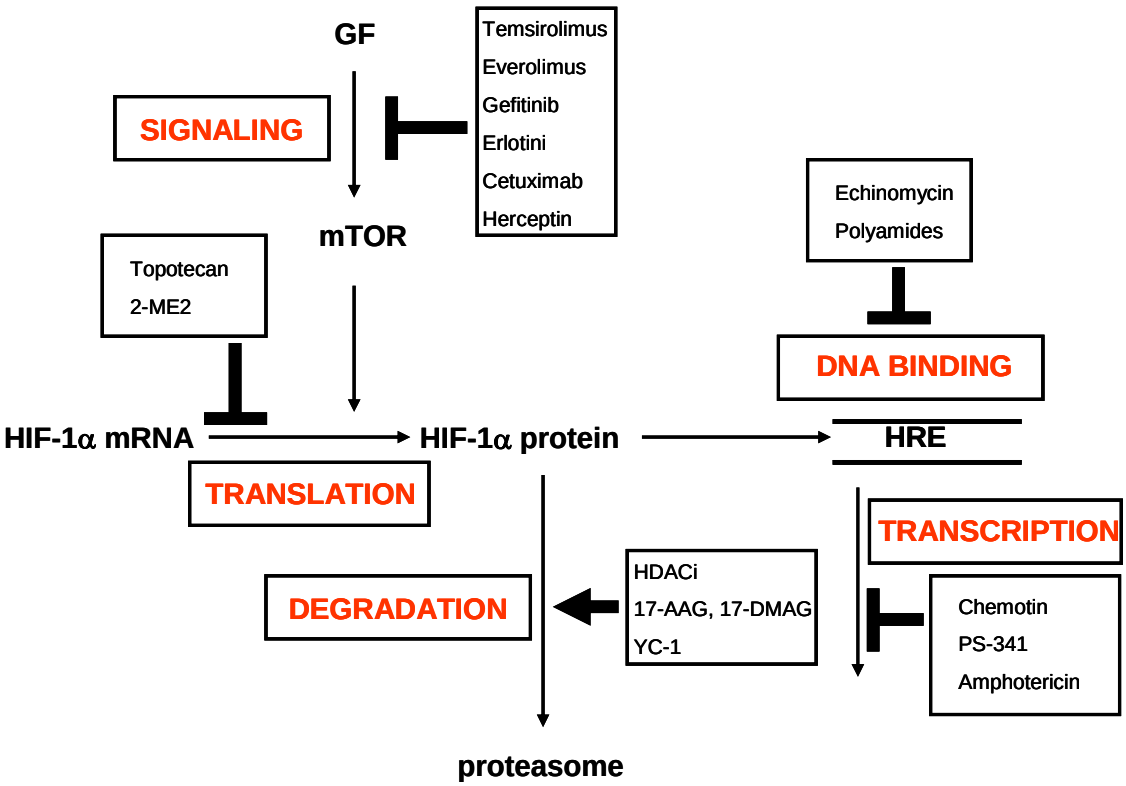


Figure 6| Possible targets of HIF-1α inhibition by small molecules.

Figure adapted from Mellilo, Cancer Metastasis Rev. 2007 Jun;26(2):341-52.

3.2. Role of Histone Deacetylase Inhibitors on HIF-1α

Two opposing groups of enzymes regulate the acetylation levels of histone and non-histone proteins. Histone deacetylases (HDACs) remove acetyl-groups from specific lysine residues of proteins, whereas acetyltransferases perform the opposite reaction. The acetylation level of histone proteins is relevant for DNA chromatin conformation and is a main determinant of gene transcription. Increased acetylation levels of histone proteins are generally associated with increased gene transcription, whereas HDACs are associated with gene silencing. The acetylation level of non-histone proteins, in particular of transcription factors such as HIF-1α, is important for its stability and transcriptional activity. HDAC inhibitors (HDACi) have been shown to inhibit HIF-1α, however several different mechanisms have been postulated. What is consistent among these different studies is that HDACs are promoting HIF-1α stability, whereas the inhibition of HDACs by HDACi is associated with decreased HIF-1α stability due to a decrease in its proteasomal degradation. The first study suggested that HDACi increase the transcription of p53 and VHL, two tumor suppressor proteins that are known to promote HIF-1α degradation. A later report implied that HDACi lead to a direct acetylation of HIF-1α protein, which was associated with increased interaction with VHL, leading to proteasomal degradation of HIF-1α protein. However, more recent studies have revealed that HDACi increase HIF-1α proteasomal degradation in a VHL-independent pathway. One group showed that HDAC6 inhibition by HDACi leads to hyperacetylation of HSP90, a chaperone protein required for the maturation of HIF-1α. Hyperacetylated HSP90 is unfunctional and is unable to promote maturation of HIF-1α, thereby leading to its proteasomal degradation. Interestingly, another report demonstrated a role of HDAC6 and HDAC4 in stabilizing HIF-1α by direct interaction with HIF-1α. Their inhibition led to an increase in HIF-1α acetylation, which was associated with increased proteasomal degradation.

4. SIRT1 protein deacetylase

SIRT1 is a NAD^+-dependent protein deacetylase, which has been implicated in a variety of cellular processes such as metabolism, longevity and cancer (reviewed in (132-137)). SIRT1 influences these processes, by deacetylating several key transcription factors (Figure 7). The acetylation level of transcription factors is a critical determinant of their stability and transcriptional activity (138).

It is known for a long time that calorie restriction increases lifespan in several organisms. Recent studies in regard of calorie restriction have demonstrated a pivotal role of SIRT1 in mediating key metabolic adaptations, which underlie the benefical effects of calorie restriction on extending lifespan and preventing age-related diseases (139, 140). In lower organisms it is well recognized that the SIRT1 homolog Sir2 promotes longevity and recent studies suggest that SIRT1 has similar effects in humans (141). However, besides calorie restriction, SIRT1 responds to a variety of stress conditions such as oxidative stress, DNA damage and oncogenic stress. In response to DNA damage, SIRT1 deacetylates and inhibits p53, thus negatively regulating p53-dependent apoptosis (142, 143). SIRT1 targets several other transcription factors, which are critically involved in growth regulation, stress response, DNA repair and apoptosis (Figure 7). There is an intense discussion ongoing to whether SIRT1 acts as a tumor promoter or tumor suppressor and it seems evident that there is no simple answer to this question. On one hand SIRT1 promotes longevity by promoting key metabolic adaptations, on the other hand SIRT1 inhibits apoptosis in response to DNA damage, which increases the risk of accumulating mutations, ultimately causing cancer.

SIRT1 is one of seven homologues in mammals (SIRT1-SIRT7). SIRT1 is by far the most studied and best characterized sirtuin. However, more recently, new insights about the other mammalian sirtuins were gained (Table 1, reviewed in (137, 144, 145)). For my thesis work, I have focused on SIRT1 and did not investigate the role and function of the other sirtuins. The seven mammalian sirtuins differ from each other in regard to several aspects, one of the most obvious ones being their cellular localization: SIRT1, SIRT6 and SIRT7 are classified as nuclear sirtuins, however it is now apparent that SIRT1 can shuttle between the nucleus and the cytoplasm; SIRT3, SIRT4 and

SIRT5 are located in the mitochondria and SIRT2 is found predominantly in the cytoplasm. (For more information, see Table 1).

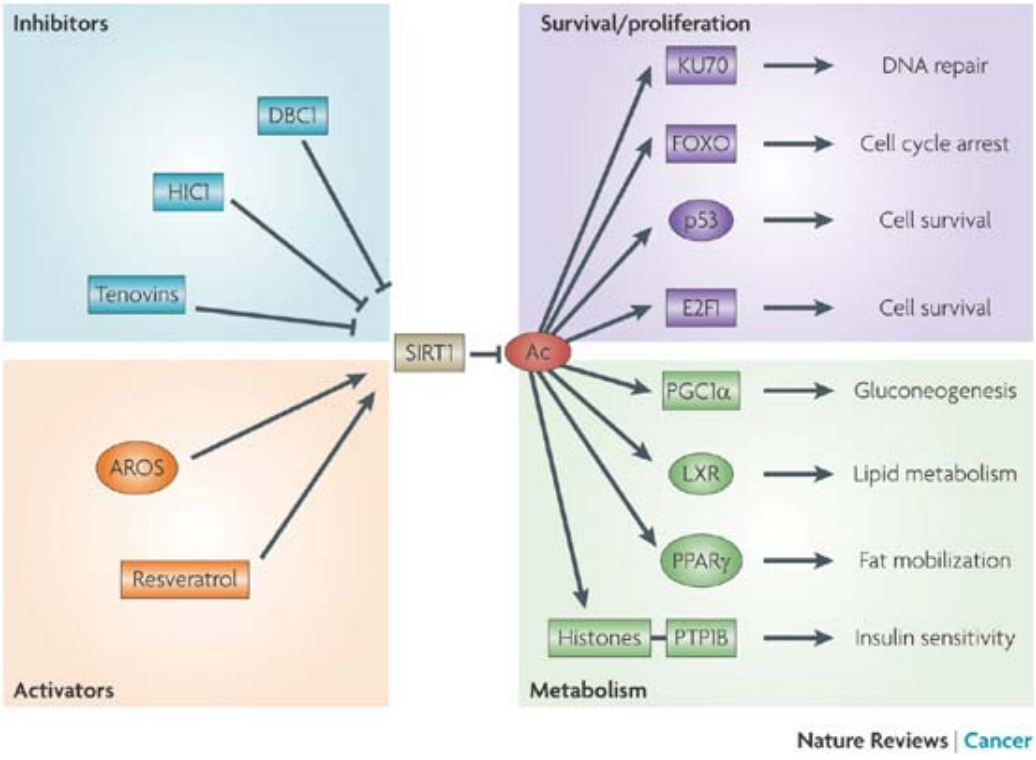


Figure 7| SIRT1 pathway overview.

Brooks, Nat Rev Cancer. 2009 Feb;9(2):123-8.

SIRT1 is an NAD+-dependent histone deacetylase that catalyses the removal of acetyl (Ac) groups from a number of non-histone targets. The downstream effects of target deacetylation include changes in cellular metabolism (lipid metabolism, insulin sensitivity, reverse cholesterol transport and gluconeogenesis) as well as cell survival and senescence effects (cell survival and DNA repair). Several protein regulators and small-molecule compounds that can activate or inhibit SIRT1 function have also been described. AROS, active regulator of SIRT1; DBC1, deleted in breast cancer 1; FOXO, forkhead box; HIC1, hypermethylated in cancer 1; LXR, liver X receptor; PGC1, PPAR coactivator 1; PPAR, peroxisome proliferator-activated receptor-; PTP1B, protein-tyrosine phosphatase 1B.

Sirtuin	Disease area	Therapeutic strategy	Substrates/ interactors	Overexpression/knockout model summary
SIRT1	Metabolic, neurological, cardiovascular, renal, cancer, mitochondrial	Activation	p53, FOXO1, FOXO4, COUP-TF, CTIP2, NF-κB-p65, NCOR, histone H1, histone H4, KU70, p300, BCL11A, Tat, PGC1α, MEF2, eNOS, ACS1, E2F1, AR, p73, SMAD7, NBS1, RB, TLE1, IRS2, LXR, AROS, SUV39H1, WRN, DBC1, TORC2	• Efficacy observed in preclinical models of diabetes with small-molecule SIRT1 activators[24] • Transgenic overexpression of SIRT1 is cardioprotective against oxidative stress and heart ageing[57] • *Sirt1*-overexpressing mice show some phenotypes of calorie-restricted mice[7] • *SIRT1* overexpression shows beneficial effects in Alzheimer's disease and Huntington's disease models[59,63] • Knockout mice have genomic instability and developmental defects[58,61] • SIRT1 activates PGC1α by deacetylation and is involved in mitochondrial biogenesis[10]
SIRT2	Neurological, metabolic, cancer	Inhibition/ activation?	Tubulin, HOXA10, FOXO, histone H4, 14-3-3 protein	• Efficacy observed in a cellular and *Drosophila melanogaster* model of Parkinson's disease with small-molecule SIRT2 inhibitors[62]
SIRT3	Metabolic, mitochondrial	Activation	ACS2	• *Sirt3*-knockout mice have hyperacetylated proteins in mitochondria[60]
SIRT4	Metabolic, mitochondrial	Inhibition?	GDH, IDE, ANT2, ANT3	• *Sirt4*-knockout mice are viable and fertile; pancreatic mitochondrial lysates from knockout animals show higher GDH activity[31]
SIRT5	Neurological	Unknown	Unknown	• Increased expression of *Sirt5* observed in frontal cortex of brains from serotonin receptor knockout mice[64]
SIRT6	Cancer	Activation	Histone H3	• Knockout mice have genomic instability, premature ageing phenotype and predisposition to developing cancer[52]
SIRT7	Cardiovascular	Activation	RNA polymerase I, p53	• Knockout mice have decreased lifespan with inflammatory cardiac hypertrophy[56]

Table 1| Protein substrates and interactions of mammalian sirtuins*

Lavu et al., Nat Rev Drug Discov. 2008 Oct;7(10):841-53.

*As mentioned in the table, discovery of cellular substrates as well as overexpression and knockout models provide validation and therapeutic strategy to target sirtuins in various diseases of ageing. Development of small-molecule modulators of sirtuin activity would validate the genetic lead obtained in animal models. ACS, acetyl-CoA synthetase; ANT, ADP/ATP carrier protein; AR, androgen receptor; AROS, active regulator of SIRT1; BCL11A, B-cell CLL/lymphoma 11A (zinc finger protein); COUP-TF, chicken ovalbumin upstream promoter-transcription factor (also known as NR2F1); CTIP2, COUP-TF interacting protein 2 (also known as BCL11B); DBC1, deleted in breast cancer 1; E2F1, E2F transcription factor 1; eNOS, endothelial nitric oxide synthase; GDH, glutamate dehydrogenase; IDE, insulin-degrading enzyme; IRS2, insulin receptor substate 2; LXR, liver X receptor; MEF2, myocyte-specific enhancer factor 2; NBS1, Nijmegen breakage syndrome 1; NCOR, nuclear receptor co-repressor; NF-κB, nuclear factor-κB; PGC1α, peroxisome proliferator-activated receptor-γ co-activator 1α; RB, retinoblastoma protein; SUV39H1, suppressor of variegation 3-9 homologue 1; TLE1, transducin-like enhancer of split 1; TORC2, transducer of regulated cAMP response element binding protein 2; WRN, Werner syndrome protein.

4.1. SIRT1 in metabolism

In lower organisms calorie restriction increases lifespan (140). Recent studies showed that SIRT1 protein levels increase upon calorie restriction and promote key metabolic adaptations, which are potentially underlying the effects of calorie restriction on longevity. Several mechanisms of SIRT1 regulation have been demonstrated. Calorie restriction seems to upregulate SIRT1 protein independent of its transcription. In hepatocytes, pyruvate levels increase in response to calorie restriction and this metabolite is thought to induce SIRT1 protein translation (146). Another central mechanism of SIRT1 regulation that impacts on the activity of SIRT1 are fluctuations in NAD^+ levels or changes in the ratios of NAD^+ / NADH. During fasting, the NAD^+-levels increase, which induces the enzymatic activity of the NAD^+-dependent SIRT1 (146).

SIRT1 deacetylates a number of targets that are involved in metabolism and energy homeostasis. Deacetylation of the transcriptional coactivator PGC-1α (peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α) by SIRT induces gluconeogenic gene transcription and represses glycolytic genes, thereby enhancing the hepatic glucose output in response to calorie restriction (146, 147). This process might be of pivotal importance, since blood glucose levels must be kept in a narrow range due to the dependency of certain organs on glucose as energy substrate. Furthermore by deacetylating PGC-1α, SIRT1 induces mitochondrial biogenesis in various tissues (146). In addition, hepatic SIRT1 controls systemic and hepatic cholesterol levels by increasing the expression of liver X receptor (LXR), which decreases cholesterol absorption (148). SIRT1 improves insulin use and resistance and by promoting insulin secretion in pancreatic β-cells, SIRT1 stimulates fat mobilization in white adipose tissue (149, 150). Taken together, the SIRT1-mediated metabolic adaptations in response to calorie restriction are likely to improve health and to cause an increase in lifespan in higher organisms.

4.2. SIRT1 in cancer

The role of SIRT1 in regard to whether it acts as a tumor promoter or a tumor suppressor has become increasingly complex and remains controversial. Whether SIRT1 promotes or suppresses tumors seems to be largely dependent on the cell-type and the genetic and cellular context. SIRT1 has been involved in the anti-aging process in several organisms. Since the incidence of most cancers correlates with an increase in age, SIRT1 was initially thought to increase the risk of cancer development. Several studies demonstrated a significant increase of SIRT1 protein levels in various cancers, supporting the idea of SIRT1 being a tumor promoter (151-154). Consistent with these studies, we have observed elevated SIRT1 protein levels in two different hepatocellular carcinoma cell lines (Hep3B and HepG2) compared to SIRT1 protein expression in human primary hepatocytes (data not shown). However, recently it was reported that certain cancer types show reduced levels of SIRT1 protein (155). These studies rather imply SIRT1 as a tumor suppressor. A recent study from our group (Chapter 2, submitted for publication) provided new evidence that SIRT1 can act as a tumor promoter by deacetylating and stabilizing a transcription factor, which is critically involved in tumor progression.

An increasing amount of reviews discuss the controversial role of SIRT1 in cancer. This section focuses on the role of SIRT1 in tumorigenesis by describing three examples, which illustrate the complexicity of SIRT1 function in cancer.

SIRT1 and p53

One of the first discovered targets for SIRT1-mediated deacetylation was p53, which is inactivated by deacetylation (143, 156-158). It was demonstrated that SIRT1 represses p53-dependent cell cycle arrest and apoptosis in response to DNA damage and oxidative stress (142, 143, 157). These experiments were the first evidence that SIRT1 is involved in the process of tumorigenesis as a tumor promoter. Two recent studies demonstrated that the tumor suppressor DBC1 (deleted in breast cancer), physically interacts with SIRT1 and inhibits SIRT1 activity. DBC1-mediated inhibition of SIRT1 leads to increased levels of p53 acetylation and thus to an increase of p53-mediated functions. The knockdown of DBC1 (similar to the situation that occurs in certain breast cancers) consistently increases SIRT1 deacetylase activity and leads to decreased

levels of p53 acetylation and to a reduction of p53-mediated apoptosis in response to genotoxic stress. Taken together these data clearly suggest a role of p53 in promoting tumorigenesis.

Interestingly, two repressive p53-binding sites are located in the SIRT1 promoter. Consistent with this finding, several p53-null tumor cell lines display increased levels of SIRT1 protein and p53-null mice show increased basal expression of SIRT1 in some tissue types (158, 159). Given the ability of SIRT1 deacetylase to inactivate other stress response proteins such as Foxo transcription factors, this may have further implications of SIRT1 on promoting tumorigenesis in the absence of p53-null tumors.

SIRT1 and E2F1

A similar negative feedback loop as observed between p53 and SIRT1, exists for E2F1. The transcription factor E2F1 induces transcription of different apoptotic genes and mediates apoptosis upon DNA damage. Recently, E2F1 has been shown to induce SIRT1 expression upon etoposide-mediated DNA damage (160). Interestingly, SIRT1 protein acts on E2F1 in a negative feedback loop by deacetylating E2F1 and thereby inactivating E2F1-mediated transcription. By repressing E2F1, which is a potent activator of several apoptotic genes, SIRT1 is likely to prevent E2F1-mediated apoptosis in response to DNA damage.

SIRT1 and HIC1

The tumor suppressor HIC1 (hypermethylated in cancer 1) physically interacts with SIRT1 protein. The HIC1-SIRT1 protein complex binds to the SIRT1 promoter and represses SIRT1 expression. Through the interaction with HIC1, SIRT1 represses its own transcription. As observed for p53 and E2F1, the interaction between SIRT1 and HIC1 is complex. In cancers in which HIC1 is epigenetically silenced through hypermethylation of HIC1 promoter, SIRT1 levels increase in the presence of oncogenic stress. $HIC1^{+/-}$ mice have an increased incidence of tumors and display a block of p53-mediated apoptosis in response to DNA damage due to an increase of SIRT1 expression (161).

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Chapter 2

SIRT1 Physically Interacts with Hypoxia-Inducible Factor-1 alpha (HIF-1α) and Is Required for HIF-1α Protein Stabilization

Submitted for publication

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Abstract

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that plays a key role in promoting tumor cell adaptation and survival in hypoxic tumors. The activity of HIF-1 is mainly regulated by posttranslational modifications that alter the stability of its α-subunit, whereas its β-subunit is constitutively expressed. In this study, we investigated the effect of deacetylation on HIF-1α stability by examining the role of SIRT1. SIRT1, a NAD^+-dependent protein deacetylase, has been shown to promote cell survival under conditions of stress by deacetylating key transcription factors. Our results demonstrate the importance of SIRT1 deacetylase function on HIF-1α stability and its transcriptional activity. Treatment with sirtinol (a specific small molecule inhibitor of SIRT1) or knockdown of SIRT1 by shRNA led to a decrease of HIF-1α protein and resulted in a dose-dependent inhibition of HIF-1 transcriptional activity in hepatocellular carcinoma cell lines. Sirtinol repressed HIF-1α in a VHL-independent mechanism which is mediated by the proteasome system. Furthermore, by co-immunoprecipitation assays, we demonstrate that SIRT1 physically interacts with HIF-1α and provide evidence that HIF-1α is deacetylated by SIRT1. Knockdown of SIRT1 led to a hyperacetylation of HIF-1α. This study suggests that SIRT1 and HIF-1α synergistically interact to promote cell survival under hypoxic conditions. Therefore, the targeted inhibition of SIRT1 might be a promising therapeutic approach in HIF-1α expressing cancers.

Introduction

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that is responsible for cellular adaptations promoting cell survival under hypoxic conditions (1). HIF-1 consists of two subunits, HIF-1α and HIF-1β. HIF-1α is oxygen-sensitive, whereas HIF-1β is constitutively expressed. At normoxic conditions, HIF-1α is hydroxylated by specific prolyl hydroxylase domain (PHDs) enzymes that utilize oxygen and α-ketoglutarate as substrates. Hydroxylation of two prolyl residues (P402 and P564) within the oxygen-dependent degradation (ODD) domain of HIF-1α serve as recognition site for von Hippel-Lindau (VHL) tumor suppressor protein, a subunit of an E3 ubiquitin ligase. Ubiquitination of HIF-1α leads to its degradation by the 26S proteasome (2-4). Under hypoxic conditions, the oxygen-dependent PHDs are inactive, HIF-1α is stabilized, accumulates and translocates to the nucleus where it dimerizes with HIF-1β and initiates a complex transcriptional program. HIF-1 binds to specific nucleotide sequences (hypoxia response elements, HRE) in target genes, thereby increasing their transcription (5).

In rapidly growing solid tumors hypoxia is a common feature due to insufficient tumor vasculature. Hypoxic tumor cells accumulate HIF-1, thereby inducing HIF-1 target genes to promote metabolic adaptations, angiogenesis, erythropoiesis and metastasis (1). In certain cancer cells HIF-1α can be stabilized at normoxia due to altered oncogenic signaling pathways. In renal cell

carcinomas, inactivation of the VHL gene is often leading to constitutively expressed HIF-1α protein (6).

HIF-1α is mainly regulated at the posttranslational level. Besides the well established process of hydroxylation, more recently other posttranslational modifications of HIF-1α, such as acetylation and deacetylation, have been reported (7). Two groups of enzymes, which are classified as histone acetyltransferases (HATs) and histone deacetylases (HDACs) due to their initially discovered ability to modify histone proteins, have been recognized to target an increasing amount of non-histone proteins. Among these non-histone proteins, there are several transcription factors, whose acetylation level was described to influence their stability and transcriptional activity (8). Conflicting data concerning the acetylation of HIF-1α was reported (7). In a yeast two-hybrid assay, interaction of HIF-1α with an acetyltransferase termed mouse ARD1 (mARD1), was shown to enhance acetylation of a specific lysine residue (K532) within the ODD of HIF-1α. Furthermore the same group described enhanced binding of VHL to acetylated HIF-1α, thus leading to an increase in its proteasomal degradation (9). However, it was shown by several other studies, that the human variant of ARD1 (hARD1), does not acetylate HIF-1α (10, 11). Several reports consistently demonstrated a role of certain class I and II HDACs in the stabilization and/or transcriptional activation of HIF-1α. However, different mechanisms were suggested (7, 12).

HDACs are classified into 4 groups: class I (HDAC1, 2, 3 and 8), class II (HDAC4, 5, 6, 7, 9 and 10), class IV (HDAC11) contains only one member, which shares features of both class I and II HDACs, and class III (also known as sirtuins) (13). Sirtuins are a highly conserved protein family from bacteria to humans (14). Sir2 (Silent mating type Information Regulator 2) was the first discovered member of sirtuins and found in yeast (15). SIRT1 is the human homolog of Sir2, on which a lot of interest has been focused due to its ability to promote longevity in lower organisms (16-18). SIRT1 is one of seven members of class III HDACs in humans (SIRT1 to SIRT7). One important difference between SIRT1 and class I, II and IV HDACs, is the SIRT1 dependency on nicotinamide adenine dinucleotide (NAD^+), thus directly linking SIRT1 enzymatic activity with metabolism (16, 19).

SIRT1 deacetylase activity influences the stability and activation potential of a broad range of transcription factors. One of the first transcription factors described to be deacetylated by SIRT1 is p53 (20, 21). Other targets for SIRT1-mediated deacetylation are Foxo transcription factors, Ku70, NF-κB, E2F1 and the transcriptional co-activator PGC-1α (22). Under conditions of stress as in calorie restriction, SIRT1 protein levels increase and promote cellular adaptations and survival (23). The role of SIRT1 in tumorigenesis is discussed controversially (24). Several studies showed that SIRT1 protein is expressed at high levels in different cancer cell lines and that inhibition of SIRT1 induces growth arrest and apoptosis. For example, SIRT1 inhibition by cambinol (a specific SIRT1 inhibitor) decreased growth of Burkitt lymphoma xenografts in mice (25).

Thus far the role of SIRT1 on HIF-1α stability has not been investigated. Therefore the aim of our study was to determine whether SIRT1 influences HIF-1α protein stability and transcriptional activity. Our results demonstrate that SIRT1 inhibition by sirtinol destabilizes HIF-1α protein and represses its transcriptional activity. We further show that SIRT1 physically interacts with HIF-1α and provide evidence that SIRT1 targets HIF-1α for deacetylation, since knockdown of SIRT1 leads to hyperacetylation of HIF-1α.

Materials and Methods

Cell cultures

Hep3B and HepG2 cells were purchased from ATCC (LCG Promochem, Molsheim, France). Huh7 cells were kindly provided by J-F. Dufour (University of Bern, Bern, Switzerland). RCC4 VHL-/- and RCC4 VHL+/+ cells were generously provided by G. Camenisch (University of Zurich, Zurich, Switzerland). Cells were cultured in DMEM medium with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Life Technology, Paisley, Scotland) at 37°C in a humified incubator with 5% CO_2. Hypoxic culture conditions were performed in a microaerophilic system (Ruskinn, Biotrace International, Bridgend, UK) at 1.5% O_2, 5% CO_2 and 93.5% N_2.

Chemicals and reagents

Dimethyloxaloylglycine (DMOG) and sirtinol were purchased from Alexis Biochemicals (Lausen, Switzerland). The proteasome inhibitor MG132 (Z-LLL-CHO) was from Sigma-Aldrich (Basel, Switzerland). All these chemicals were dissolved in DMSO as stock solutions (DMOG: 100 mmol/L; sirtinol: 50 mmol/L; MG132: 25 mmol/L) and diluted with cell culture medium for the experiments.

Western blotting

Western blot was performed as previously described (26). Briefly, whole cell lysates were prepared by direct lysis in modified radioimmunoprecipitation

(RIPA) buffer, proteins were separated by SDS-PAGE. Nitrocellulose membranes were incubated with primary antibodies overnight at 4°C. HRP-conjugated secondary antibodies were incubated for 1 hour at room temperature and membranes were developed with enhanced chemiluminescent substrate from Perkin Elmer (Schwerzenbach, Switzerland).

Primary antibodies were purchased as follows: rabbit polyclonal anti-SIRT1 and mouse monoclonal anti-alpha-tubulin from Santa Cruz Biotechnology (Heidelberg, Germany), rabbit polyclonal anti-acetyl-lysine from Cell Signaling (Allschwil, Switzerland), mouse monoclonal anti-HIF-1α from Alexis Biochemicals (Lausen, Switzerland). Chicken polyclonal anti-HIF-1α antibody (27) was a courtesy by M. Gassmann (University of Zurich, Zurich, Switzerland).

HRP-conjugated secondary antibodies were purchased as follows: goat anti-rabbit (Dako, Baar, Switzerland), goat anti-mouse (Perbio Science S.A, Lausanne, Switzerland) and rabbit anti-chicken (Promega, Dubendorf, Switzerland).

Immunoprecipitation

For cytoplasmic and nuclear protein fractionation cells were lysed with lysis buffer [10 mmol/L Hepes, pH 7.9; 10 mmol/L KCL; 0.1 mmol/L EDTA; 0.1 mmol/L EGTA; 1 mmol/L DDT; phosphatase inhibitors (Na_3VO_4, NaF, PMSF); a protease inhibitor mix (Sigma-Aldrich) and 0.5% NP40]. The lysates were quickly centrifuged at high speed and the supernatant was transferred in a new tube (cytoplasmic fraction). For the extraction of nuclear proteins, a modified buffer

than used for cell lysis (modification: 20 mmol/L Hepes, pH 7.9; 400 mmol/L KCL) was added to the pellets and incubated for 15 minutes on a vortex at 4°C. Then tubes were centrifuged for 5 min at 2000 rpm and the supernatant was kept (nuclear fraction). For the immunoprecipitation of SIRT1, cytoplasmic proteins (1250 μg) and nuclear extracts (250 μg) were incubated with 2 μg and 1 μg of rabbit polyclonal anti-SIRT1 antibody, respectively (Santa Cruz Biotechnology) or similar amounts of rabbit IgG1 control antibody overnight at 4°C. The same procedure was performed for immunoprecipitation of HIF-1α with a mouse monoclonal anti HIF-1α antibody (Alexis Biochemicals) or mouse IgG1 control antibody. The lysates were then incubated for a further hour at 4°C together with 50 μl (cytoplasmic fraction) or 30 μl (nuclear fraction) of Protein G magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and loaded on μcolumns (Miltenyi Biotec) according to the manufacturer`s protocol. Beads were first extensively washed with lysis buffer (composed of 2/3 of lysis buffer and 1/3 of nuclear extraction buffer) and then boiled with Laemmli sample buffer for five minutes and analyzed by SDS-PAGE as described before.

To check the acetylation level of HIF-1α, cells were lysed with a lysis buffer containing 1% Triton-X 100, 0.5% NP-40, 10 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, phosphatase inhibitors (Na_3VO_4, NaF, $ZnCl_2$, Na_2MoO_4) and a protease inhibitor mix (Sigma-Aldrich). Of each sample, 2 mg of protein were incubated with 2 μg of mouse monoclonal HIF-1α antibody overnight at 4°C. Five percent of whole cell lysates were saved as input controls.

For immunoprecipitation of HIF-1α the above described procedure with Protein G magnetic microbeads (50 µl/ sample) was performed and samples were analyzed by SDS-PAGE.

Plasmids, transfections and reporter gene assay

We cloned three tandem repeats of the HIF-1 binding site (HBS) from the transferrin promoter in the pGL3 luciferase vector (Promega, Dubendorf, Switzerland). A HBS is part of the HRE of HIF-1 target genes. This plasmid was cotransfected with Renilla luciferase control plasmid (pRL-TK) with effectene reagent according to the manufacturer's protocol (Qiagen, Hombrechtikon, Switzerland). Twenty-four hours after cotransfection, cells were treated with sirtinol and incubated under hypoxia for 24 hours. Absolute luminescence was measured according to the Dual-Luciferase® Reporter Assay protocol (Promega). The firefly luciferase values were normalized to the corresponding renilla luciferase control values.

SIRT1 wt (wildtype) and SIRT1 H363Y (point-mutated) plasmids (28) were a kind gift from Dr. Michael Potente (University of Frankfurt, Frankfurt, Germany). Cells were transfected as described above and 24 hours after transfection they were incubated under hypoxia for 4 hours. pcDNA 3.1 (Promega) was used as negative control.

RNA extraction and quantitative RT-PCR

RNA was isolated and analyzed by quantitative RT-PCR as previously

described (29). Human probes for 18S rRNA (# 4310893E), HIF-1α (# Hs00153153_m1), EPO (# Hs00171267_m1) and CA IX (# 00154208_m1) were obtained from ABI (Applied Biosystems, Rotkreuz, Switzerland). In figure 1B, PCR products obtained from the quantitative analysis of HIF-1α mRNA and 18S rRNA are shown.

Lentiviral vectors expressing shRNAs

Lentivirus production, titer determination and transduction were carried out as described before (30). Two different clones of shRNA against SIRT1 (clone 1, ID: NM_012238.3-1958s1c1 termed shSIRT1.1958 and clone 2, ID: NM_012238.3-3206s1c1 termed shSIRT1.3206) and a non-target shRNA control vector (shScr) were obtained from Sigma-Aldrich (Basel, Switzerland). Five to seven days after infection with the lentiviruses, experiments were performed.

Results

SIRT1 inhibition represses HIF-1α protein stabilization and decreases HIF-1 transcriptional activity.

We first questioned whether inhibition of SIRT1 influences HIF-1α protein levels. To inhibit the enzymatic activity of SIRT1, sirtinol was used at increasing concentrations to treat the hepatocellular carcinoma (HCC) cell line Hep3B. Sixteen hours after sirtinol administration, cells were exposed to hypoxia (1.5% O_2) for 4 hours to induce HIF-1α protein stabilization. As expected, in normoxic Hep3B cells HIF-1α protein was degraded and nearly undetectable by Western blotting, whereas 4 hours of hypoxia efficiently induced HIF-1α protein. The sirtinol-mediated inhibition of SIRT1 activity led to a dose-dependent repression of HIF-1α protein, with a maximal effect at 100 μM (Fig. 1A). Similar results were obtained when cells were treated with sirtinol and HIF-1α protein was stabilized by the non-hypoxic HIF-1α activator dimethyloxaloylglycine (DMOG) (data not shown).

To determine the kinetics of sirtinol-mediated HIF-1α protein repression, Hep3B cells were either incubated with sirtinol for 16, 2 and 0 hours before exposing them 4 hours to hypoxia, or sirtinol was added to the cells 2 hours after exposure to hypoxia for a total of 4 hours. Addition of sirtinol at the time of exposure to hypoxia inhibited the induction of HIF-1α protein. A stronger repressive effect was observed when cells were pretreated with sirtinol for 2 to 16

hours. Interestingly, addition of sirtinol 2 hours after incubating the cells under hypoxia showed only a minor reduction in HIF-1α protein levels (Fig. 1B, upper panel). The same experiment was performed to analyze HIF-1α mRNA and revealed no markedly differences in HIF-1α mRNA levels (Fig. 1B, lower panel). Therefore, we concluded that sirtinol does not inhibit HIF-1α at the transcriptional level. Taken together, these results suggest that SIRT1 enzymatic activity is required for the stabilization and accumulation of HIF-1α protein. However, SIRT1 inhibition does not seem to affect preformed (mature) HIF-1α protein.

To confirm that the sirtinol-mediated repression of HIF-1α protein is specific to the inhibition of SIRT1, Hep3B cells were infected with lentiviruses carrying shRNA targeting SIRT1. Targeted disruption of SIRT1 with shSIRT1.1958 led to a nearly complete knockdown and the use of shSIRT1.3206 resulted in a partial knockdown of SIRT1 protein compared to parental and shScr infected controls. The efficiency of SIRT1 knockdown correlated with the suppression of HIF-1α protein, with the stronger effect achieved in cells treated with shSIRT1.1958 (Fig. 1C). These results provide further evidence that SIRT1 is required for the stabilization of HIF-1α protein, since SIRT1 knockdown repressed HIF-1α.

Next, we investigated the consequence of sirtinol-mediated HIF-1α protein repression on HIF-1 transcriptional activity. Hep3B cells were transfected with a HIF-1 binding site (HBS)-driven firefly luciferase reporter gene. Twenty-four hours after transfection, cells were treated with increasing doses of sirtinol, followed by

a 24 hour incubation under hypoxia. Hypoxia increased reporter activity 35-fold compared to a normoxic controls. Sirtinol treatment resulted in a decrease of HIF-1 transcriptional activity in a dose-dependent manner (Fig. 1D). A reduction of 35% and 70% of reporter activity was observed with 50 μM and 100 μM sirtinol, respectively.

We next verified that inhibition of SIRT1 influences the expression of representative HIF-1 target genes such as erythropoietin (EPO) and carbonic anhydrase IX (CA IX). SIRT1 was either inhibited with sirtinol or knocked down with shSIRT1.1958. In control cells, 12 hours of hypoxia led in average to a 74-fold and a 24-fold induction of EPO and CA IX mRNA, respectively (data not shown). Pretreatment with sirtinol as well as SIRT1 knockdown significantly reduced hypoxic induction of EPO mRNA by 88% (Fig. 1E). Likewise, sirtinol reduced the hypoxic induction of CA IX mRNA by 81% and shSIRT1.1958 treatment resulted in a 75% decrease of CA IX mRNA (Fig. 1E). Our data demonstrate that inhibition of SIRT1 leads to a strong suppression of HIF-1 target genes.

Finally, to prove that the inhibition of SIRT1 represses HIF-1α in a cell line independent manner, two additional HCC cell lines (HepG2 and Huh7) were tested. As in Hep3B cells, sirtinol pretreatment decreased hypoxia-induced HIF-1α protein levels (Fig. 1F).

Sirtinol-induced repression of HIF-1α protein is independent of VHL and is mediated by the proteasome system.

To determine whether the sirtinol-induced repression of HIF-1α protein is dependent on VHL, we used a VHL-deficient renal cell carcinoma (RCC4 VHL-/-) cell line. RCC4 VHL-/- cells express HIF-1α protein constitutively, whereas in RCC4 VHL-competent (RCC4 VHL+/+) cells, HIF-1α protein is rapidly degraded under normoxic conditions and stabilized by hypoxia (6, 31). Exposure of RCC4 VHL+/+ cells to hypoxia for 4 hours, led to the induction of HIF-1α protein. Two hours of sirtinol pretreatment strongly inhibited hypoxia-induced HIF-1α stabilization (Fig. 2A, left panel). In RCC4 VHL-/- cells, sirtinol repressed HIF-1α protein under both normoxic and hypoxic conditions, thus demonstrating that sirtinol-mediated HIF-1α repression is independent of VHL (Fig. 2A, middle panel). Interestingly, RCC4 VHL-/- cells required a longer treatment time with sirtinol (16 hours) in order to decrease HIF-1α protein levels. The shorter incubation time of 2 hours was insufficient to observe this effect (Fig. 2A, right panel). This observation is consistent with our findings in Hep3B cells (Fig. 1B), indicating that sirtinol-mediated repression of HIF-1α is rather due to a decrease of newly stabilized HIF-1α protein, than due to enhanced degradation of preformed (mature) HIF-1α.

To further investigate the molecular mechanism by which SIRT1 inhibition leads to a repression of HIF-1α protein, we checked whether this effect is

dependent on the proteasome system. Hep3B cells were pretreated with sirtinol for 2 hours, followed by treatment with a proteasome inhibitor (MG132) and continuous exposure to normoxia for 6 hours or incubation under hypoxia for 6 hours. In normoxic cells, addition of MG132 led to a strong accumulation of HIF-1α protein independent of sirtinol pretreatment (Fig. 2B). These results suggest, that sirtinol has no major effect on HIF-1α translation, since sirtinol had no influence on HIF-1α protein accumulation in the presence of MG132. Similar results were obtained, when cells were pretreated with sirtinol and exposed to hypoxia in the presence of MG132. Two hours of sirtinol pretreatment inhibited the hypoxic accumulation of HIF-1α. This effect was completely reversed by addition of MG132. From this experiment we conclude, that the sirtinol-induced inhibition of HIF-1α protein is mediated by the proteasome system. Furthermore these results provide evidence that the sirtinol-mediated repression of HIF-1α takes place at the posttranslational level of HIF-1α regulation.

SIRT1 physically interacts with HIF-1α in the cytoplasm.

Our previous observations in Hep3B and RCC4 VHL-/- cells implicated a function of SIRT1 in the stabilization of newly synthesized HIF-1α protein (Fig. 1B and 2A). We hypothesized that SIRT1 and HIF-1α protein might interact in the cytoplasm, the cellular compartment where HIF-1α protein is translated and stabilized. Therefore, we prepared cytoplasmic and nuclear protein fractions of Hep3B cells that were previously treated with DMOG for 5 hours to induce HIF-

1α. The separated fractions of cytoplasmic and nuclear proteins were immunoprecipitated with an anti-SIRT1 antibody and precipitates were immunoblotted with an anti-HIF-1α antibody. Hereby we could verify our hypothesis that endogenous HIF-1α coimmunoprecipitates with endogenous SIRT1 in the cytoplasmic protein fraction (Fig. 3A, left panel). Interestingly, coimmunoprecipitation of HIF-1α was also detected in the nuclear fraction (Fig. 3A, right panel). A similar experiment was performed by immunoprecipitating HIF-1α. Western blot of the precipitates with a SIRT1 antibody confirmed coimmunoprecipitation of SIRT1 and HIF-1α in the cytoplasm (Fig. 3B, left panel). However, even though HIF-1α was abundantly immunoprecipitated in the nuclear cell extracts, SIRT1 was not detected by Western blot (Fig. 3B, right panel). To confirm efficient fractionation of cytoplasmic and nuclear proteins, input controls were immunoblotted for α-tubulin and Sp1 (Fig. 3C). In summary, we show for the first time that the endogenous proteins SIRT1 and HIF-1α physically interact.

SIRT1 knockdown leads to hyperacetylation of HIF-1α protein.

Having demonstrated that SIRT1 and HIF-1α physically interact, we investigated whether SIRT1 targets HIF-1α for deacetylation. Hep3B cells were infected with lentiviruses containing shRNA against SIRT1 or shSCR as negative control. As shown before (Fig. 1C), SIRT1 knockdown led to a repression of HIF-1α protein. Therefore, to investigate a possible change in the acetylation level of

HIF-1α protein in the SIRT1 knockdown cells, we added the proteasome inhibitor MG132, to avoid proteasomal degradation of HIF-1α. Immunoprecipitation with a HIF-1α antibody demonstrated similar HIF-1α levels in the shSCR treated and SIRT1 knockdown cells (Fig. 4), confirming our previous observation that HIF-1α degradation induced by SIRT1 inhibition is mediated by the proteasome and can be blocked by MG132 (Fig. 2B). Immunoblotting with a polyclonal anti-acetyl-lysine antibody revealed strong acetylation of HIF-1α protein in the SIRT1 knockdown cells compared to the shScr infected control (Fig. 4). From this experiment we conclude that SIRT1 deacetylates HIF-1α protein, as knockdown of SIRT1 led to a strong increase of HIF-1α acetylation.

SIRT1 overexpression enhances hypoxic stabilization of HIF-1α protein.

Finally, we explored whether the overexpression of SIRT1 has a stabilizing effect on HIF-1α levels. Hep3B cells were transfected with SIRT1 wt plasmid and SIRT1 H363Y plasmid, which is point-mutated in the catalytic domain of SIRT1 and therefore enzymatically inactive. The transfected cells were incubated under hypoxia for 4 hours or kept at normoxia. Cell lysates were analyzed by Western blot and showed that overexpression of SIRT1 wt (and to a lower extent overexpression of SIRT1 H363Y) increased HIF-1α levels compared to the control (Fig. 5). Since SIRT1 wt showed a stronger effect on HIF-1α stabilization than SIRT1 H363Y, these results further demonstrate that SIRT1 deacetylase activity plays a role in the stabilization of HIF-1α. However, we cannot exclude an

additional function of SIRT1 in stabilizing HIF-1α, independent of its enzymatic activity. Under normoxic conditions, overexpression of SIRT1 did not lead to HIF-1α accumulation.

Discussion

HIF-1α plays a key role in promoting tumor cell adaptation and survival under hypoxic conditions, a common feature of solid and fast growing tumors. Therefore, the regulation and inhibition of HIF-1α is a major interest in tumor biology. The hydroxylation of HIF-1α protein and its consecutive degradation under normoxia is the best known and most explored posttranslational regulation of HIF-1α (32). More recently it has been demonstrated that HIF-1α can be modified by acetylases and deacetylases. The acetylation level of HIF-1α influences its stability and transcriptional activity (7, 12). To our knowledge herein we present the first study that investigates the impact of SIRT1 on HIF-1α. SIRT1 belongs to the class III HDACs and besides its role as a histone deacetylase, it was shown to modify several key transcription factors (22). Our results clearly demonstrate that SIRT1 is required for HIF-1α stabilization, since chemical inhibition of SIRT1 or its knockdown led to a repression of HIF-1α protein. In addition, SIRT1 overexpression enhanced the hypoxic induction of HIF-1α. Moreover, we identified SIRT1 as a new binding partner for HIF-1α by coimmunoprecipitation assays of endogenous proteins. These observations are consistent with other studies that reported a role of certain class II histone deacetylases in the stabilization of HIF-1α. HDAC4 and HDAC6 were demonstrated to physically interact with HIF-1α and their inhibition repressed HIF-1α (33). One mechanism by which HDAC6 inhibition leads to HIF-1α repression, was postulated to involve hyperacetylation of HSP90, thereby

impairing HSP90 chaperone function which is required for the maturation of HIF-1α (34).

Our results strongly suggest that SIRT1 deacetylates HIF-1α directly, since SIRT1 knockdown enhanced HIF-1α acetylation. Similar to our observation, knockdown of HDAC4 led to a hyperacetylation of HIF-1α (33) and a more recent study suggested a role for HDAC1 in HIF-1α deacetylation (35). Conflicting data in regard of HIF-1α acetylation have been reported (7). In yeast two hybrid systems mARD1 associates with human HIF-1α and thereby promotes its acetylation. It was postulated by the same group that HIF-1α acetylation enhances the binding to VHL, subsequently leading to its ubiquitination and degradation (9). However, several other studies revealed that the human variant of ARD1 (hARD1), does not acetylate HIF-1α (10, 11). Our experiments in RCC4 VHL-deficient cells (Fig. 2A), support different studies showing a VHL-independent mechanism of HIF-1α repression by HDAC inhibitors (33, 34). The exact mechanism of how SIRT1 inhibition leads to proteasomal degradation of HIF-1α remains to be determined. A recent report demonstrated an O_2/VHL-independent mechanism of HIF-1α ubiquitination and proteasomal degradation that is mediated by RACK1 (36). Interestingly, the same group demonstrated that an acetyltransferase termed SSAT1 interacts with HIF-1α and RACK1 and is required for RACK1-mediated HIF-1α ubiquitination (37).

The fact that inhibition of HIF-1α required prolonged sirtinol treatment in the constitutively expressing HIF-1α RCC4 VHL-/- cell line implies that

preformed, mature HIF-1α is not the primary target of SIRT1. These results suggest that SIRT1 plays an important role in the maturation of HIF-1α, a process taking place in the cytoplasm. Our coimmunoprecipitation experiments with cytoplasmic and nuclear protein fractions confirmed physical interaction of HIF-1α and SIRT1 in the cytoplasm. This finding might be surprising, since SIRT1 was initially described as an exclusively nuclear protein (38). However, our data are consistent with more recent reports, demonstrating cytoplasmic localization of SIRT1 in certain cell lines (39, 40). Interestingly, our experiments indicate that SIRT1 and HIF-1α also coimmunoprecipitate in the nucleus. Recently, others have shown that SIRT1 can shuttle between the cytoplasm and the nucleus and they identified nuclear export signals which are involved in this transport process (40, 41). Whether there is a potential role for SIRT1 in the HIF-1α translocation to the nucleus is speculative. However, HDAC7 has been shown to physically interact with HIF-1α in the cytoplasm and translocate to the nucleus along with HIF-1α under hypoxic conditions (42).

SIRT1 was reported to promote lifespan and prevent age-associated diseases (43), however, the role of SIRT1 in tumorigenesis remains to be defined (24, 44). In various tumor cells SIRT1 is overexpressed and its knockdown leads to growth arrest and apoptosis (45). SIRT1 seems to be a key promoter of cell survival in certain tumors, such as Burkitt lymphoma xenografts in mice, in which SIRT1 inhibition by cambinol markedly decreased tumor growth (25). Likewise, inhibitors of class II HDACs have displayed antitumoral properties in clinical trials

(46, 47) and repressed HIF-1α in cell culture studies. To our surprise, until now no study addressed the role of SIRT1 on HIF-1α.

In conclusion, here we demonstrate that SIRT1 is required for HIF-1α stability and transcriptional activity. SIRT1 inhibition leads to hyperacetylation of HIF-1α and to its subsequent proteasomal degradation. Our results further show that SIRT1 overexpression, as it exists in certain cancers enhances the hypoxic induction of HIF-1α protein. We identify the physical interaction of SIRT1 and HIF-1α, two proteins that have a central role in promoting cell survival under conditions of stress. Therefore, it is tempting to speculate that SIRT1 inhibition in cancer cells has an antitumoral effect by the repression of HIF-1α. Further studies to address the potential therapeutic effect of SIRT1 inhibition in HIF-1α expressing cancers are required. Given the function of SIRT1 in regulating metabolism and its association with longevity, it will be of great importance to selectively inhibit SIRT1 in tumors to avoid side effects.

Acknowledgements

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Figure legends

Figure 1. SIRT1 inhibition represses HIF-1α protein and HIF-1 transcriptional activity.

A. Dose-dependent HIF-1α protein inhibition by sirtinol.

Hep3B cells were treated with increasing doses of sirtinol for 16 hours and then exposed to hypoxia [H (1.5% O_2)] for 4 hours. Whole cell lysates (100 μg) were analyzed by Western blotting using chicken polyclonal anti-HIF-1α antibody and α-tubulin as loading control. N, normoxia. D, DMSO.

B. Sirtinol does not affect HIF-1α mRNA, but it rapidly inhibits HIF-1α protein.

Hep3B cells were treated with 100μM sirtinol for 16, 2 and 0 hours before and 2 hours after the exposure to hypoxia for a total of 4 hours. HIF-1α was analyzed by Western blotting (upper panel) and by quantitative RT-PCR (lower panel).

C. shRNA against SIRT1 inhibits HIF-1α protein.

The parental cell line Hep3B was infected with lentiviral vectors containing shRNA against SIRT1 (shSIRT1.1958 or shSIRT1.3206). The same vector expressing scrambled shRNA (shScr) served as negative control. Five days after infection, cells were exposed to hypoxia for 4 hours and SIRT1 and HIF-1α were analyzed by Western blot.

D. Dose-dependent inhibition of HIF-1 transcriptional activity.

Hep3B cells were cotransfected with HBS-driven firefly luciferase vector and renilla luciferase control plasmid. Twenty-four hours after the transfection, cells were treated with increasing doses of sirtinol and exposed to hypoxia for 24

hours. Dual luciferase activity was measured and firefly values were normalized by renilla values. *Columns*, mean of triplicates from one representative experiment (n=3). *Bars*, SD.

E. Inhibition of HIF-1 target genes.

To inhibit SIRT1, Hep3B cells were either treated with 100 μM sirtinol or infected with lentiviral vectors containing shSIRT1.1958. Cells were exposed to hypoxia for 12 hours. Quantitative RT-PCR with specific primers for EPO and CA-IX was performed. The relative mRNA expression of hypoxic controls (DMSO or shScr) was considered as 100%.

Graphs representing sirtinol data: *columns*, mean of 3 independent experiments; *bars*, SD. *, $P<$ 0.05 (Mann-Whitney U test). Knockdown experiment with shSIRT1.1958 was performed once to confirm sirtinol data.

F. Sirtinol-mediated HIF-1α repression is cell line independent.

HepG2 and Huh7 cells were treated with 100 μM sirtinol for 16 hours and incubated under hypoxia for 4 hours. HIF-1α was analyzed by Western blotting.

Figure 2. Sirtinol-induced repression of HIF-1α protein is independent of VHL and mediated by the proteasome system.

A. VHL-independency.

RCC4 VHL+/+ and RCC4 VHL-/- cells were pretreated with 100 μM sirtinol (or DMSO) for 2 or 16 hours, followed by 4 hours exposure to hypoxia (H) or

continuous incubation under normoxia (N). Whole cell lysates were analyzed by Western blotting.

B. Proteasome inhibitor MG132 blocks the sirtinol-mediated HIF-1α repression. Hep3B cells were pretreated with 100 μM sirtinol for 2 hours, followed by continuous exposure to normoxia (N) for 6 hours or exposure to hypoxia (H) for 6 hours in the presence (N, *lane 3*; H, *lane 3*) or absence (N, *lane 2*; H, *lane 2*) of 10 μm MG132. Effect of 6 hours treatment with 10 μm MG132 alone at normoxia (N, *lane 4*) and hypoxia (H, *lane 4*). Whole cell lysates were analyzed by Western blotting.

Figure 3. SIRT1 and HIF-1α coimmunoprecipitate.

A. Hep3B cells were treated with 125 μm DMOG for 5 hours. DMSO was used as negative control. Cytoplasmic and nuclear protein fractionation was prepared. Cytoplasmic proteins (1250 μg) (left panel) and nuclear extracts (250 μg) (right panel) were immunoprecipitated with a rabbit polyclonal SIRT1 antibody or a rabbit IgG1 control antibody. The immunoprecipitates were subjected to Western blot analysis with anti-SIRT1 and chicken anti-HIF-1α antibodies.

B. Cytoplasmic proteins (1250 μg) and nuclear extracts (250 μg) from the same experiment as described in (*A*) were immunoprecipitated with a mouse monoclonal HIF-1α antibody or a mouse IgG1 control antibody. Precipitates were blotted for HIF-1α and SIRT1.

C. From the same experiment as described in (*A* and *B*), 10% of the protein amount that was subjected to immunoprecipitation was saved as input control (25 μg nuclear proteins and 125 μg cytoplasmic proteins). Input controls were blotted for SIRT1, HIF-1α, Sp1 and α-tubulin.

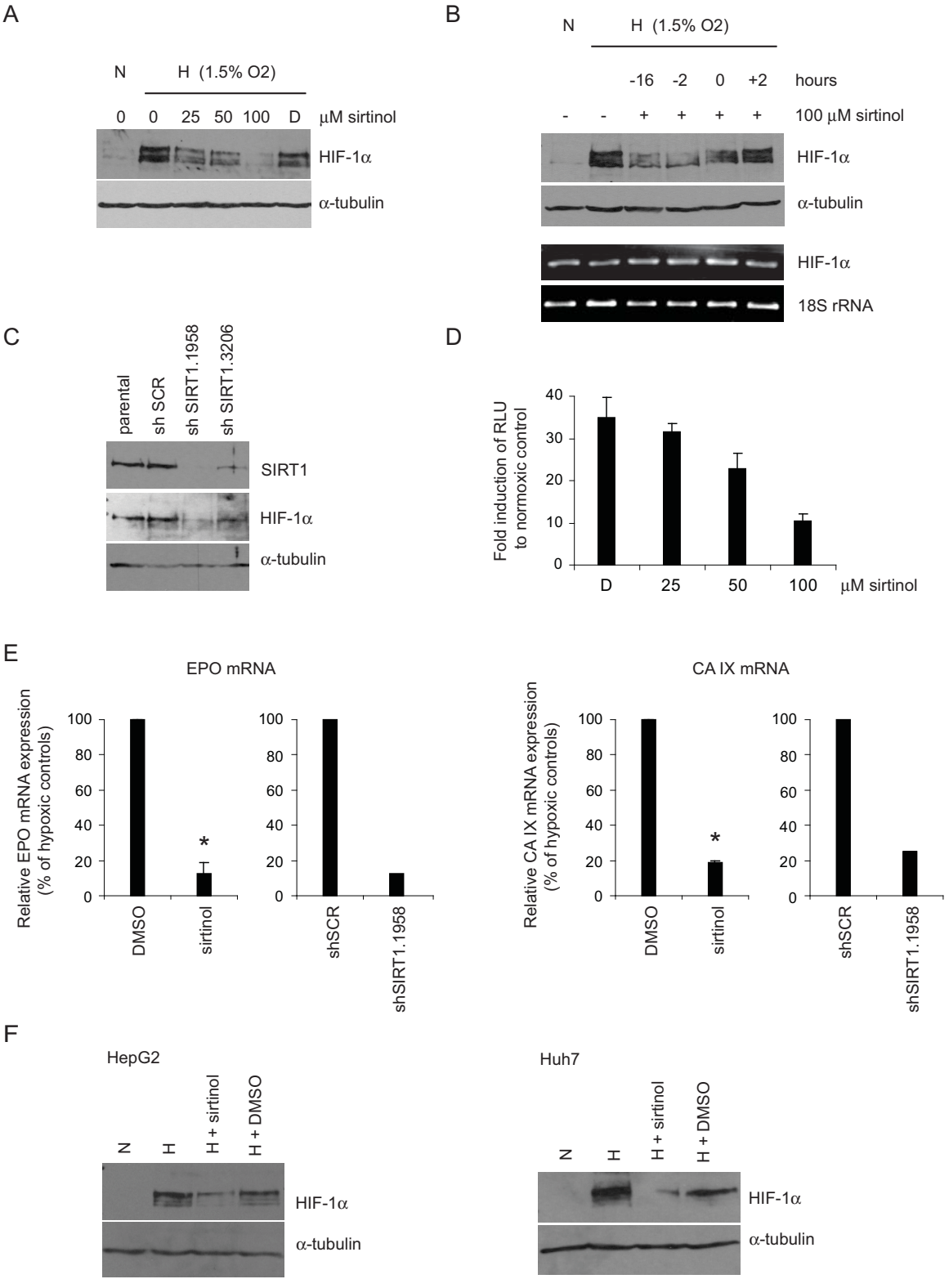
Figure 4. SIRT1 inhibition leads to increased acetylation of HIF-1α.

Hep3B cells were infected with lentiviral vectors containing shRNA against SIRT1 (shSIRT1.1958 or shSIRT1.3206) or scrambled shRNA (shScr) as a negative control. Seven days postinfection, cells were incubated under hypoxia for 5 hours in the presence of 10 μm MG132. Whole cell lysates (2 mg) were immunoprecipitated with a mouse monoclonal HIF-1α antibody (2 μg) and precipitates were analyzed by Western blotting with a rabbit polyclonal anti-acetyl-lysine and a polyclonal chicken anti-HIF-1α antibody. Five percent of whole cell lysates (100 μg) were used as input controls and were analyzed for SIRT1 and α-tubulin.

Figure 5. SIRT1 overexpression stabilizes HIF-1α protein.

Hep3B cells were transfected with SIRT1 wt, SIRT1 H363Y or pcDNA 3.1 (control plasmid) and 24 hours after transfection cells were either exposed to hypoxia for 4 hours or continuously kept at normoxic conditions for 4 hours. Whole cell lysates were analyzed for SIRT1, HIF-1α and α-tubulin by Western blotting.

Figure 1


A
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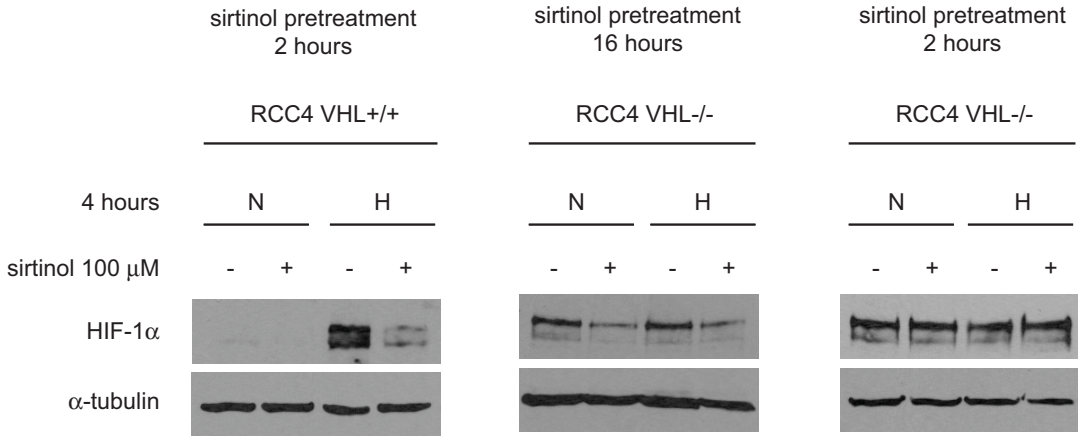
H (1.5% O2)
0 0 25 50 100 D µM sirtinol
HIF-1α
α-tubulin
B
N
H (1.5% O2)
-16 -2 0 +2 hours
- - + + + + 100 µM sirtinol
HIF-1α
α-tubulin
HIF-1α
18S rRNA
C
parental
sh SCR
sh SIRT1.1958
sh SIRT1.3206
SIRT1
HIF-1α
α-tubulin
D
Fold induction of RLU to normoxic control
40
30
20
10
0
D 25 50 100 µM sirtinol
E
EPO mRNA
Relative EPO mRNA expression (% of hypoxic controls)
100
80
60
40
20
0
*
DMSO
sirtinol
shSCR
shSIRT1.1958
CA IX mRNA
Relative CA IX mRNA expression (% of hypoxic controls)
F
HepG2
N
H
H + sirtinol
H + DMSO
HIF-1α
α-tubulin
Huh7
N
H
H + sirtinol
H + DMSO
HIF-1α
α-tubulin

Figure 2

A


sirtinol pretreatment
2 hours
sirtinol pretreatment
16 hours
sirtinol pretreatment
2 hours
RCC4 VHL+/+
RCC4 VHL-/-
RCC4 VHL-/-
4 hours
N
H
N
H
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H
sirtinol 100 μM
- + - +
- + - +
- + - +
HIF-1α
α-tubulin

B

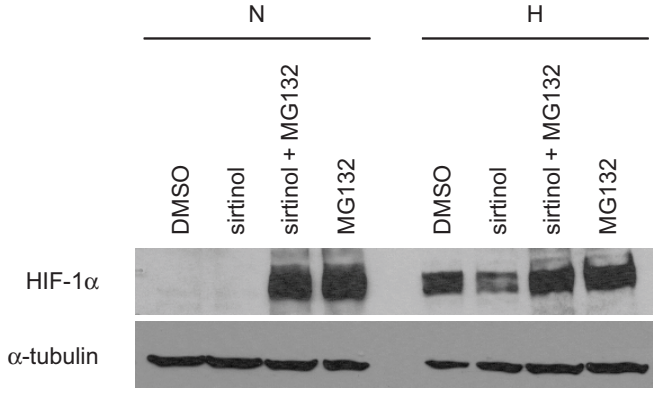
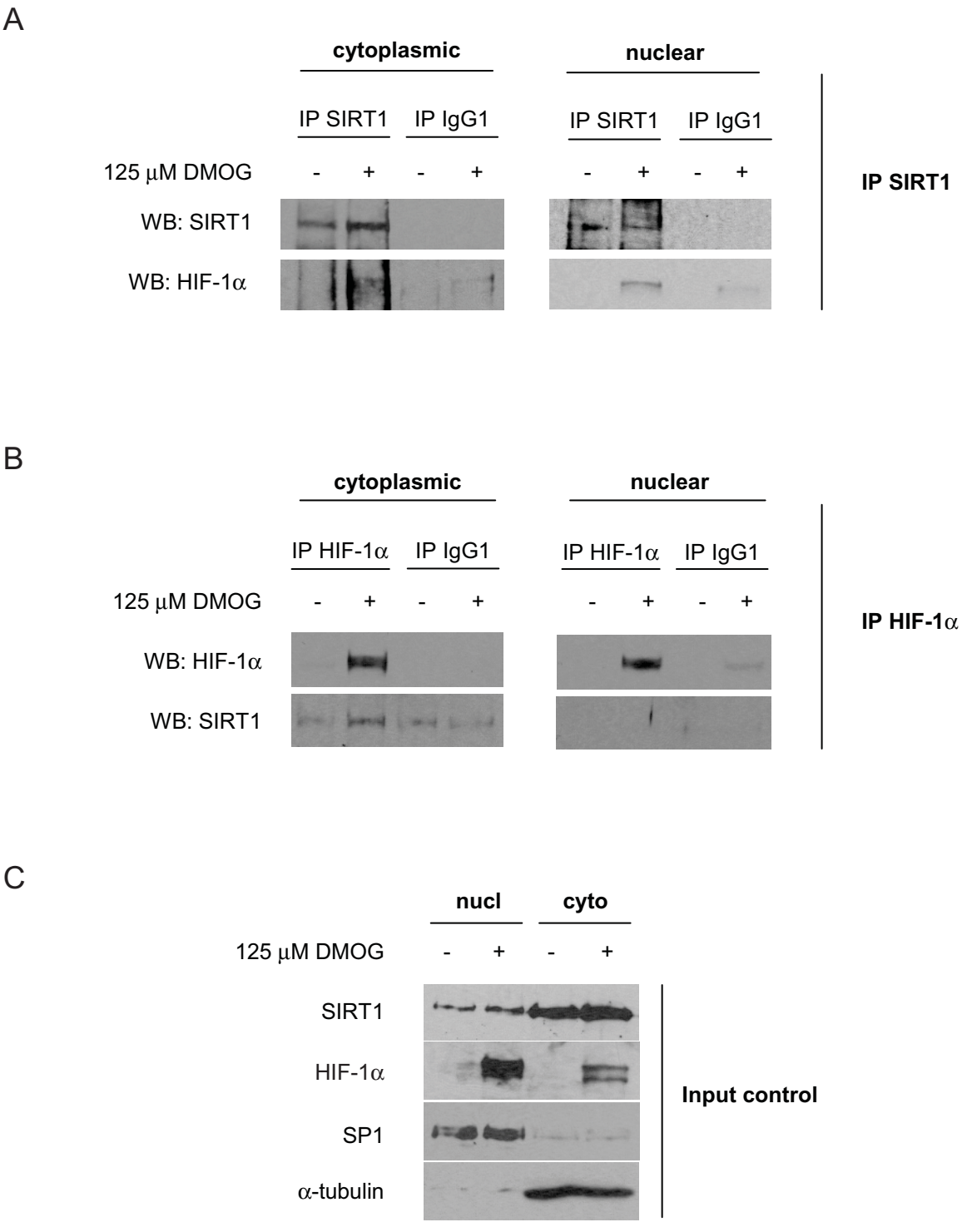

N
H
DMSO
sirtinol
sirtinol + MG132
MG132
DMSO
sirtinol
sirtinol + MG132
MG132
HIF-1α
α-tubulin

Figure 3


A
cytoplasmic
nuclear
IP SIRT1
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125 μM DMOG
- + - +
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WB: SIRT1
WB: HIF-1α
IP SIRT1
B
cytoplasmic
nuclear
IP HIF-1α
IP IgG1
IP HIF-1α
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WB: HIF-1α
WB: SIRT1
IP HIF-1α
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- + - +
SIRT1
HIF-1α
SP1
α-tubulin
Input control

Figure 4

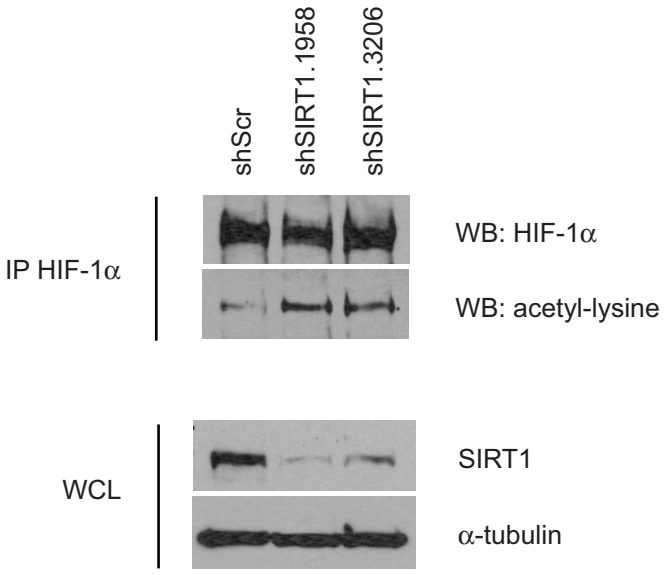
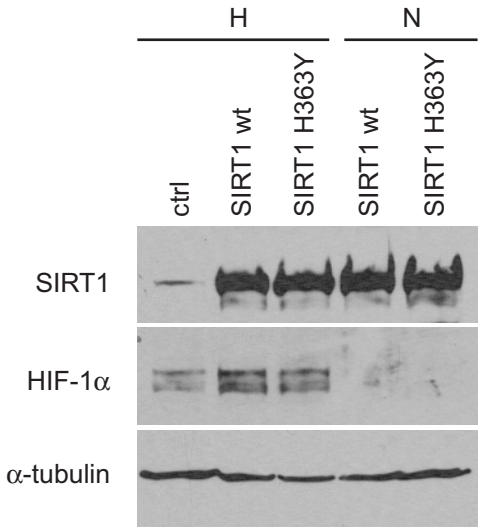

shScr
shSIRT1.1958
shSIRT1.3206
IP HIF-1α
WB: HIF-1α
WB: acetyl-lysine
WCL
SIRT1
α-tubulin

Figure 5


H
N
ctrl
SIRT1 wt
SIRT1 H363Y
SIRT1 wt
SIRT1 H363Y
SIRT1
HIF-1α
α-tubulin

Chapter 3

The role and regulation of the NAD^+-dependent protein deacetylase SIRT1 upon hypoxic stress

In preparation for submission

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The role and regulation of the NAD^+-dependent protein deacetylase SIRT1 upon hypoxic stress

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Running title: SIRT1 upon hypoxic stress

Key Words: SIRT1, Hypoxia, Hepatocytes, Hepatocellular Carcinoma, p53

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Abstract

SIRT1 is a protein deacetylase and its yeast homolog Sir2 promotes longevity in lower organisms in response to calorie restriction. SIRT1 protein increases upon several stress conditions and by deacetylating different transcription factors it promotes cell survival. In response to oxidative stress and DNA damage SIRT1 deacetylates and inhibits the activity of p53, thereby inhibiting p53-mediated apoptosis. Although the role of SIRT1 in tumorigenesis is discussed controversial, there is evidence that SIRT1 acts as a tumor promoter in certain cancers.

Little is known about the response of SIRT1 to hypoxic stress. However, in a recent study we demonstrated that SIRT1 physically interacts with hypoxia-inducible factor 1α (HIF-1α) and is required for the stabilization of HIF-1α protein. HIF-1α is part of a transcription factor, which promotes survival by inducing various target genes upon hypoxic stress.

In this study we investigated the regulation of SIRT1 expression in response to hypoxia. Our results demonstrate that SIRT1 mRNA and protein do not increase upon hypoxia in human primary hepatocytes and two hepatocellular carcinoma cell lines. However, more importantly, we found that SIRT1 inhibition by sirtinol (specific small molecule inhibitor) leads to a growth arrest in Hep3B cells. These results are in line with our previous finding that inhibition of SIRT1 leads to a reduction of HIF-1α, which is required for cell growth and survival upon hypoxic stress.

Introduction

SIRT1 is a protein deacetylase and its yeast homolog Sir2 has been shown to promote longevity in lower organisms (1-3). Unlike other histone/protein deacetylases, SIRT1 enzyme activity depends on NAD^+ as a cosubstrate, thereby directly linking its activity with metabolism (1, 4). Calorie restriction has been demonstrated to increase lifespan in various species. SIRT1 protein markedly increases upon calorie restriction and promotes key metabolic adaptations (5, 6). The exact mechanism of how SIRT1 promotes longevity is still under extensive investigations, however an increase of SIRT1 protein levels is associated with prolonged lifespan (2). Calorie restriction is the most explored stress condition, which has been demonstrated to increase SIRT1 protein levels and deacetylase activity.

In addition to nutrient withdrawal, other stress conditions such as oxidative stress and DNA damage have been shown to increase SIRT1 levels (7-10). One mechanism by which elevated SIRT1 levels favor cell survival in response to DNA damage and oxidative stress, is the deacetylation and consecutive inhibition of p53 activity and p53-mediated apoptosis (7, 8). It is now evident that the initial discovery of SIRT1 to promote cell survival in response to different cellular stresses, has implications in cancer biology. The inhibitory effect of SIRT1 on p53-mediated apoptosis in response to DNA damage, strongly suggested a role of SIRT1 in promoting tumorigesis. Consistent with the SIRT1-mediated inhibition of p53, other tumor suppressors such as E2F1 (11), HIC-1 (12) and Foxo3a (13) are deacetylated and inhibited by SIRT1 in response to stress. In response to calorie restriction, SIRT1 is well recognized to promote metabolic adaptations in favour of increasing lifespan. However, although the role of SIRT1 in cancer remains highly controversial, it is certain that with an increase in lifespan, an organism is more likely to achieve genetic mutations, which ultimately can cause cancer.

Little is known about the regulation and function of SIRT1 upon hypoxic stress conditions. One well known and key mechanism how cells respond to and cope with hypoxic stress, is mediated by the hypoxia-inducible factor 1α. HIF-1α is a transcription factor, which is induced in response to hypoxia and initiates the transcription of various target genes to promote cell survival under hypoxic

conditions. Our previous results (paper submitted, Chapter 2) demonstrated that SIRT1 is required for the the stabilization of the hypoxia-inducible factor 1α (HIF-1α).

Another group reported an increase of SIRT1 protein levels in primary human trophoblasts upon exposure to hypoxia (14). This group suggested a possible role of SIRT1 protein in promoting cell survival under hypoxic conditions. This suggestion based on the observation that SIRT1 inhibition led to a decrease of N-myc down-regulated 1 (NDRG1) protein in hypoxic trophoblasts. The exact function of NDRG1 remains to be established. However, it is known that NDRG1 is strongly induced by hypoxia and NDRG1 was shown to inhibit p53 expression, thereby attenuating hypoxic cell injury.

In this study, we investigated whether SIRT1 expression (mRNA and protein levels) is induced in response to hypoxia. Therefore, we exposed human primary hepatocytes and hepatocellular carcinoma (HCC) cell lines to moderate (1.5% O_2) and severe hypoxia (0.1% O_2) and analyzed SIRT1 mRNA and protein levels. In addition, we examined whether SIRT1 inhibition upon hypoxia influences cell survival. Interestingly, SIRT1 mRNA and protein levels remained largely constant even under severe and prolonged hypoxic conditions. However, the inhibition of SIRT1 in hypoxic cells showed a decrease of G1-cell cycle phase and an increase in G2-phase, consistent with a G2-arrest.

Materials and Methods

Liver Specimens and Cultivation of Human Primary Hepatocytes and HCC cell lines

Normal human liver tissue was taken from the periphery of liver specimens from patients undergoing surgical resection for colorectal metastases that were HBV and HCV negative and had no apparent liver disease. Informed consent of the patients was obtained in accordance with institutional guide lines and the local ethics committee. Human primary hepatocytes were isolated from the liver specimens following a two-step perfusion protocol as described previously (15). Briefly, a wedge of liver was perfused with an EGTA solution followed by an enzyme solution containing collagenases. The liver was coarsely chopped and manipulated to dissociate cells and then filtered. Hepatocytes were cultured for 24 to 48 hours prior to being used for experiments. The hepatocytes were cultured on collagen coated plastic in arginine-free Williams E medium supplemented with insulin (0.015 IU/ml), hydrocortisone (5 μmol/L), penicillin (100 IU/mL), streptomycin (100 μg/mL), glutamine (2 mmol/L), and ornithine (0.4 mmol/L). Human hepatocellular carcinoma cell lines Hep3B and HepG2 were purchased from ATCC (LCG Promochem, Molsheim, France). Cell lines were cultured in Dulbecco`s modified Eagle`s medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/mL), streptomycin (100 μg/mL). All cell cultures were incubated at 37° C in a humified incubator with 5% CO_2. Experiments with hypoxic conditions were performed in a hypoxic chamber (Hypoxia Workstation, Ruskinn Technology Limited, West Yorkshire, UK), in which the O_2 level can be set. For our studies we incubated cells either at 1.5% O_2 (intermediate hypoxia) or at 0.1% O_2 (severe hypoxia) for the indicated time.

RNA extraction and quantitative RT-PCR

Total RNA was extracted with Trizol (Life Technologies, Paisley, Scotland) and analyzed by quantitative RT-PCR as previously described (16). Brievly, 500 nanograms of total RNA was reverse transcribed into cDNA with a commercial kit (Qiagen, Hilden, Germany). Human probes for the internal control 18S rRNA (#

4310893E) and human probes for SIRT1 (# Hs00202021_m1) and EPO (# Hs00171267_m1) were obtained from ABI (Applied Biosystems, Rotkreuz, Switzerland). Real-time PCR was performed using ABI PRISM 7000 Sequence Detector System (Applied Biosystems). Mean cycle threshold (C_t) values were calculated for 18S, SIRT1 and EPO. C_t values for SIRT1 and EPO were normalized against the internal control 18S rRNA to calculate Δ C_t values. $\Delta\Delta$ C_t values were calculated by substracting the Δ C_t values of normoxic control cells from the Δ C_t values of hypoxia-exposed cells. Fold change of mRNA expression was calculated using the formula $2^{-(\Delta\Delta Ct)}$.

Protein extraction and Western blotting

Western blot was performed as previously described (17). Briefly, whole cell lysates were prepared by direct lysis in modified radioimmunoprecipitation (RIPA) buffer, proteins were separated by SDS-PAGE. Nitrocellulose membranes were incubated with primary antibodies overnight at 4°C. HRP-conjugated secondary antibodies were incubated for 1 hour at room temperature and membranes were developed with enhanced chemiluminescent substrate from Perkin Elmer (Schwerzenbach, Switzerland).

Primary antibodies were purchased as follows: rabbit polyclonal anti-SIRT1 from Santa Cruz Biotechnology (Heidelberg, Germany) and rabbit anti-β-actin from Sigma (Sigma, Buchs, Switzerland). HRP-conjugated goat anti-rabbit secondary antibody was purchased from DAKO (Dako, Baar, Switzerland).

FACS

Cells and supernatants were harvested and washed in 1 x PBS, fixed in 70% ethanol for 1 hour at -20°C, then resuspended in propidium iodide (PI) solution (50 μg/mL) containing 0.1% Triton X-100, 0.1% sodium citrate and RNAse A (10 μg/mL) (Sigma). Cells were then analyzed by FACS (Becton Dickinson AG, Basel, Switzerland) for PI incorporation. The different cell cycle phase populations (sub-G1 (<G1), G1, S and G2) were determined using FlowJo software (FlowJo, Ashland, OR).

Results and Discussion

We first exposed isolated human primary hepatocytes from 4 individual patients to moderate hypoxic conditions (1.5% O_2) for 12 and 24 hours. Hypoxic hepatocytes did not show any significant difference in SIRT1 mRNA expression compared to normoxic control cells (Fig. 1A). A similar experiment was performed in the hepatocellular carcinoma (HCC) cell line Hep3B, in which exposure to hypoxia (1.5% O_2) did neither reveal a significant change of SIRT1 mRNA expression (Fig. 1B).

The O_2 concentration in human hepatocytes under physiological conditions is dependent on the localization of the cells within the liver. The O_2 concentration is in the range from approximately 13% O_2 in the arterial zone to 5% - 7% O_2 in the perivenous zone (18). However, an O_2 concentration of 1.5% is not uncommon under certain physiological/ pathophysiological conditions in vivo and cells within solid tumors can be exposed to severe hypoxic or anoxic conditions. Therefore, in order to increase the applied stress to the cells, we incubated human primary hepatocytes to severe hypoxic conditions (0.1 % O_2) for 12 and 24 hours. In the cultured hepatocytes, which in average are exposed to higher O_2 concentrations, a prolonged exposure (>24 hours) to severe hypoxia led to cell death as assessed by microscopy (data not shown). We therefore set the maximal time of exposure to severe hypoxic conditions at 24 hours, which was just tolerated by the hepatocytes. Interestingly, even under severe hypoxic conditions we did not observe a change in SIRT1 mRNA expression in human hepatocytes (Fig. 2A). To verify that this lack of effect on SIRT1 mRNA expression upon severe hypoxia was not due to decreased cell viability, we investigated the mRNA expression of erythropoietin (EPO). In human hepatocytes hypoxia was previously shown to induce EPO mRNA expression (19). Consistently, our results show that 24 hours of severe hypoxia induced EPO mRNA expression more than 50-fold compared to normoxic controls (Fig. 2A). With this experiment we demonstrate that hepatocytes were still able to induce hypoxia-regulated genes under the given conditions. A similar experiment was performed in the HCC cell lines Hep3B (Fig. 2B) and HepG2 (Fig. 2C). Since these cell lines derive from human hepatocellular carcinomas, in which the O_2 concentrations are likely to be lower than in normal liver tissue, it is not surprising that these cell lines tolerated longer incubation times under severe hypoxic conditions (data not shown).

Whereas SIRT1 mRNA expression in hypoxic Hep3B cells remained constant, 24 hours of severe hypoxia induced EPO mRNA more than 100-fold (Fig. 2B). Likewise, in HepG2 cells hypoxia did not influence SIRT1 mRNA expression, however, we measured a 5-fold increase of EPO mRNA levels upon hypoxia (Fig. 2C).

Our results clearly demonstrate that even severe hypoxic conditions do not affect SIRT1 mRNA expression. One group investigated the regulation of SIRT1 upon caloric restriction (CR) in mice. The researchers demonstrated that SIRT1 protein is induced in liver during fasting and returned to nearly control levels upon refeeding. It was further shown that SIRT1 mRNA was not regulated (6). The authors demonstrated that pyruvate, which increases upon CR, increases SIRT1 protein synthesis. The finding that nutrient deprivation increases SIRT1 at the protein level and does not influence SIRT1 mRNA, was confirmed by another group (20). The regulatory mechanism of SIRT1 is complicated and multifaceted. SIRT1 can be strongly regulated at the transcriptional, translational as well as the posttranslational level.

To determine possible changes in SIRT1 protein levels in response to hypoxia, we next incubated human hepatocytes under moderate hypoxic conditions for 12 and 24 hours and performed Western blot analysis. SIRT1 protein levels remained constant after 12 and 24 hours of hypoxia (Fig. 3A). This result was surprising, considering that SIRT1 protein increases in response to various cellular stresses including oxidative stress. Another group demonstrated an upregulation of SIRT1 protein in primary human trophoblasts after exposure to similar hypoxic conditions as we used in our experiment (14). In addition, a recent study showed an increase of SIRT1 protein levels upon 3% O_2 concentration. However, in this report two different cell lines were exposed to low oxygen conditions for 5 weeks. They further demonstrated a slight induction of DNA damage upon prolonged hypoxia, which gives an explanation for the moderate increase of SIRT1 protein levels (21). We next investigated the effect of prolonged hypoxia (12, 24 and 48 hours) on SIRT1 protein levels in the p53-deficient cell line Hep3B (Fig. 3B) and in the p53 wildtype cell line HepG2 (Fig. 3C). Similar to our results in human primary hepatocytes, we did not observe any markedly differences in SIRT1 protein levels upon hypoxia in neither of the two HCC cell lines. One interesting finding of this experiment is that we detected similar SIRT1 protein levels in Hep3B and HepG2 cells (Fig. 3B). Since p53

represses SIRT1 transcription and Hep3B cells are p53-deficient, we would have expected elevated SIRT1 levels in Hep3B cells compared to the p53-expressing HepG2 cells. In various p53-deficient cell lines SIRT1 protein levels are increased and p53-/- mice show increased basal expression of SIRT1 in certain tissues (13). However, in these p53-/- mice an applied stress such as nutrient withdrawal did not further elevate SIRT1 levels (13). As mentioned in the introduction the interplay between SIRT1 and p53 is very complex and dependent on many unpredictable factors. Moreover, it is important to note that p53 itself is upregulated in response to hypoxia.

Finally, we treated the p53-deficient Hep3B cell line with sirtinol, a specific small molecule inhibitor of SIRT1. Sirtinol does not affect SIRT1 protein levels, it simply inhibits SIRT1 deacetylase activity (data not shown). Simultaneously to the sirtinol treatment, cells were exposed to hypoxia (1.5% O_2) for 1 to 3 days. FACS analysis by PI staining of Hep3B cells demonstrated that the inhibition of SIRT1 led to a G2-arrest and to a modest increase of the sub-G1 phase (apoptotic cell fraction) (Fig. 4). Interestingly, even under normoxic conditions, SIRT1 inhibition led to a G2-arrest (data not shown). Our results are consistent with the findings of other studies, which have demonstrated growth arrest of cancer cells by SIRT1 inhibition (9, 22, 23). Experiments to assess differences of sirtinol treatment on cell cycle phases under normoxic and hypoxic conditions are currently performed in our lab.

The central mechanism how cells respond to hypoxia, is the induction and stabilization of the hypoxia-inducible transcription factor 1 (HIF-1), which induces a large set of target genes to promote cellular adaptations and survival. NDRG1 is a HIF-1 target gene and is strongly induced upon hypoxia. The role of NDRG1 remains to be fully determined. However, NDRG1 was demonstrated to promote cell survival under hypoxic conditions in certain cancer cell lines. One report suggested a role of SIRT1 in promoting cell survival upon hypoxia through the regulation of NDRG1. When SIRT1 was inhibited with sirtinol, they found a decrease in NDRG1 levels upon hypoxia (24). This result is consistent with our finding in various HCC cell lines and primary hepatocytes, in which the inhibition of SIRT1 led to a decrease of NDRG1 protein (data not shown). As we have shown previously, SIRT1 is required for the stabilization and maturation of HIF-1α protein. Inhibition of SIRT1 led to a decrease

of HIF-1α protein and to a markedly reduced transcriptional activity of HIF-1α (paper submitted, Chapter 2). In our hands, several tested HIF-1 target genes were reduced by the inhibition of SIRT1. We provided evidence that the sirtinol-mediated inhibition of NDRG1 is mediated via the inhibition of HIF-1α protein and the concomitant repression of transcriptional activity.

In conclusion, we did not detect any significant changes of SIRT1 mRNA expression in response to severe hypoxic stress in human primary hepatocytes and two HCC cell lines. SIRT1 protein levels neither showed any markedly differences upon moderate hypoxia. Western blots to assess SIRT1 protein expression in response to severe hypoxic conditions are currently performed in our laboratory. Although, we did not observe an increase of SIRT1 protein upon hypoxia, we provided further evidence that cells require SIRT1 to appropriately respond to hypoxia. Inhibition of SIRT1 led to a growth arrest in Hep3B cells.

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Figure legends

Figure 1. Moderate Hypoxia (1.5% O_2) does not alter SIRT1 mRNA expression
A. Human primary hepatocytes of four different patient livers were exposed to hypoxia (1.5% O_2) for 12 and 24 hours. Controls were kept at normoxic conditions (20% O_2). Total RNA was extracted and RQ-PCR was performed to analyze SIRT1 mRNA expression. *Columns* show the mean fold change of SIRT1 mRNA expression upon hypoxia (12 h and 24 h) compared to normoxic controls (0 h), (n=4). *Bars*, SD. **B.** A similar experiment as described in *A* was done in Hep3B cells, (n=3).

Figure 2. Severe Hypoxia (0.1% O_2) does not change SIRT1 mRNA expression
A. Human primary hepatocytes were exposed to severe hypoxia (0.1% O_2) for 12 and 24 hours. Total RNA was extracted and RQ-PCR was performed to analyze SIRT1 mRNA expression. EPO mRNA expression was analyzed to verify the induction of an established hypoxia-inducible gene upon severe hypoxic conditions. *Columns* show the fold change of mRNA expression upon hypoxia compared to normoxic controls. **B**. A similar experiment as described in *A* was done in Hep3B cells. **C**. HepG2 cells were treated and analyzed as in *A* and *B*.

Figure 3: SIRT1 protein levels remain constant upon prolonged hypoxia (1.5% O_2)
A. Human primary hepatocytes were exposed to hypoxia (1.5% O_2) for 12 and 24 hours. Controls were kept at normoxic conditions (20% O_2). Proteins were extracted and whole cell lysates (120 μg) were analyzed by Western blot for SIRT1. β-actin served as loading control. **B**. A similar experiment as performed in *A* was done in Hep3B cells, which were exposed to hypoxia (1.5% O_2) for the 12, 24 and 48 hours. Whole cell lysates (40μg) were analyzed by Western blot for SIRT1. **C**. A similar experiment as in *B* was performed in HepG2 cells.

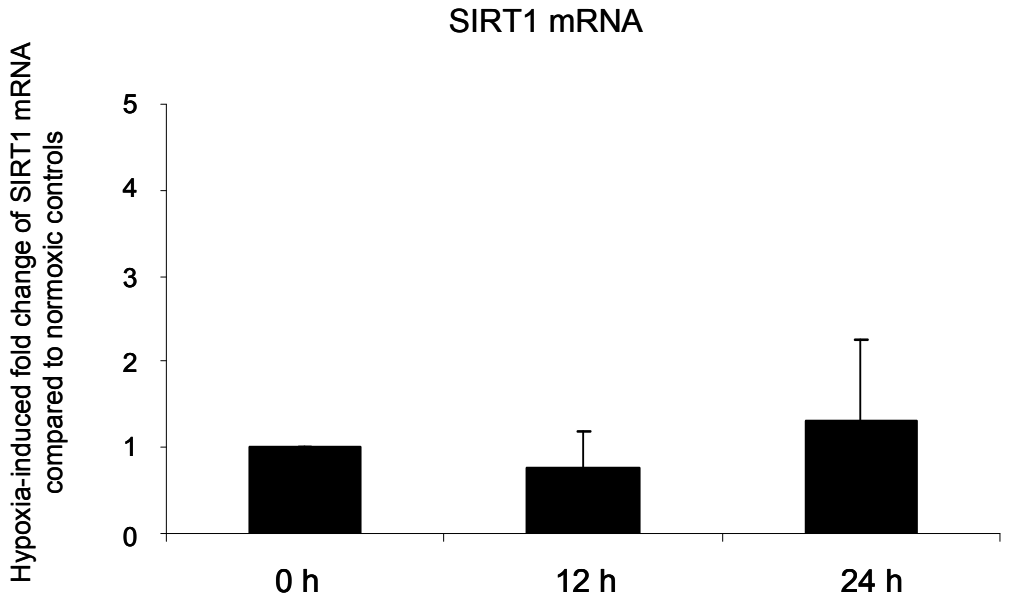
Figure 4: SIRT1 inhibition in hypoxic Hep3B cells leads to G2 cell cycle arrest
Hep3B cells were treated with 100 μm sirtinol (specific SIRT1 inhibitor) for 1d, 2d and 3d and kept at hypoxic conditions (1.5% O_2). Controls were treated with DMSO for

3d while exposing them to hypoxia. Cells were harvested and analyzed by FACS for propidium iodide (PI) incorporation. Columns represent cell cycle phases (sub-G1 (<G1), G2, S and G1) which are indicated in percent.

Figures

Figure 1

A



B

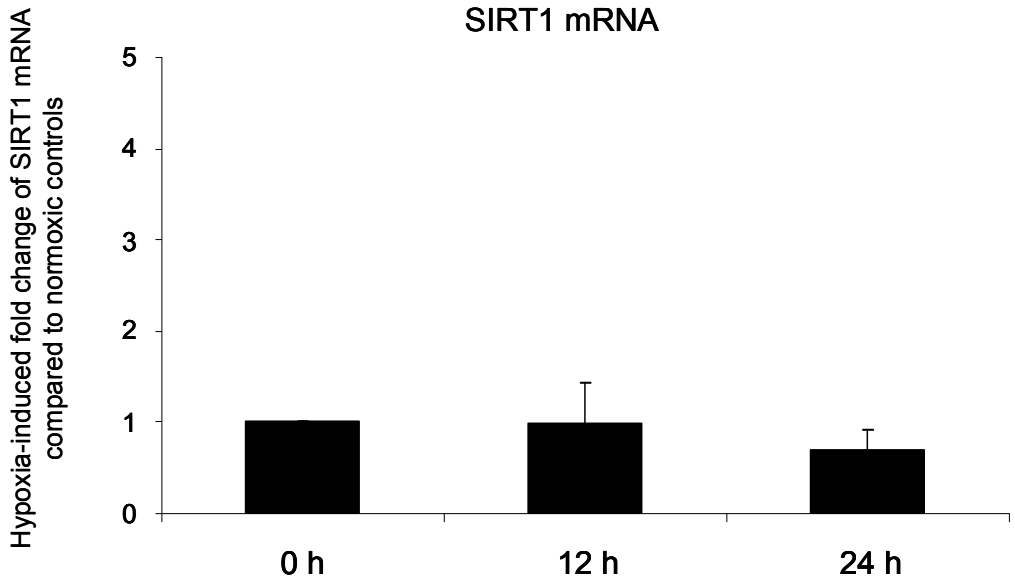


Figure 2

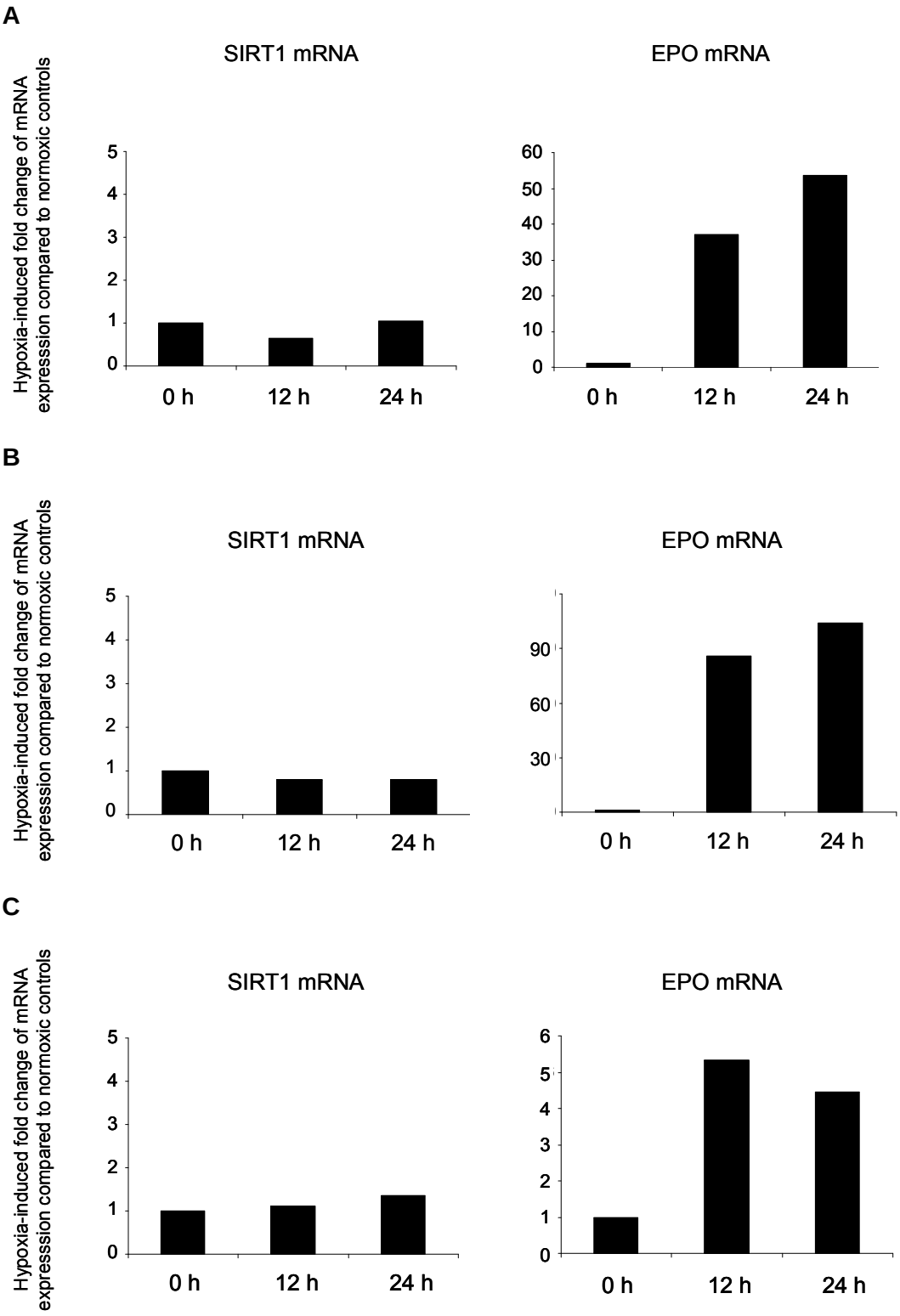

A
SIRT1 mRNA
EPO mRNA
Hypoxia-induced fold change of mRNA expresssion compared to normoxic controls
0 h
12 h
24 h
B
SIRT1 mRNA
EPO mRNA
Hypoxia-induced fold change of mRNA expresssion compared to normoxic controls
0 h
12 h
24 h
C
SIRT1 mRNA
EPO mRNA
Hypoxia-induced fold change of mRNA expresssion compared to normoxic controls
0 h
12 h
24 h

Figure 3

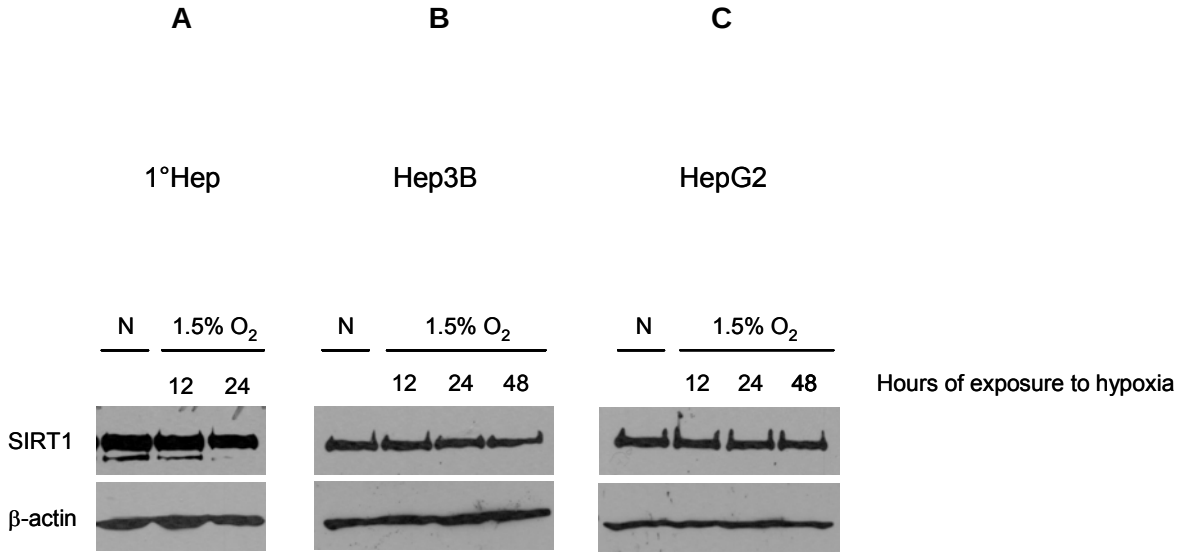
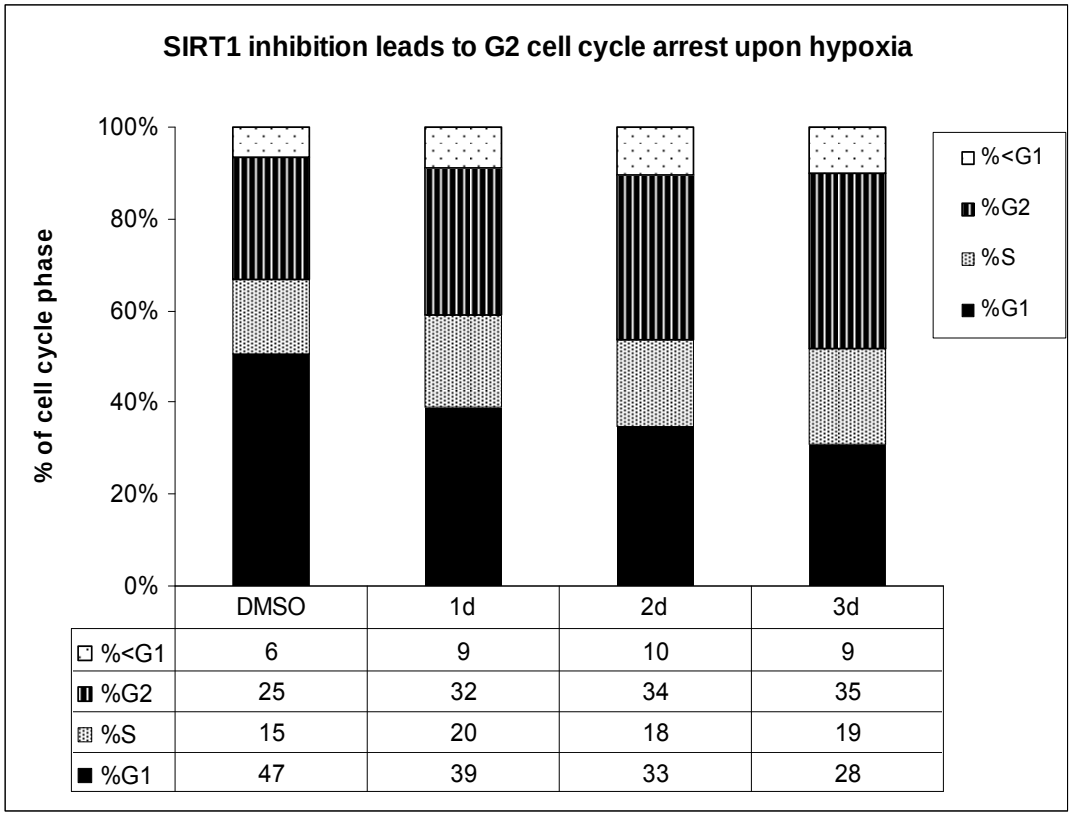

A
B
C
1°Hep
Hep3B
HepG2
N
1.5% O2
12
24
48
Hours of exposure to hypoxia
SIRT1
β-actin

Figure 4



	DMSO	1d	2d	3d
%<G1	6	9	10	9
%G2	25	32	34	35
%S	15	20	18	19
%G1	47	39	33	28

Chapter 4

Discussion and Perspectives

Discussion

Hypoxia is a common feature in solid tumors. Activation of hypoxia-inducible factor 1α (HIF-1α) initiates key cellular adaptations to promote cell survival in the hypoxic tumor microenvironment. Several animal experiments and clinical studies validate HIF-1 as a target for anti-cancer therapy. SIRT1 is a protein deacetylase, which is induced in response to various stress conditions such as calorie restriction, DNA damage, oxidative and oncogenic stress. SIRT1 also induces key cellular adaptations upon these stress conditions and thereby promotes cell survival.

In my thesis, I have focused on the complex interplay of HIF-1α and SIRT1 in hypoxic cancer cells. Given the role of SIRT1 to promote survival in response to stress conditions by deacetylating and regulating key transcription factors and given the central role of HIF-1 to promote survival in response to hypoxic stress, we investigated the effect of SIRT1-mediated deacetylation on HIF-1α stability and transcriptional activity.

Our results demonstrate that SIRT1 deacetylase activity is required for HIF-1α protein stabilization, since chemical inhibition of SIRT1 (with sirtinol) or its knockdown (with shRNA) led to a repression of HIF-1α protein (Chapter 2, Figure 1). Consistent with these data, we showed that SIRT1 inhibition resulted in a dose-dependent inhibition of HIF-1 transcriptional activity. To further verify these results, we investigated the functional consequence of SIRT1 inhibition on the expression of HIF-1 target genes and found a significant reduction of EPO and CA IX mRNA expression (Chapter 2, Figure 1). By coimmunoprecipitation assays we identified the physical interaction of the two endogenous proteins SIRT1 and HIF-1α (Chapter 2, Figure 3). We further provided strong evidence that SIRT1 deacetylates and thereby stabilizes HIF-1α protein, since SIRT1 knockdown resulted in hyperacetylation and consecutive degradation of HIF-1α (Chapter 2, Figure 4). Our findings are in line with the observations of recent studies, which suggested a role of certain class II HDACs in the stabilization of HIF-1α protein (30-33). Several studies consistently reveal that increased acetylation of HIF-1α protein is associated with its proteasomal degradation (30, 32-34). However, different mechanisms were suggested to be involved in this process. Conflicting data in regard of the exact mechanism of how HIF-1α acetylation leads to proteasomal degradation have been reported (reviewed in (15, 16)). The first study that investigated the acetylation of

HIF-1α protein, was demonstrating that an acetyltransferase termed mouse ARD1 (mARD1) associates with human HIF-1α and thereby promotes its acetylation. It was further postulated by the same group that HIF-1α acetylation enhances the binding to VHL (similar to the recognition of prolyl-hydroxylation by VHL), subsequently leading to its ubiquitination and degradation (34). However, several other studies failed to confirm a role for the human variant of ARD1 (hARD1) in the acetylation process of HIF-1α (35, 36). Whereas it is certain that the acetylation of HIF-1α has an important impact on its degradation, it remains to be determined which acetyltransferase(s) perform the acetylation. Another important aspect, which remains to be fully determined is the exact mechanism of how HIF-1α acetylation is linked to its proteasomal degradation. Our results demonstrate that HIF-1α acetylation leads to proteasomal degradation of HIF-1α protein in a VHL-independent mechanism (Chapter 2, Figure 2). This result is in line with the findings of several other studies (30, 31). However, what else could it be, if it is not VHL, which recognizes and mediates ubiquitination and degradation of acetylated HIF-1α protein? A recent report gave a potential answer to this question by demonstrating an O_2/VHL-independent mechanism of HIF-1α ubiquitination and proteasomal degradation that is mediated by a protein termed RACK (37). Interestingly, the same group additionally provided a potential answer to the question, which acetyltransferase mediates acetylation of HIF-1α protein. They demonstrated that an acetyltransferase termed SSAT1 interacts with both HIF-1α and RACK1 and is required for the RACK1-mediated HIF-1α ubiquitination and degradation (38).

SIRT1 promotes lifespan and prevents age-associated diseases under certain circumstances (reviewed in (39)). However, the role of SIRT1 in tumorigenesis remains to be defined (reviewed in (24, 25). SIRT1 is overexpressed in various cancers and its inhibition was shown to induce growth arrest and apoptosis (40-43). In certain tumors SIRT1 seems to be a key promoter of cell survival such as in Burkitt lymphoma xenografts in mice, in which SIRT1 inhibition by a small molecule inhibitor markedly decreased tumor growth (29). Likewise, inhibitors of class II HDACs have displayed antitumoral properties in clinical trials and repressed HIF-1α in cell culture studies (44). Surprisingly, until now no study addressed the role of SIRT1 on HIF- 1α.

In conclusion, we demonstrated that SIRT1 is required for HIF-1α stability and transcriptional activity. SIRT1 inhibition led to hyperacetylation of HIF-1α and to its

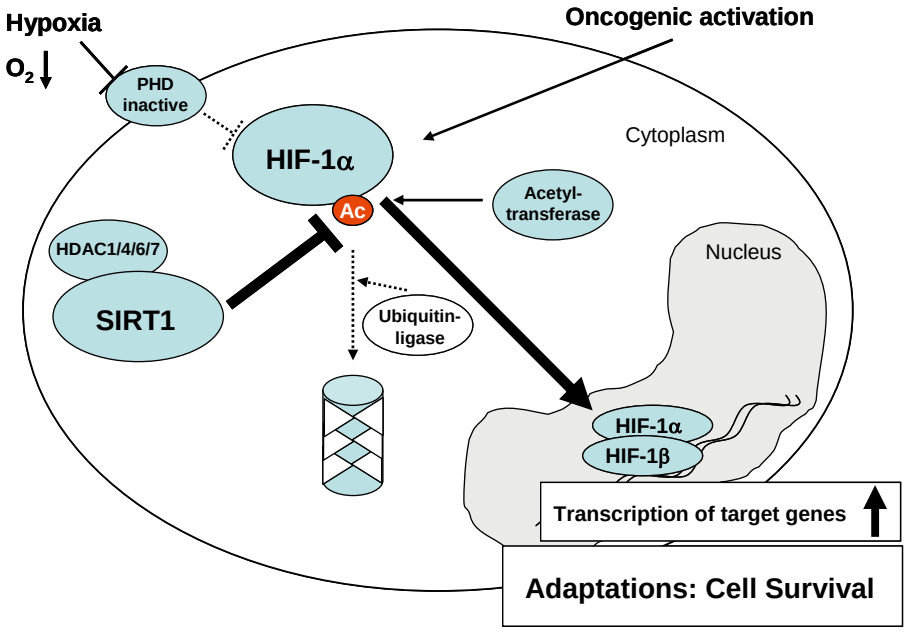
subsequent proteasomal degradation (Figure 1/ Discussion). We have further shown that SIRT1 overexpression, as it exists in certain cancers enhances the hypoxic induction of HIF-1α protein. We identified the physical interaction of SIRT1 and HIF-1α, two proteins that both have a central role in promoting cell survival under conditions of stress (Figure 2/ Discussion). We further found evidence that the inhibition of SIRT1 in cancer cells has an antitumoral effect by the repression of HIF-1α. Importantly, the repressive effect of SIRT1 inhibition on HIF-1α protein seems to be independent of VHL and p53, two tumor suppressors that are frequently mutated in cancers. Further studies to address the potential therapeutic effect of SIRT1 inhibition in HIF-1α expressing cancers are required. Given the function of SIRT1 in regulating various cellular processes such as metabolism (Figure 3/ Discussion), and taking in account its association with longevity, it will be of great importance to selectively inhibit SIRT1 in tumors. Considering the role of SIRT1 and HIF-1α in the cardiovascular system and in metabolism, this synergistic interaction might have implications far beyond the field of tumorigenesis. Future studies will address these questions and possibly provide additional significance of this protein interaction.

Perspectives

Further studies to detail the mechanism of how SIRT1 stabilizes HIF-1α protein are currently being performed in our laboratory. Specifically, we are investigating, which lysine residues of HIF-1α protein are targeted for deacetylation. Another tempting question is which acetyltransferase(s) is (are) targeting HIF-1α. In addition, we are performing in vitro studies to further assess the outcome of SIRT1 inhibition on HIF-1α expressing cancer cells. Finally, using two different models of hepatocellular carcinomas in mice (see List of Publications; Roh, Laemmle, et al.; Dual induction of PKR with E2F-1 and IFN-alpha to enhance gene therapy against hepatocellular carcinoma. *Cancer Gene Ther.* 2008 Oct;15(10):636-44.) we will investigate the role of SIRT1 inhibition in these tumors.

Figure 1| SIRT1 inhibition is a potential strategy to target HIF-1 in cancer therapy

SIRT1 stabilizes HIF-1α protein and is required for the HIF-1-mediated adaptations



SIRT1 inhibition leads to HIF-1α protein hyperacteylation and to its proteasomal degradation

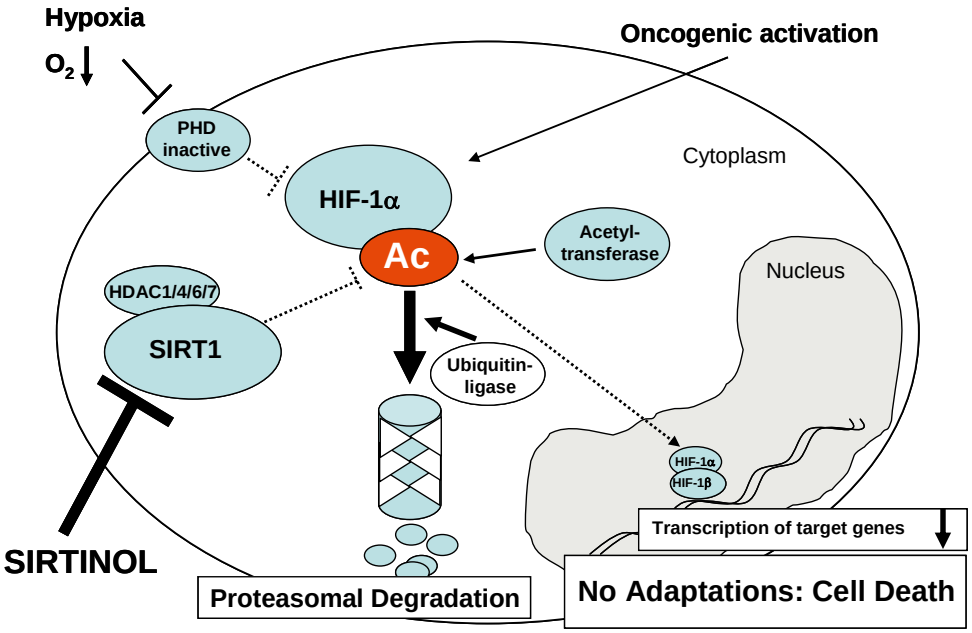


Figure 2A| A proposed model for the function of NDRG1 in hypoxic placental injury

Chen, J Biol Chem. 2006 Feb 3;281(5):2764-72.

Up-regulation of NDRG1 by hypoxia reduces p53 expression, enhances trophoblast differentiation, and thereby attenuates cell injury. SIRT1 is up-regulated in hypoxia and enhances NDRG1 expression. The direct influence of p53 on NDRG1 promoter was shown by Stein *et al.* (61). The influence of SIRT1 on p53 expression was shown by Luo *et al.* (67).

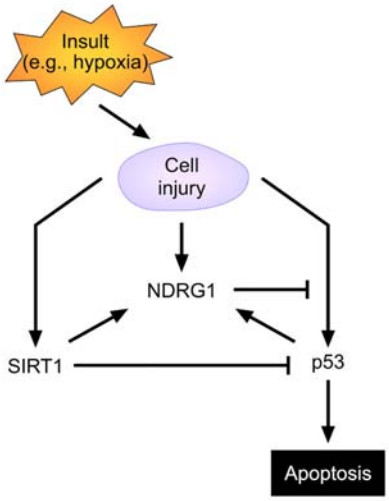


Figure 2B| An adapted integrative model for the key role of HIF-1α and SIRT1 in hypoxia

HIF-1α is induced by hypoxia and initiates the transcription of several target genes (EPO, VEGF, Glut1, CAIX, NDRG1), which promote adaptations and survival in response to hypoxic stress. SIRT1 is not regulated in response to hypoxia in liver cells, however SIRT1 is required for the stabilization of HIF-1α protein.

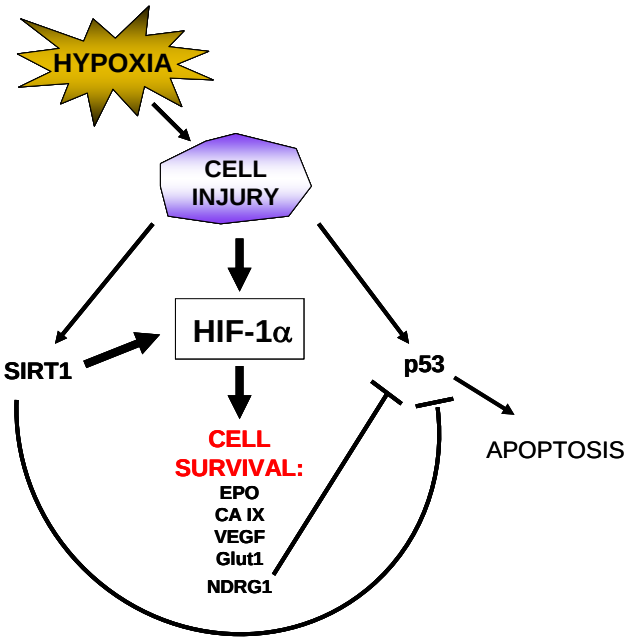
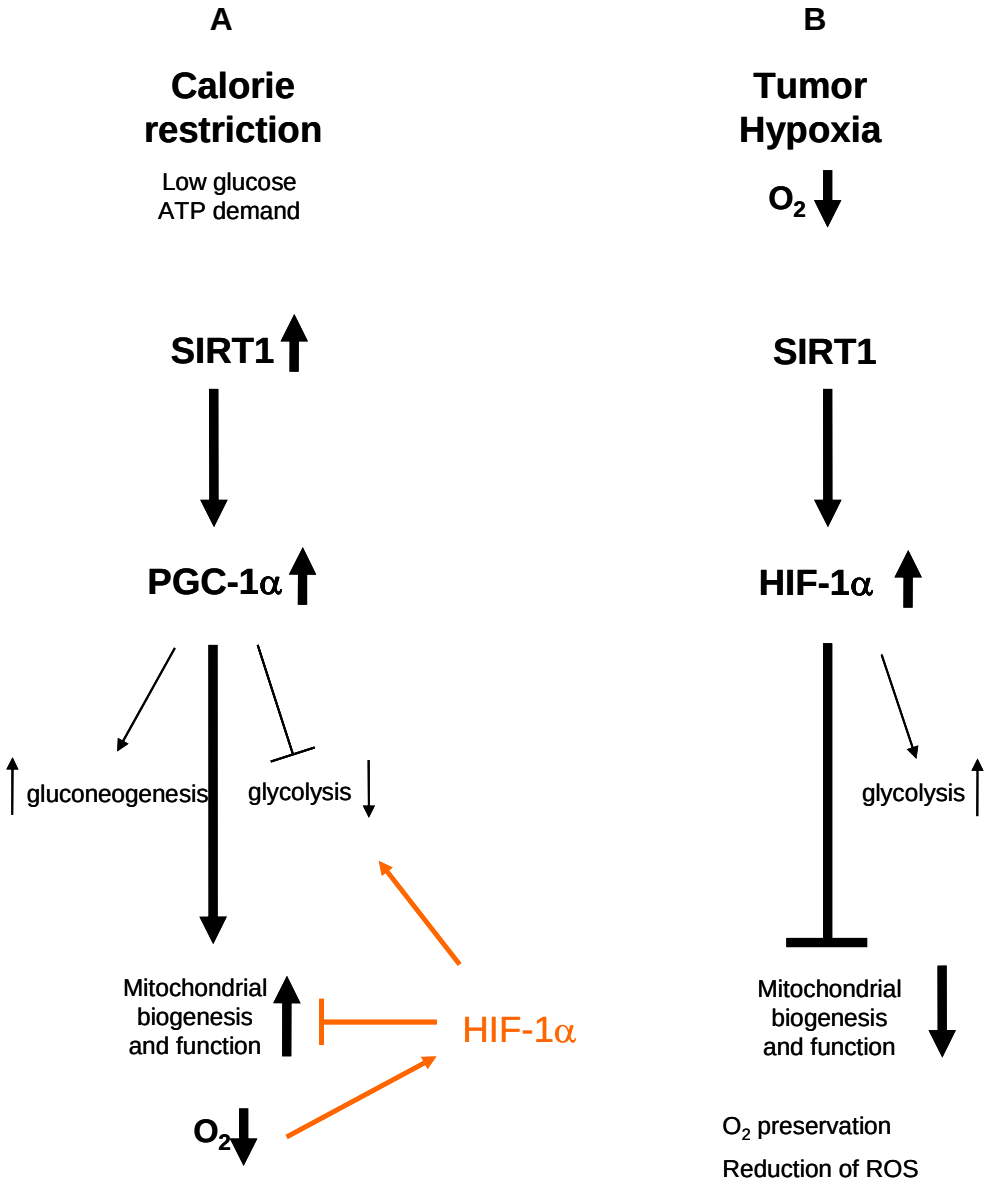


Figure 3| SIRT1 as key regulator of mitochondrial and glucose metabolism - a hypothetical model



A SIRT1 levels increase in response to calorie restriction. SIRT1-mediated deacetylation of PGC-1α increases mitochondrial biogenesis and reduces glucose utilization. Increased oxidative phosphorylation can induce intracellular hypoxia and activation of HIF-1 (red arrows). The SIRT1-mediated adaptations upon calorie restriction are thought to promote longevity. **B** SIRT1 levels remain constant in response to hypoxia. However, SIRT1-mediated deacetylation is required for HIF-1α activation (Chapter 2 and 3). In hypoxia, HIF-1 is inhibiting mitochondrial biogenesis and function, thereby conserving O_2 and reducing the formation of ROS (for more detail, see Chapter 1). This model proposes that SIRT1 regulates metabolic pathways in a specific and stress-dependent manner by regulating different transcription factors (PGC-1α, HIF-1α).

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Appendices

Curriculum Vitae

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Education

1993-1998	High School with Matura Typus E, Gymnasium Kirchenfeld Bern
1998-2004	Medical Studies, University of Bern 1999 1. Propaedeutikum 2000 2. Propaedeutikum 2001 1. part swiss medical licensing exam 2003 2. part swiss medical licensing exam, 1. serial 2004 2. part swiss medical licensing exam, 2. serial and 3. part swiss medical licensing exam
01/2005 – 08/2005	Start of medical dissertation („Gene Therapy in Cirrhotic Livers“) in the group of Dr. S. Vorburger and Dr. D. Stroka, Department of Clinical Research, Laboratory of Visceral and Transplantation Surgery 09/2005: Switch to the MD – PhD doctoral program
09/2005 – 03/2009	MD – PhD doctoral program at the Graduate School, University of Bern Experimental Research in the group of Dr. D. Stroka and Dr. S. Vorburger, Department of Clinical Research, Laboratory of Visceral and Transplantation Surgery, University Hospital Bern, Inselspital Bern

Languages

German	native
English	very good
French	good
Italian	basics
Spanish	basics

Award

Keystone Symposia Scholarship Award
Keystone Symposium: Molecular, Cellular, Physiological, and Pathogenic Responses to Hypoxia; Vancouver, British Columbia, 01/2008

Publications

1. Published

Roh V, **Laemmle A**, Von Holzen U, Stroka D, Dufour JF, Hunt KK, Candinas D, Vorburger SA. Dual induction of PKR with E2F-1 and IFN-alpha to enhance gene therapy against hepatocellular carcinoma. *Cancer Gene Ther.* 2008 Oct;15(10):636-44.

2. Submitted

Laemmle A, Roh V, Tschan MP, Keogh A, Candinas D, Vorburger SA, Stroka D. SIRT1 Physically Interacts with Hypoxia-Inducible Factor-1 alpha (HIF-1α) and Is Required for HIF-1α Protein Stabilization.

Vorburger SA, **Laemmle A**, Strehlen M, Roh V, Wagner M, Winiger I, Dufour JF, Hunt K K, Stroka D, Egger B, Candinas D. Monitoring of tumor growth in animals with serum TIMP-1 (tissue inhibitor of metalloproteinases–1).

Wampfler J, Tschan MP, Shan D, **Laemmle A**, Oppliger Leibundgut E, Baerlocher GM, Stroka D, Fey MF., Britschgi C. SIRT1 is down-regulated during neutrophil differentiation of acute promyelocytic leukaemia cells.

3. In preparation for submission

Laemmle A, Roh V, Keogh A, Candinas D, Vorburger SA, Stroka D. The role and regulation of the NAD^+-dependent protein deacetylase SIRT1 upon hypoxic stress.

Tiffon C, **Laemmle A**, Keogh A, Portmann J, Tschan MP, Candinas D, Stroka D. Inhibition of histone deacetylases induce pancreatic cancer cell differentiation independent of N-myc downstream regulated gene-1.

Roh V, Portmann J, **Laemmle A**, Tschan MP, Candinas D, Vorburger SA, Stroka D. E2F1 regulates NDRG1 in a bimodal manner.

Abstracts

06/ 2008 Oral presentation: Targeting SIRT1 for anti-tumor therapy: Inhibition of SIRT1 down-regulates HIF-1α. Annual Meeting of the Swiss Society of Surgery, Basel, Switzerland, May 2008.

01/ 2008 Poster: Targeting SIRT1 for anti-tumor therapy: Inhibition of SIRT1 down-regulates HIF-1α. Keystone Symposium: Molecular, Cellular, Physiological, and Pathogenic Responses to Hypoxia, Jan 15 - Jan 20, 2008, Vancouver, British Columbia.

09/ 2007 Poster: Targeting SIRT1 for anti-tumor therapy: Inhibition of SIRT1 down-regulates HIF-1α. Inaugural ILCA (International Liver Cancer Association) Annual Conference, 5-7 October 2007, Barcelona, Spain.

06/ 2007 Oral poster presentation: Targeting SIRT1 for anti-tumor therapy: Inhibition of SIRT1 downregulates HIF1α.
Annual Meeting of the Swiss Society of Surgery, Lausanne, Switzerland, June 2007.

04/ 2007 Poster: Dual induction of PKR (dsRNA activated protein kinase) with E2F1 Gene therapy and Interferon alpha to enhance apoptosis in a hepatocellular carcinoma model
Vincent Roh, Alexander Laemmle, Deborah Stroka, Kelly K. Hunt, Daniel Candinas, Stephan A. Vorburger
American Association of Cancer Research, Los Angeles, USA, April 2007.

06/ 2006 Oral presentation: Tumor monitoring in animal models by the tissue inhibitor of metalloproteinases (TIMP-1)
Annual Meeting of the Swiss Society of Surgery, Lugano, Switzerland, June 2006.

List of publications

1. Published / SEE NEXT PAGE

Roh V, **Laemmle A**, Von Holzen U, Stroka D, Dufour JF, Hunt KK, Candinas D, Vorburger SA. Dual induction of PKR with E2F-1 and IFN-alpha to enhance gene therapy against hepatocellular carcinoma. *Cancer Gene Ther.* 2008 Oct;15(10):636-44.

2. Submitted

Laemmle A, Roh V, Tschan MP, Keogh A, Candinas D, Vorburger SA, Stroka D. SIRT1 Physically Interacts with Hypoxia-Inducible Factor-1 alpha (HIF-1α) and Is Required for HIF-1α Protein Stabilization.

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Roh V, Portmann J, **Laemmle A**, Tschan MP, Candinas D, Vorburger SA, Stroka D. E2F1 regulates NDRG1 in a bimodal manner.

Cancer Gene Therapy (2008), 1–9

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ORIGINAL ARTICLE

Dual induction of PKR with E2F-1 and IFN-α to enhance gene therapy against hepatocellular carcinoma

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Overexpression of the transcription factor E2F-1 induces apoptosis in tumor cells. This apoptotic effect is partly mediated through the induction of the double-stranded RNA-activated protein kinase (PKR). Here, we investigate if agents that upregulate PKR could enhance the apoptotic effect of E2F-1 overexpression in liver tumors. In human hepatocellular carcinoma (HCC) cells (Hep3B, HepG2, Huh7), adenovirus-mediated overexpression of E2F-1 (AdCMV-E2F) transcriptionally increased PKR mRNA. The subsequent increase of total and phosphorylated PKR protein was followed by induction of apoptosis. When AdCMV-E2F was combined with the PKR modifier interferon α (IFNα), PKR was additionally upregulated and both PKR activation and apoptosis were increased. Subcutaneous xenograft tumors were selectively targeted using an adenoviral vector expressing E2F-1 under the control of the human telomerase reverse transcriptase (hTERT) promoter (AdhTERT-E2F). Weekly systemic administration of AdhTERT-E2F inhibited tumor growth. The tumor suppressive effect of AdhTERT-E2F therapy was further enhanced in combination with IFNα.Our results demonstrate that PKR activating agents enhance the anti-tumor effect of E2F-1 overexpression in HCC *in-vitro* and *in-vivo*. Hence, modulation of PKR is a potential strategy to increase the efficacy of PKR-dependent anti-tumor therapies.
Cancer Gene Therapy advance online publication, 6 June 2008; doi:10.1038/cgt.2008.34

Keywords: PKR; E2F-1; HCC; interferon

Introduction

E2F-1 is a member of the E2F family of transcription factors that holds a key role in cell-cycle progression through the G1/S checkpoint, by regulating G1 to S phase transition.[1,2] Unlike the other members of the E2F family, E2F-1 exhibits a dual role, as it can also selectively induce apoptosis in tumor cells (reviewed in[3–8]). We previously showed that E2F-1-induced apoptosis is mediated partly through the activation of the dsRNA-activated protein kinase (PKR).[9]

Activation of PKR is a major host defense mechanism against viral infections. Signals produced by viral infection, such as interferon (IFN) or double-stranded RNA induce PKR dimerization, auto-phosphorylation and subsequent activation.[10] A major downstream target of activated PKR is the α subunit of the eukaryotic initiation factor 2 (eIF2α). Phosphorylation of eIF2α impairs protein synthesis at the translation initiation step and results in cell growth arrest and apoptosis.[11,12] PKR has been found to be downregulated in various tumors. Its low expression correlates with worse prognosis and enhanced tumorigenicity, suggesting that activated PKR has a tumor-suppressor function.[13]

Inhibitors of heat-shock protein (HSP)-90 such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) and radicicol have gained recent attention due to their anti-tumoral function and selective activity in malignant cells. These agents, which are currently being tested in phase I clinical trials, are also activators of PKR.[14,15] Another PKR modulator, the polyanion heparin, can mimic the effect of double-stranded RNA leading to autophosphorylation and subsequent activation of PKR.[16,17]

The most prominent activator of PKR is IFN. Interferon stimulates IFN response elements and γ-activated sequences (GAS) within the PKR promoter.[18–20] IFN mainly induces the transcriptional increase of PKR mRNA and protein, while other post-transcriptional modifications regulate PKR activity.[21,22]

PKR plays a central role in IFN-based therapies against hepatitis and resistance to IFN is correlated with lower PKR expression.[23] Worldwide, hepatitis B and C infections are the most important etiologic factors in the

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development of hepatocellular carcinoma (HCC).[24] HCC is the most frequent type of primary liver cancer and has a dire prognosis and low survival probability due, in part, to a lack of efficient anti-tumor therapies.

The purpose of this study was to evaluate if overexpression of E2F-1 induces apoptosis in HCC and if E2F-1 gene therapy inhibits HCC tumor growth. Furthermore, we assessed whether PKR upregulating agents could enhance the effect of E2F-1 overexpression. Ultimately, we tested the combined effect of E2F-1 and PKR upregulation in a mouse xenograft model using an E2F-1 expression vector under control of a tumor-specific promoter (human telomerase reverse transcriptase, hTERT).

Materials and methods

Antibodies and reagents

E2F-1 (KH95) and PKR (K-17) antibodies were purchased from SantaCruz Biotechnology (SantaCruz Biotechnology Inc., Heidelberg, Germany), β-actin[20–33] from Sigma (Sigma, Buchs, Switzerland), phospho-PKR (pT451) from Biosource (Biosource Europe S A, Nivelles, Belgium). HRP-conjugated secondary antibodies were purchased from DAKO for goat anti-rabbit (Dako, Baar, Switzerland) and Pierce for goat anti-mouse (Perbio Science SA, Lausanne, Switzerland). Interferon α (Roferon-A) and Heparin (Liquemin) were obtained from Roche (Roche Pharma AG, Reinach, Switzerland), Radicicol and 17-allylamino-17-demethoxygeldanamycin (17-AAG) from AG Scientific (AG Scientific Inc., San Diego, CA).

Cell culture

Human hepatocellular carcinoma cell lines Hep3B (p53-deleted), HepG2 (p53-wild type) and Huh7 (p53-mutated) were purchased from ATCC (LCG Promochem, Molsheim, France). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Life Technology, Paisley, Scotland) and incubated at 37 °C in a humidified incubator with 5% CO_2.

Vectors

Replication incompetent adenovirus type 5 with deletions in the region E1/E3 were used to overexpress the transgenes E2F-1 and firefly luciferase under the control of the cytomegalovirus promoter CMV (adenovirus-mediated overexpression of E2F-1 (AdCMV-E2F) (from Dr KK Hunt (MD Anderson Cancer Center, Houston); AdCMV-Luc (from Dr Sunil Chada (Introgen Therapeutics, Inc., Houston)). For tumor-selective (telomerase-dependent) transgene expression, we constructed a vector in which the human telomerase reverse transcriptase (hTERT) core promoter was used to control E2F-1 expression (AdhTERT-E2F). In previous experiments with a β-Gal expressing adenovirus, we determined that more than 90% of the cells expressed the transgene when they were infected with a multiplicity of infection (MOI) of 2000 viral particles (vp) per cell (data not shown). The virus particles to plaque forming units (pfu) ratios of AdCMV-Luc, AdCMV-E2F and AdhTERT-E2F virus were: 10 vp/pfu (5.5×10^{12} vp/ml; 5.5×10^{11} pfu/ml), 50 vp/pfu (1.7×10^{12} vp/ml; 3.4×10^{10} pfu/ml) and 92 vp/pfu (4.15×10^{12} vp/ml; 4.5×10^{10} pfu/ml) respectively.

Protein extraction and western blotting

Total cell lysates were prepared by lysis in RIPA buffer (50 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl and 1% NP-40 supplemented with 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 1 × protease inhibitor cocktail (P-8340, Sigma)). Total protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Reinach, Switzerland) and equal amounts (30 μg) of protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% gel. Proteins were transferred to nitrocellulose membrane using a semidry transfer system (Bio-Rad) and blocked with 5% nonfat dry milk in PBS. Primary antibodies were diluted (PKR and pPKR: 1/500, E2F-1 and β-actin: 1/1000) in PBS 5% milk and added to the membrane for overnight incubation. After three washing steps in PBS-0.5% Tween 20, membranes were incubated for 1 h with the secondary HRP-conjugated antibodies (diluted 1/2000 in PBS 5% milk). After another three washing steps, membranes were developed with an enhanced chemoluminescent substrate (LiteAblot, Euroclone SpA, Lugano, Switzerland). For phosphorylated PKR detection, the cells were directly lysed in a solution of RIPA buffer containing 50% Laemmli sample buffer (Bio-Rad). Lysates were sonicated for 5 s and immediately loaded on a gel for SDS-PAGE.

RNA extraction and real time PCR

Total RNA was extracted with TRIzol (Life Technologies, Paisley, Scotland) according to the manufacturer's instructions. One microgram of total RNA was DNase-treated (Promega, Madison, WI) and reverse transcribed into cDNA with a commercial kit (Qiagen, Hilden, Germany). FAM dye-labelled TaqMan probes and polymerase chain reaction (PCR) primers for human PKR were purchased at Applied Biosystems (Rotkreuz, Switzerland). As internal positive control, ribosomal 18S was used with a VIC dye-labelled TaqMan probe. Real-time PCR was performed using ABI PRISM 7000 Sequence Detector System (Applied Biosystems). The amplification conditions were as follows: 40 cycles at 95 °C for 15 s (denaturation step) and 60 °C for 1 min (combined annealing–extension step). Each experiment was carried out in triplicate. Mean cycle threshold (C_t) values were calculated for 18S and PKR. C_t values for PKR were normalized against the internal ribosomal RNA (18S) control probe to calculate ΔC_t values. $\Delta\Delta C_t$ values were calculated by subtracting the ΔC_t values of untreated control cells from the ΔC_t value of treated cells. Fold increase was calculated using the formula $2^{-(\Delta\Delta Ct)}$.

Cell viability assay

AlamarBlue (Biosource Europe) is a redox dye that yields a fluorescent signal in response to a metabolic activity. The assay is based on the ability of metabolically active cells to convert the reagent into a fluorescent indicator (emitting at 590 nm). AlamarBlue was diluted in culture medium to a final concentration of 10%. After 2 hours incubation, AlamarBlue reduction was quantified using a Tecan Infinite 200-plate reader set at excitation and emission 544/590 nm respectively. Cell viability was determined as a percentage of AlamarBlue reduction in the treated cells normalized to the reduction in untreated control cells. All combination treatments have been carried out by adding each vector and the agents into the media at the same time. During the treatment the cells media was not changed. Cell viability was assessed in triplets in at least three independent experiments.

FACS

Cells and supernatants were harvested and washed in 1 × PBS, then resuspended in propidium iodide (PI) solution (50 μg/ml) containing 0.1%Triton X-100, 0.1% sodium citrate and RNAse A (10 μg/ml, Sigma). Cells were analyzed by FACS (Becton Dickinson AG, Basel, Switzerland) for PI incorporation. Sub-G1 phase cell population was determined using FlowJo software (FlowJo, Ashland, OR). FACS analysis was performed with at least three independent experiments.

Mouse model

Experiments were performed according to the National Institute of Health's *Guidelines for the Care and Use of Laboratory Animals* and with the approval of the local Animal Ethics Committee. 32 Hsd:Athymic Nude-*Foxn1*^{nu} mice (male, 4 weeks) were purchased from Harlan (Harlan, Horst, The Netherlands). After 1 week of acclimation, 5×10^5 HepG2 hepatocellular carcinoma cells diluted in 25 μl of medium were injected subcutaneously in the right flank of each mouse. One week after operation, the mice were randomized by 'randomly permutated blocks method' through www.randomization.com and distributed into four groups (PBS, interferon α (IFNα), E2F-1, combination IFNα + E2F). The following systemic treatment was administrated for a period of 7 weeks: animals received weekly tail-vein injections of AdhTERT-E2F (5×10^8 pfu) diluted in 50 μl PBS (control group received PBS only), and/or 40 000 Units (U) of IFNα in 100 μl PBS intra-peritoneally three times per week (control group received PBS only). Tumor growth was evaluated by measuring the tumor diameters in two dimensions (dim1 and dim2), and mean tumor growth per day was calculated with the formula $(((\pi/6) \times [(\text{dim1start} + \text{dim2start})/2]^3) - ((\pi/6) \times [(\text{dim1end} + \text{dim2end})/2]^3))/$ days treatment (start to end (= 49 days)). For direct intratumoral treatment, 20 μl AdCMV-E2F (1×10^7 pfu) or PBS was injected into subcutaneous tumors. This intratumoral treatment was initiated at a mean tumor diameter of 5 mm and continued over a period of 17 days. The human hepatocellular xenografts were harvested and fixed in formalin 4% for 24 h, then incubated in ethanol 70% for another 48 h before embedding in paraffin for immunohistochemistry.

Immunohistochemistry

Paraffin-embedded samples were cut into 5 μm-thin sections for analysis. Deparaffinized sections were microwave-heated for 20 min in 0.01 M Na-citrate, pH 6.4, for antigen retrieval. Endogenous peroxidases were blocked by 0.3% H_2O_2 in methanol for 30 min. Sections were incubated with PKR (K-17) antibody (1/100) overnight at 4 °C. Samples were washed in PBS before incubation with the secondary antibody (biotinylated goat anti-rabbit 1/200, Vectastains (Vector Laboratories Inc., Burlingame, CA)) for 1 h. Sections were further developed with components of the Vectastains Kit (Vector Laboratories Inc.) according to the manufacturer's instructions. Immunoreactivity was developed using 3,3′-diaminobenzidine (Sigma Fast) as the peroxidase substrate and nuclei were counterstained with hematoxylin.

Statistical analysis

Mann–Whitney rank sum tests were performed to assess the significance of all differences observed in the *in-vitro* cell viability assays. The significance of the differences between two treatment groups in the *in-vivo* study was determined with the students *t*-test and difference over all groups was determined by ANOVA. *P*-values below 0.05 were considered to be statistically significant. All *in vitro* experiments were performed at least three independent times.

Results

E2F-1 up-regulates PKR and induces apoptosis in HCC cell lines

To investigate the effect of E2F-1 overexpression in HCC, HCC cell lines were treated with two adenoviral vectors, AdCMV-E2F or AdhTERT-E2F. Cell viability assays demonstrated that overexpression of E2F-1 had a cytotoxic effect in HCC cell lines. 72 h after infection with AdCMV-E2F, the viability of Hep3B, HepG2 and Huh7 cells was reduced to 36, 66 and 33%, respectively, compared to uninfected cells (Figure 1a). When infected with an adenovirus expressing E2F-1 under the control of a tumor-specific, telomerase-dependent promoter (AdhTERT-E2F), the cytotoxic effect was less pronounced, as cell viability was reduced to only 48% (Hep3B), 87% (HepG2) and 84% (Huh7) of the control cells (Figure 1a).

Quantification of propidium iodide incorporation by FACS analysis demonstrated that the sub-G1 cell population increased after E2F-1 overexpression, indicating that cell-death occurred by apoptosis (Figure 1b). In all cell lines, adenovirus-mediated E2F-1 overexpression resulted in a 5- to 10-fold increase of the sub-G1 population. Apoptosis was apparent after 48 h of infection in Hep3B and Huh-7 cells (Figure 1b); however, in HepG2 cells apoptosis was delayed, becoming apparent after 72 h. Further analysis revealed that infection of HCC cells with AdCMV-E2F upregulated PKR protein

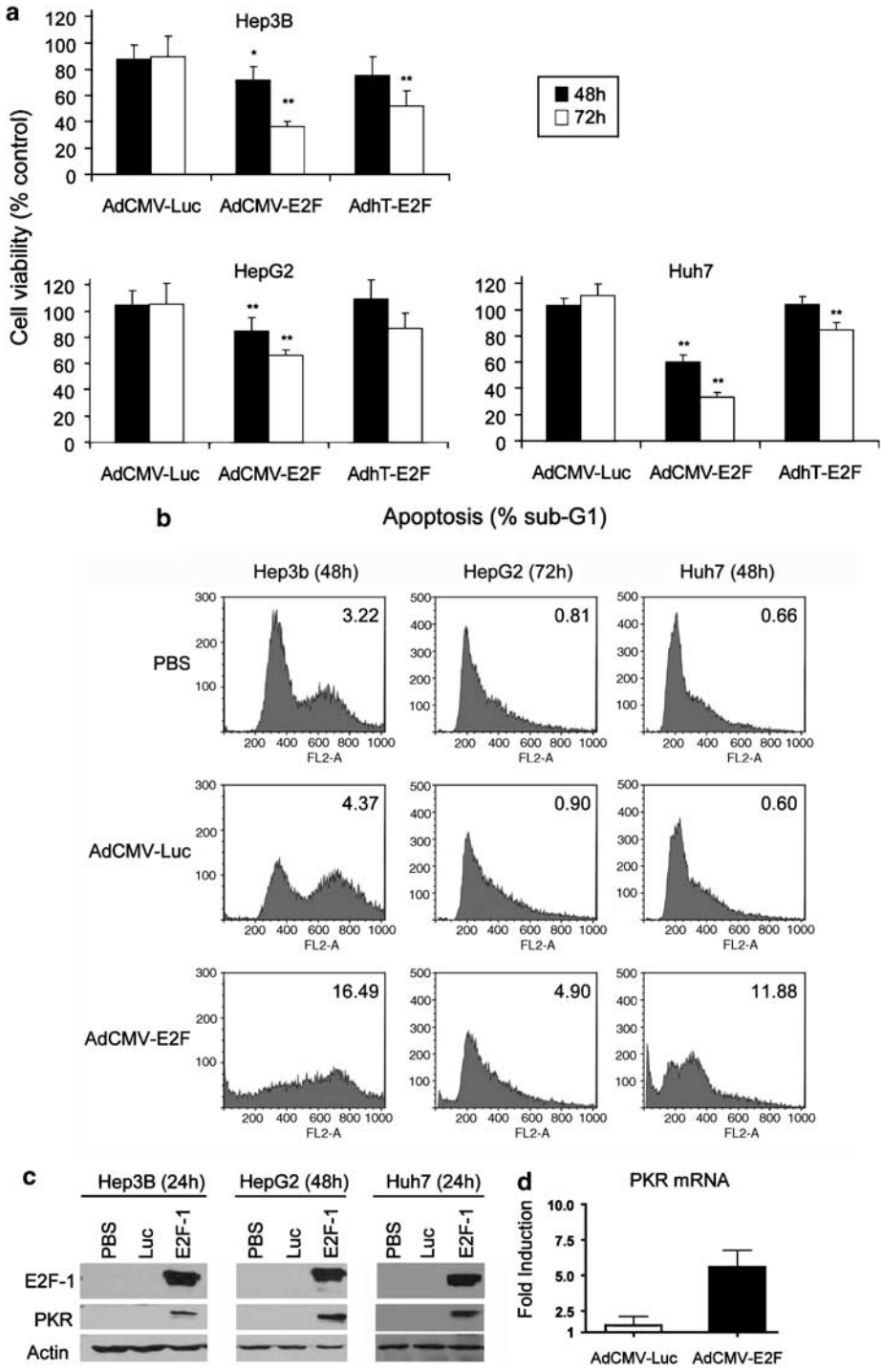

Figure 1 E2F-1 upregulates PKR and induces apoptosis in HCC cells. Hep3B, HepG2 and Huh7 cells were infected with recombinant adenovirus-expressing luciferase or E2F-1 under the control of an unspecific CMV promoter (AdCMV-Luc; AdCMV-E2F) or a telomerase-specific promoter (AdhTERT-E2F) at an MOI of 2000 vp per cell. (**a**) Bars represent cell viability determined by AlamarBlue assays 48 and 72 h after treatment compared to untreated cells (asterisks indicate significant difference to the corresponding luciferase control $*P<0.02$; $**P<0.01$; Mann–Whitney *U*-test). (Data from at least three independent experiments, in triplicates. Standard deviations were plotted as error bars. (**b**) FACS analysis of PI-stained cells 48 h (Hep3B, Huh7) and 72 h (HepG2) after PBS, AdCMV-Luc, or AdCMV-E2F (MOI 1000 vp per cell) treatment. Percentages of sub-G1 phase cells (apoptotic cells) are indicated in the upper right corner of each sample. (**c**) Western blot analysis of E2F-1 and PKR expression in whole-cell lysates 24 h (Hep3B, Huh7) or 48 h (HepG2) after treatment with PBS, AdCMV-Luc or AdCMV-E2F (MOI 2000 vp per cell). (**d**) Bar graph represents relative PKR mRNA levels in Hep3B determined by quantitative real-time PCR 24 h after indicated treatments.

(Figure 1c). Real time PCR analysis confirmed that the increase of PKR protein was due to the increased levels of PKR mRNA (Figure 1d). Treatment with the control luciferase-expressing vector (AdCMV-Luc) did not increase PKR expression. Hence, PKR upregulation was specific to E2F-1 overexpression and independent of the adenoviral vector backbone. PKR protein increased after 24 h in Hep3B and Huh-7 cells (Figure 1c). Consistent with the delay of apoptosis observed in HepG2 cells (Figure 1b), PKR upregulation was also delayed in this cell line, becoming apparent only after 48 h (Figure 1c).

These results show that E2F-1 overexpression in HCC cells result in decreased cell viability due to apoptosis, which is associated with upregulation of PKR, both at protein and at mRNA levels.

PKR-activating agents show a time- and dose-dependent toxicity in HCC cells

Interferon α, heparin and the heat-shock protein 90 inhibitors, 17-AAG and radicicol modify PKR activity.[20,17,25,14] To determine their effect on HCC cells, viability assays were performed in the Hep3B cell line. Concentrations were chosen based on previously reported studies.[25–29] Increasing concentrations of the PKR modifiers radicicol, heparin and 17-AAG reduced cell viability in a time and dose-dependent manner (Figures 2a–c), whereas IFNα showed no cytotoxic effect (Figure 2d).

Combination of E2F-1 overexpression with radicicol or IFNα enhances cytotoxicity in Hep3B cells

We next questioned whether the combination of PKR-modifying agents with E2F-1 overexpression would lead to an enhanced cytotoxic effect in HCC cells. To answer this, the viability of Hep3B cells was measured after treatment with the PKR modifiers radicicol or IFNα, either alone or in combination with E2F-1 overexpression. Treatment with radicicol (0.05 μM) alone resulted in 51% cytotoxicity compared to untreated cells. Overexpressing E2F-1 by AdCMV-E2F or AdhTERT-E2F resulted in a cytotoxicity of 64 and 48%, respectively, which was significantly increased to 75 and 62% when combined with radicicol ($P<0.03$) (Figure 3a). Likewise, combining AdCMV-E2F or AdhTERT-E2F with IFNα (1000 U/ml) significantly increased cytotoxicity to 72 and 55%, respectively ($P<0.03$) (Figure 3b). The increased cytotoxicity obtained by combination with radicicol or IFNα was specific to E2F-1 overexpression and independent of the adenoviral vector backbone, because in cells infected with the luciferase expressing vector (AdCMV-Luc) cytoxicity was not increased. IFNα as a single-agent or in combination with AdCMV-Luc had no significant toxicity; however, IFNα strongly enhanced the cytotoxic effect of E2F-1 overexpression. Taken together, the combination of PKR modifiers with E2F-1 overexpression leads to enhanced cytotoxicity in Hep3B cells.

Increased apoptosis by combination of E2F-1 overexpression with IFNα correlates with higher PKR mRNA and protein levels, as well as enhanced PKR activation

We next wanted to demonstrate that the enhanced cytotoxic effect of E2F-1 and IFNα is correlated with increased apoptosis and PKR activation. Consistent with its cytotoxic effect on Hep3B cells, E2F-1 overexpression

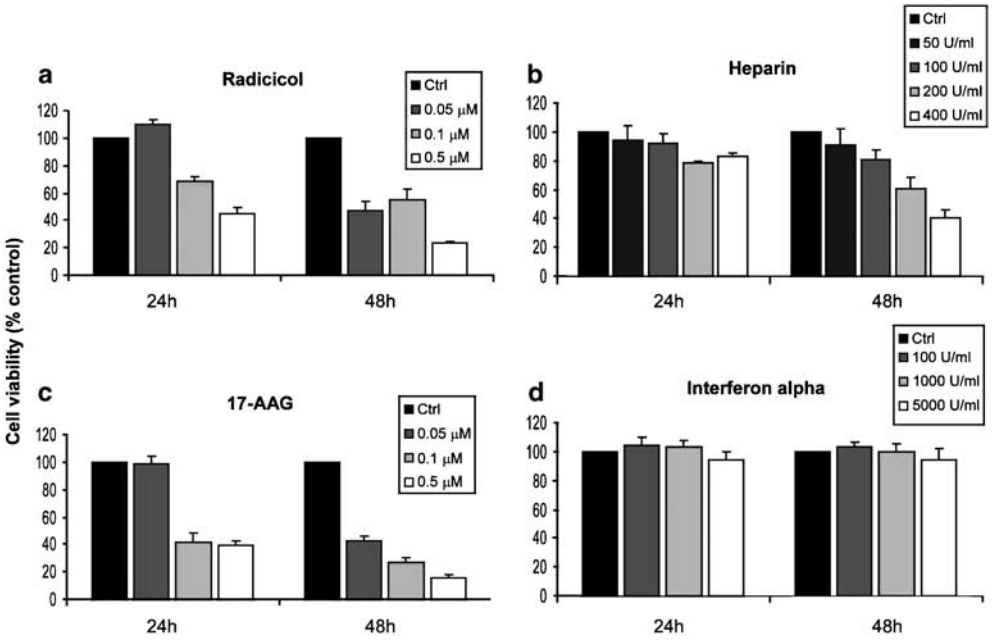

Figure 2 PKR modifiers show dose- and time-dependent cytotoxicity in HCC cells. Bars represent cell viability of Hep3B cells determined by AlamarBlue assay 24 and 48 h after treatment with (**a**) radicicol, (**b**) heparin, (**c**) 17-AAG, or (**d**) IFNα. Cells were treated with indicated agents at the given concentrations. Experiments were done in triplicates and repeated three times.

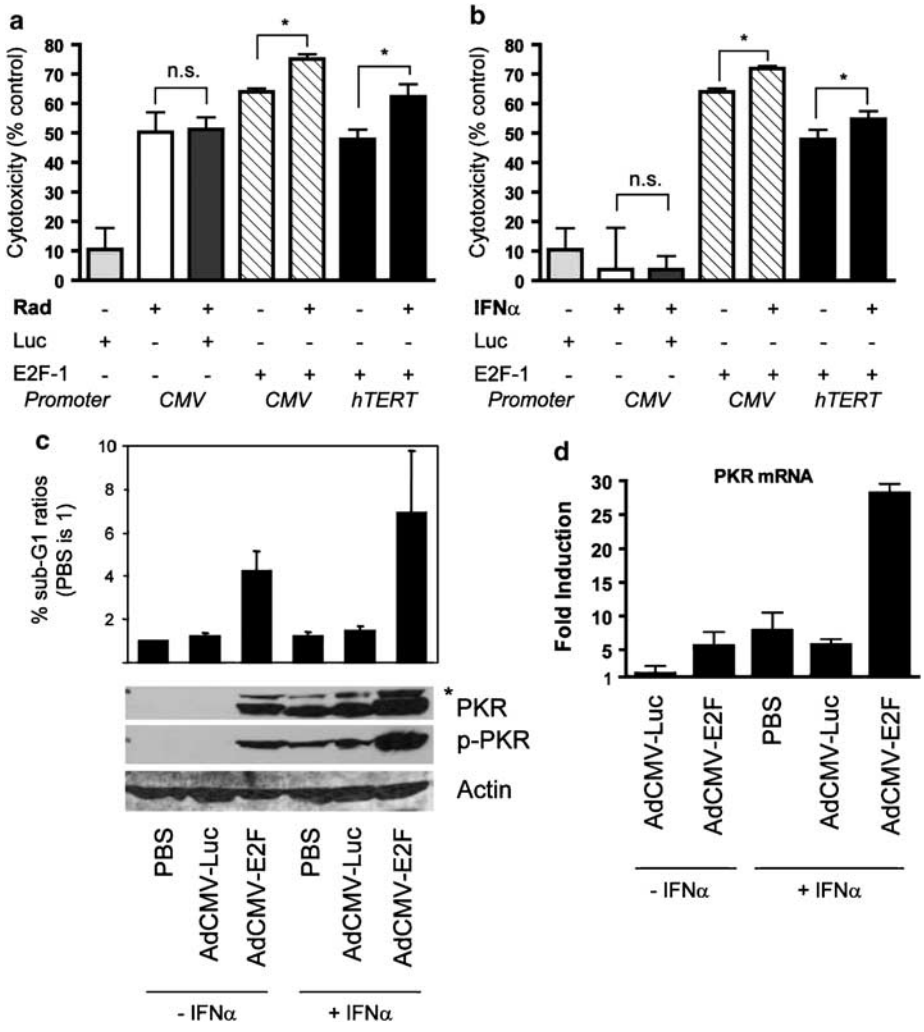

Figure 3 Combination of E2F-1 with radicicol or IFNα enhances PKR upregulation, PKR activation and apoptosis. (**a**) Hep3B cells were infected with AdCMV-E2F, AdhTERT-E2F, AdCMV-Luc (MOI of 2000 vp per cell) or left uninfected (PBS), either alone or in combination with radicicol (0.05 μM) (Rad) (**a**) or IFNα (1000 U/ml) (**b**). Bar graphs indicate cytotoxicity after 72 h as determined by AlamarBlue assay (asterisks indicate significant difference between means ($*P < 0.03$, Mann–Withney *U*-test)). Data from at least three independent experiments are presented. Each point was performed in triplicate, and s.d. were plotted as error bars. (**c**) Bar graph represents the relative increase of cells undergoing apoptosis 48 h after indicated treatments as determined by FACS analysis. The plotted ratios are calculated by dividing the percentage of sub-G1 phase cells in the treatment sample by the percentage of sub-G1 phase cells in the untreated control sample. The graph is representative of three independant experiments. In the lower panel western blots of total cell lysates for total PKR protein and for phosphorylated (active) PKR 24 h after treatments is shown (*corresponds to a residual phosphorylated PKR signal from the initial immunoblotting). (**d**) Bars represent induction of PKR mRNA 24 h after indicated treatments as determined by quantitative real-time PCR (error bars are s.d.).

induced apoptosis, as shown by an increase of the sub-G1 cell population 48 h after infection. E2F-1 overexpression alone resulted in a four-fold increase of the apoptotic fraction. The combination with IFNα further induced this fraction up to a seven-fold increase (Figure 3c, bar graph). IFNα alone or in combination with AdCMV-Luc had no apoptotic effect.

Coherent with its induced apoptosis, E2F-1 overexpression increased both PKR and phospho-PKR protein levels (Figure 3c, western blot). PKR upregulation and PKR activation were further amplified, when E2F-1 overexpression was combined with IFNα. IFNα treatment alone also increased both total and phosphorylated PKR protein; however, at lower levels than after E2F-1 treatment. Therefore, interestingly, IFNα alone did not induce apoptosis despite upregulated PKR protein levels.

PKR protein levels were reflected by PKR mRNA levels after treatment with AdCMV-E2F or IFNα alone or in combination (Figure 3d). When cells were treated with IFNα alone or in combination with AdCMV-Luc, PKR mRNA increased fivefold compared to the PBS control level. The combination of IFNα with AdCMV-E2F resulted in a 27-fold increase of PKR mRNA (Figure 3d).

Hence, we demonstrate that PKR mRNA, PKR protein and activated PKR relate with the extent of apoptosis and cytotoxicity after AdCMV-E2F treatment alone or in combination with IFNα.

Suppression of HCC tumors by E2F-1 gene therapy is enhanced by the combination with IFNα

Next, we studied the anti-tumoral effect of EF2-1 overexpression alone and in combination with IFNα *in vivo*. Preliminary experiments established HepG2 cells as a standard for our HCC tumor xenograft model, because they efficiently develop into subcutaneous tumors when injected into the flank of immunodeficient mice. Therefore, we injected HepG2 cells into nude mice, and the following treatments were initiated 7 days after tumor cell injection and continued for 7 weeks: PBS, IFNα, AdhTERT-E2F or a combination of IFNα and AdhTERT-E2F. Tumor sizes were monitored by measuring the tumor diameters in two dimensions, and mean tumor growth per day was calculated.

Tumors in animals treated with AdhTERT-E2F showed a trend to slower progression (Figure 4a) and smaller tumors at the end of the experiments than in control-treated animals. However, the large variance in tumor growth in the PBS control group did not allow it to reach statistical significance levels. In contrast, treatment with AdhTERT-E2F alone or in combination with IFNα showed a significant suppressive effect on HCC tumor growth compared to IFNα treatment alone ($P = 0.02$). Moreover, these results indicate that the combination therapy was the most effective anti-tumor treatment.

During the course of the experiment no acute or chronic toxicity was observed. This is consistent with previous experiments where systemic AdhTERT-E2F application at doses up to 5×10^9 plaque forming units (pfu) per week was not toxic to the liver (no elevation of liver enzymes) (data not shown).

Combination of adenovirus mediated E2F-1 overexpression with IFNα increases PKR expression in HCC tumors

We used the same subcutaneous xenografted HepG2 tumor model to study PKR expression in tumor tissue. Mice received either PBS or IFNα by intraperitoneal injection and, additionally, AdCMV-E2F or PBS intratumorally. PKR expression in the xenograft tumors was then evaluated by immunohistochemistry. Whereas, PKR was expressed at very low levels in tumors treated with PBS, both IFNα and AdCMV-E2F treatment resulted in increased staining for PKR expression. The combination of intratumoral AdCMV-E2F with systemic IFNα further increased PKR expression in the tumors compared to single-agent treatment (Figure 4b).

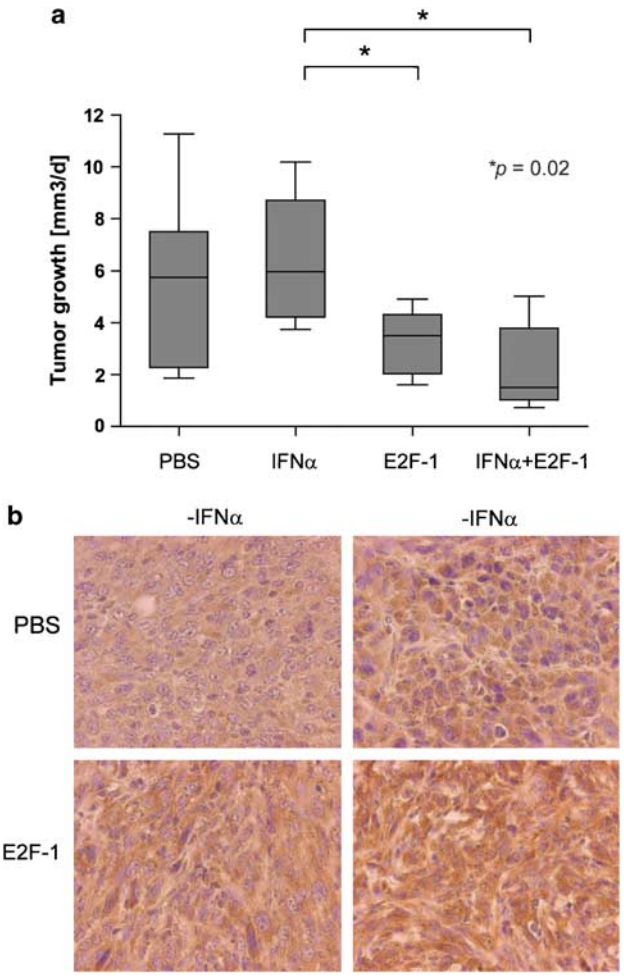

Figure 4 Combination of systemic E2F-1 therapy with IFNα decreases tumor growth and enhances PKR upregulation *in vivo*. HepG2 subcutaneous tumors in nude mice were treated with AdhTERT-E2F (5×10^8 pfu) (E2F-1) or PBS. IFNα (40 000 U), was given as a single-agent or in combination with AdhTERT-E2F (IFNα + E2F-1). Box plots represent tumor growth per day over a period of 7 weeks. Combination treatment group (IFNα + E2F-1), as well as E2F-1 single agent treatment group (E2F-1), were significantly different from IFNα group (*$P = 0.02$, *t*-test). (**b**) Immunohistochemistry for PKR in subcutaneous HepG2 xenografted tumors. Treatment consisted of intraperitoneal injection of PBS or IFNα (40 000 U)/3 × per week, as well as intratumoral injection of AdCMV-E2F (2×10^7 pfu per week) (E2F-1) or a combination of E2F-1 and IFNα for 17 days. Brown cytoplasmic staining shows PKR expression. Counterstaining for the nuclei is blue (magnification × 400).

Discussion

We showed that adenovirus-mediated overexpression of the transcription factor E2F-1 induces cytotoxicity, PKR upregulation and apoptosis in hepatocellular carcinoma *in vitro* and *in vivo*. The less pronounced cytotoxic effect of AdhTERT-E2F compared to AdCMV-E2F can be explained by the weaker transgene expression from the hTERT promoter (10–50% of transgene expression levels reached from the unspecific CMV promoter (data not shown)). In HepG2 cells, a delay in PKR upregulation and induction of apoptosis was observed. This is consistent with studies which showed HepG2 cells to be less sensitive to cytotoxic treatments compared to Hep3B cells (probably due to active p53).[30,31]

We studied various PKR-inducing agents that showed dose-dependent cytotoxicity in HCC cells. The combination of E2F-1 overexpression with the PKR-inducing agents radicicol and IFNα resulted in a strong increase of

cytotoxicity. Although IFNα treatment alone resulted in PKR upregulation and activation (at low levels), no cytotoxicity of IFNα was observed in our experiments. The low cytotoxicity of IFNα is consistent with the existing literature[27] and suggests that PKR upregulation alone is not sufficient to induce apoptosis. However, combination of E2F-1 overexpression with IFNα resulted in a synergistic effect on PKR activation and enhanced apoptosis in HCC cell lines. The *in vivo* experiments with a tumor-specific expression vector further confirmed this enhanced anti-tumor effect of E2F-1 gene therapy in combination with IFNα. Like in our *in vitro* experiments, increased PKR expression in tumors reflected the enhanced effect of E2F-1 with IFNα on HCC growth suppression.

Our findings corroborate the described pro-apoptotic effect of E2F-1 overexpression in various other cell lines and tumors. Previously, we reported that this pro-apoptotic effect is, at least partly, mediated by PKR.[9] Here, we show that additionally increasing PKR expression and activation by PKR-modifying agents lead to increased cytotoxicity of E2F-1 overexpression on HCC *in vitro* and *in vivo*. Noticeably, these effects were not due to interference with the viral vector backbone as combination with a luciferase expressing adenovirus did not result in PKR upregulation or additional cytotoxicity. These findings correlate with an *in vitro* work by Pataer *et al*.[32] showing that treatment of lung cancer cell lines with melanoma differentiation antigen 7 (mda-7) was more efficient in combination with PKR modifiers.

The principle to re-establish, overexpress and activate PKR in tumors is attractive because PKR downregulation *per se* seems to increase tumorigenicity in cancers.[13] In those PKR downregulated tumors, which are often virally induced (like in HCC), the virus-mediated PKR inhibition could be counteracted by the therapeutic effect of E2F-1 in combination with PKR modifiers.[33]

Our findings demonstrate, that PKR modification by agents, that are clinically already in use, is an attractive and effective strategy to enhance E2F-1 gene therapy against cancer. The novelty of this work is that modulating PKR can be used to increase anti-tumor efficiency of PKR-dependent gene therapies.

Abbreviations

E2F-1, E2F transcription factor 1; HCC, hepatocellular carcinoma; IFNα, interferon α; PKR, double-stranded RNA-activated protein kinase.

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Declaration of Originality

Last name, first name: **Laemmle Alexander**

Matriculation number: **98-121-551**

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
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Bern, 31. 03. 2009 Alexander Leo Lämmle