Regulation of the Hypoxia-Inducible Factor-1 by Prolonged and Severe Low Oxygen Tension

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“Love is like oxygen.

You get too much you get too high,

not enough and you're gonna die.”

(The Sweet, 1978)

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Abbreviations

aa	amino acids
Act D	actinomycin D
Akt	protein kinase B
AMC	7-amino-4-trifluoromethyl coumarin
Apaf-1	apoptotic protease activating factor-1
ARD1	activator of RNA decay 1
ARE	adenylate- and uridylate-rich elements
Arnt	aryl hydrocarbon receptor nuclear translocator
asHIF-1α	anti-sense HIF-1α mRNA
ATM	ataxia telangiectasia-mutated
ATP	adenosine triphosphate
ATR	ATM and Rad3-related
AUBP	AU-binding protein
AUF-1	ARE/poly(U)-binding/degradation factor-1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell leucemia
bHLH	basic helix-loop-helix
BNIP3	E1B 19K/Bcl-2 binding nineteen kD interacting protein-3
BSA	bovine serum albumin
CBP	cAMP-response element-binding protein
cDNA	copy DNA
CH1-domain	first cystein-histidine rich domain
CHX	cycloheximide
CITED2	CBP/p300-interacting transactivator with ED-rich tail 2
Cyt C	cytochrome C
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
dNTP	deoxy nucleotides
DTT	1,4-dithiothreit
Earle's MEM	Earle's minimum essential medium

ECL	enhanced chemoluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eIF-4F	eukaryotic initiation factor-4F
ELAV	embryonic lethal, abnormal vision
ELB	elongin B
ELC	elongin C
Epo	erythropoietin
ERK	extracellular signal-regulated kinase
FIH-1	factor inhibiting HIF-1
FRAP	FKBP12/rapamycin-associated protein
GSH	glutathione
GSK3β	glycogen synthase kinase 3β
GSNO	S-nitrosoglutathione
HA	hemagglutinin
HBS	Hepes buffered saline
Hepes	hydroxyethylpiperazinethanesulfonic acid
HIF-1	Hypoxia-inducible factor-1
HRE	hypoxia responsive element
HRP	horseradish peroxidase
Hsp	heat shock protein
HuR	ELAV-like 1 Human antigen R
IAP-2	inhibitor of apoptosis-2
IGF	insulin-like growth factor
IL-1β	interleukin-1β
IPTG	isopropyl-β-D-thiogalacto-pyranoside
IVTT	*in vitro* transcription/translation
JNK	c-Jun NH_2-terminal kinase
LB	Luria Broth
Luc	luciferase
MAPK	mitogen-activated protein kinase
Mdm2	mouse double minute 2
MEK-1	MAP kinase kinase kinase-1

mRNA	messenger ribose nucleic acid
mTOR	mammalian target of rapamycin
NEAA	non-essential amino acids
NF-κB	nuclear factor-κB
NIX	homolog of BNIP3
NLS	nuclear localization sequence
NO	nitric oxide
NOC-18	2,2'-(Hydroxynitrosohydrazino)bisethanamine
NOS	nitric oxide synthase
OD	optical density
ODD	oxygen-dependent degradation domain
2-OG	2-oxoglutarate
ORF	open reading frame
p300	transcriptional co-factor with a molecular weight of 300 kD
p27	cyclin-dependent kinase inhibitors with a molecular weight of 27 kD
p53	tumor suppressor protein with a molecular weight of 53 kD
$p70^{s6k}$	p70 ribosomal protein S6 kinase
PAS	PER-Arnt-SIM
PBS	phosphate buffered saline
PHD	prolyl hydroxylase
PI3K	phosphatidylinositol 3-kinase
PIM	protease inhibitor mix
PMSF	phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog
pVHL	von Hippel Lindau protein
RNI	reactive nitrogen intermediate
ROS	reactive oxygen species
RT-PCR	reverse transcription – polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel
SeAP	Secreted alkaline phosphatase
TAD	transactivation domain
TBE	Tris-Borate-EDTA buffer
TGF-β1	transforming growth factor-β1
TNF-α	tumor necrosis factor-α

TTBS	tris buffered saline with tween
TTP	tristetraproline
Ubi	ubiquitin
URL	unprogrammed reticulocyte lysate
3'UTR	3' untranslated region
VEGF	vascular endothelial growth factor

1 Summary

The hypoxia inducible factor-1 (HIF-1), a heterodimer composed of HIF-1α and HIF-1β, is activated in response to low O_2 tension and serves as the master regulator for cells to adapt to hypoxia. HIF-1 is usually considered to be regulated via degradation of its α-subunit. Recent findings, however, point to the existence of alternative mechanisms of HIF-1 regulation which appear to be important for down-regulating HIF-1 under prolonged and severe O_2 depletion. The aims of my Ph.D. thesis, therefore, were to further elucidate mechanisms involved in such down-regulation of HIF-1.

The first part of the thesis addresses the impact of the severity and duration of O_2 depletion on HIF-1α protein accumulation and HIF-1 transcriptional activity. A special focus was put on the influence of the transcription factor p53 on HIF-1. I found that p53 only accumulates under prolonged anoxia (but not hypoxia), thus limiting its influence on HIF-1 to severe hypoxic conditions. At low expression levels, p53 inhibits HIF-1 transactivity. I attributed this effect to a competition between p53 and HIF-1α for binding to the transcriptional co-factor p300, since p300 overepxression reverses this inhibition. This assumption is corroborated by competitive binding of IVTT-generated p53 and HIF-1α to the CH1-domain of p300 *in vitro*. High p53 expression, on the other hand, affects HIF-1α protein negatively, i.e., p53 provokes pVHL-independent degradation of HIF-1α. Therefore, I conclude that low p53 expression attenuates HIF-1 transactivation by competing for p300, while high p53 expression negatively affects HIF-1α protein, thereby eliminating HIF-1 transactivity. Thus, once p53 becomes activated under prolonged anoxia, it contributes to terminating HIF-1 responses.

In the second part of my study, I intended to further characterize the effects induced by prolonged periods of low O_2, i.e., hypoxia, as compared to anoxia, with respect to alterations in HIF-1α mRNA. Prolonged anoxia, but not hypoxia, showed pronounced effects on HIF-1α mRNA. Long-term anoxia induced destabilization of HIF-1α mRNA, which manifests itself in a dramatic reduction of the half-life. The mechanistic background points to natural anti-sense HIF-1α mRNA, which is induced in a HIF-1-dependent manner, and additional factors, which most likely influence HIF-1α mRNA indirectly via anti-sense HIF-1α mRNA mediated *trans*-effects.

In summary, the data provide new information concerning the impact of p53 on HIF-1, which might be of importance for the decision between pro- and anti-apoptotic

mechanisms depending upon the severity and duration of hypoxia. Furthermore, the results of this project give further insights into a novel mechanism of HIF-1 regulation, namely mRNA down-regulation under prolonged anoxic incubations. These mechanisms appear to be activated only in response to prolonged anoxia, but not to hypoxia. These considerations regarding HIF-1 regulation should be taken into account when prolonged incubations to hypoxic or anoxic conditions are analyzed at the level of HIF-1 stability regulation.

2 Zusammenfassung

Der Hypoxie-induzierbare Faktor-1 (HIF-1), ein Heterodimer bestehend aus HIF-1α und HIF-1β, wird bei vermindertem Sauerstoffpartialdruck induziert und wirkt als Hauptregulator für die Anpassung von Zellen an Hypoxie. Nach gängiger Meinung wird HIF-1 hauptsächlich über den Abbau seiner α-Untereinheit reguliert. Neuere Untersuchungen zeigten jedoch alternative HIF-1 Regulationsmechanismen auf, die wichtig für die Herunterregulation von HIF-1 unter lange andauerndem und starkem Sauerstoffmangel zu sein scheinen. Das Ziel meiner Doktorarbeit war es daher, Mechanismen die in der Herunterregulation von HIF-1 eine Rolle spielen, genauer zu charakterisieren.

Im ersten Teil meiner Arbeit beschäftigte ich mich mit den Auswirkungen des Grades und der Dauer des Sauerstoffentzugs auf die Akkumulation von HIF-1α und die transkriptionelle Aktivität von HIF-1. Dabei habe ich einen Schwerpunkt auf den Einfluss des Transkriptionsfaktors p53 auf HIF-1 gelegt. Ich konnte zeigen, dass p53 nur unter lange andauernder Anoxie, nicht jedoch unter Hypoxie, akkumuliert. Sein Einfluss auf HIF-1 beschränkt sich also auf stark hypoxische Bedingungen. Wenn p53 nur zu einem geringen Maße exprimiert wird, bedingt es eine Hemmung der HIF-1 Transaktivität. Dies kann auf eine Kompetition zwischen p53 und HIF-1α um die Bindung des transkriptionellen Ko-Faktors p300 zurückgeführt werden, da die Überexpression von p300 die Hemmung antagonisierte. Dies wird durch die Beobachtung untermauert, dass in einem Bindungsassay *in vitro* generiertes p53 und HIF-1α um die Bindung an der CH1-Domäne von p300 konkurrieren. Wenn p53 jedoch verstärkt exprimiert wird, beeinflusst es das HIF-1α-Protein negativ, d.h. p53 führt zum pVHL-unabhängigen Abbau von HIF-1α. Daher schliesse ich, dass eine geringe p53 Expression über eine Kompetition um p300 zu einer verminderten HIF-1 Transaktivität führt, während höhere p53 Konzentrationen durch den abbauendend Einfluss auf das HIF-1α Protein zu einer Termination der HIF-1 Transaktivität führen. Daraus folgt, dass p53, wenn es durch andauernde Anoxie aktiviert wird, zur Antagonisierung der HIF-1-Antwort führt.

Im zweiten Teil meiner Arbeit, beschäftigte ich mich mit den Effekten lange andauernder Hypoxie im Vergleich zu Anoxie, wobei das Hauptaugenmerk auf Veränderungen der HIF-1α mRNA lag. Dabei stellte sich heraus, dass andauernde Anoxie, im Gegensatz zu Hypoxie, einen deutlichen Einfluss auf die HIF-1α mRNA hat. Langzeitinkubationen unter

Anoxie verursachten eine Destabilisierung der HIF-1α mRNA, was sich in einer massiven Verringerung der Halbwertszeit zeigte. Die Daten deuten auf einen Mechanismus hin, der zum einen die natürliche anti-sense HIF-1α mRNA, die HIF-1-abhängig reguliert wird, involviert, zum anderen aber weiterer Faktoren bedarf, welche HIF-1α mRNA wahrscheinlich indirekt über anti-sense HIF-1α mRNA in Form eines *trans*-Effekts beeinflussen.

Zusammenfassend liefern die präsentierten Daten neue Informationen über den Einfluss von p53 auf HIF-1. Dieser könnte bei der Entscheidung zwischen pro- und anti-apoptotischen Mechanismen, die in Abhängigkeit von Intensität und Dauer des hypoxischen Stimulus induziert werden, eine Rolle spielen. Darüber hinaus bieten die Ergebnisse dieser Studie weitergehende Einblicke in einen neuen HIF-1-Regulationsmechanismus, nämlich den der mRNA-Herunterregulation unter lange andauernder Anoxie. Dieser Mechanismus scheint nur durch Anoxie, jedoch nicht durch Hypoxie, aktiviert zu werden.

Die beschriebenen Unterschiede zwischen Anoxie und Hypoxie sowohl in Bezug auf p53-vermittelte Effekte auf HIF-1α-Protein und HIF-1-Transaktivität, als auch in Bezug auf mRNA-destabilisierende Einflüsse sollten bei zukünftigen Arbeiten, bei denen hypoxische oder anoxische Bedingungen auf ihren Einfluss auf die HIF-1-Stabilitätsregulation hin untersucht werden, in betracht gezogen werden, um Fehlinterpretationen zu vermeiden.

3 Introduction

3.1 Introductory remarks

It has been increasingly appreciated that the reduced availability of oxygen (O_2), i.e., hypoxia, puts a strong adaptive pressure on the affected individual. This influence manifestes itself to a large degree on the cellular level, i.e., each cell is forced to undergo changes which act towards an increase in the supply of O_2 (e.g., angiogenesis, erythropoiesis), towards an adaptation in the energy metabolism (e.g., glycolysis), and towards a prevention of cell death (Semenza, 2002; Semenza, 2003). Since a major transcription factor involved in signaling under oxygen deficient conditions is the hypoxia-inducible factor-1 (HIF-1), the present study was carried out to further clarify HIF-1 signaling mechanisms involved under prolonged and severe hypoxia.

3.2 Normoxia/Hypoxia/Anoxia

Today's atmosphere contains approximately 21% of O_2, which is considered to be normoxia by most researchers. Even this seemingly simple assumption turns out to be problematic for the work with O_2 depletion, for even though 21% of O_2 is inhaled, the O_2 concentration eventually reaching the individual cell is dramatically lower. For instance, while pulmonary alveoli still receive 16% of O_2, most other organs face a concentration of less than 6% of O_2. Consequently, this implies that "normoxia" refers to different O_2 concentrations for different tissue (Yu *et al.*, 1998).

Most experiments are done in mammalian cells cultured under laboratory conditions (i.e., 37°C, 95% relative humidity, 5% CO_2), which means that they are kept at 21% O_2. This poses a major problem for scientists in this area of research today. The conditions used in normal cell culture should probably be considered to be hyperoxic rather than normoxic. Cells most likely adapt to these conditions in the course of culturing, especially when cell lines are used. These have to be considered a rather sub-optimal system. However, experiments in the cell culture system are still useful to obtain first indications regarding possible pathways involved in intra-cellular signaling in response to O_2 depletion.

The above-mentioned restrictions of an exact definition also apply to hypoxia. Thus, the term hypoxia has been used for O_2 concentrations between 8% (Kietzmann *et al.*, 1999) and 0.1%. Sometimes even 0% of O_2 have imprecisely been termed hypoxia. More

correctly, however, the complete depletion of O_2 should be referred to as anoxia (Jiang *et al.*, 1996b).

To avoid conflicts with the terminology, I used 21% of O_2 for normoxia, 0.5% for hypoxia, and 0% for anoxia in my experiments.

3.3 Hypoxia and the hypoxia-inducible factor 1 (HIF-1)

The lack of O_2 causes a multitude of adaptive processes in the organism. On the cellular level one of the main transcription factors induced is the hypoxia-inducible factor-1 (HIF-1). HIF-1 was first described in 1992 (Semenza and Wang, 1992) and further characterized in the laboratory of Gregg Semenza in the following years (Wang and Semenza, 1993a; Wang *et al.*, 1995a). This transcription factor accumulates under conditions of O_2 depletion and induces adaptive responses (e.g., angiogenesis, increased glycolytic metabolism, erythropoietin synthesis) (Semenza, 2002; Semenza, 2003).

3.3.1 Structure of HIF-1

HIF-1 belongs to the basic helix-loop-helix (bHLH) superfamily of eukaryotic transcription factors (Wang *et al.*, 1995a). More specifically, it belongs to a part of the family which contains an additional auxiliary dimerization site, the so-called PAS domain. This domain was termed after the first three proteins in which it was found, namely the *Drosophila* proteins period (PER) and single-minded (SIM) (Citri *et al.*, 1987; Crews *et al.*, 1988; Burbach *et al.*, 1992) as well as the heterodimerization partner of the dioxin receptor/aryl hydrocarbon receptor (AhR), known as the AhR nuclear translocator (ARNT). The basic domain and the carboxy-terminus of PAS are specifically required for DNA binding of HIF-1, whereas the HLH domain and the amino-terminus of the PAS domains are responsible for dimerization (Jiang *et al.*, 1996a). HIF-1 is a heterodimer composed of one of the three alpha subunits, i.e., HIF-1α, HIF-2α (Iyer *et al.*, 1998b) or HIF-3α (Heidbreder *et al.*, 2003), and the HIF-1β subunit (Wang *et al.*, 1995b) (Figure 1). HIF-1β is constitutively expressed and its activity is not affected by hypoxia (Li *et al.*, 1996). It is identical to the above-mentioned ARNT (Hoffman *et al.*, 1991). HIF-1β does not contain an ODD and is therefore not regulated by oxygen. Nevertheless, it appears to be indispensable for HIF-1 transcriptional activity (Salceda *et al.*, 1996; Kaelin, 2002), which is assumed to depend on its C-terminal transactivation domain (C-TAD) (Corton *et al.*, 1996). HIF-1α constitutes the most prominent member of the 3 α-subunits. It contains

two nuclear localization sequences, one amino-terminal (aa 17 –33) in the bHLH domain and one carboxy-terminal (aa 718–721), which directs it to the nucleus upon accumulation (Kallio *et al.*, 1998). Its stability is largely regulated via an oxygen-dependent degradation domain (ODD) (aa 429-608) (Huang *et al.*, 1998). The transcriptional activity of HIF-1 is mediated mainly via two transactivation domains (TADs) of HIF-1α, the N-terminal TAD (N-TAD), located between amino acids 531 and 575, and the C-TAD, located between amino acids 786 and 826 (Ruas *et al.*, 2002). These TADs, besides being essential for interaction with transcriptional co-activators such as cAMP-response element-binding protein (CREB)-binding protein (CBP)/p300 (Lando *et al.*, 2002b), are targets for regulation via post-translational modifications such as phosphorylation (Richard *et al.*, 1999; Suzuki *et al.*, 2001), acetylation (Jeong *et al.*, 2002), and/or redox modifications (Huang *et al.*, 1996).

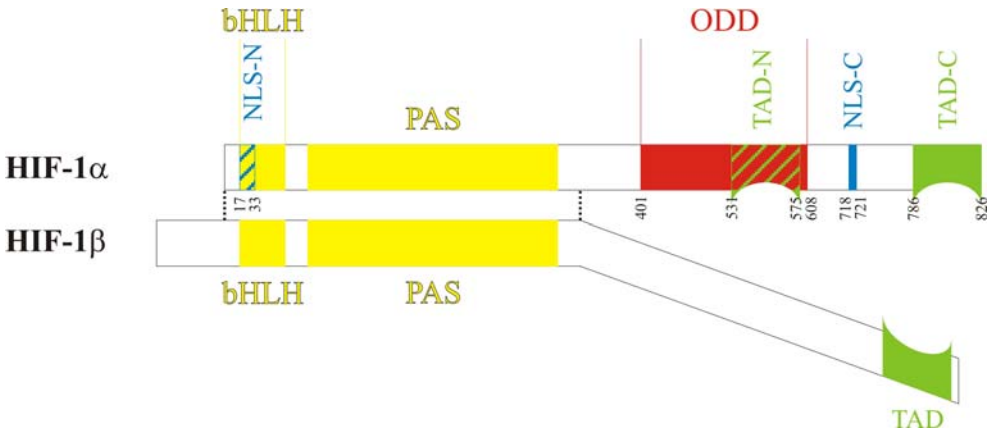

Figure 1: HIF-1 structure

The basic helix-loop-helix (bHLH) and the PER-ARNT-SIM (PAS) domains of HIF-1α and HIF-1β, indicated in yellow, are required for dimerization and DNA binding. In addition, HIF-1α contains an amino-terminal and a carboxy-terminal nuclear localization signal (NLS, blue). Regulation of the α-subunit is mediated by the oxygen dependent degradation domain (ODD, red), which contains two regulatory proline residues. Transcriptional activity of HIF-1 is mediated by two transactivation domains (TADs) in HIF-1α and by one in HIF-1β (green). The indicated numbers represent the amino acid residues at the end of the respective domains.

HIF-2α expression, which is also induced by hypoxia (Wiesener *et al.*, 2003), appears, however, to play a major role in certain cell types only (e.g., macrophages, endothelial cells). The cell-type specific pattern of HIF-1α and HIF-2α expression might allow for

differential regulation. The proposed differences in regulation remain yet to be completely elucidated. It has been described, though, that HIF-2α plays an important role in tumorgenesis of certain cancers (e.g., non-Hodgkin lymphoma, bladder cancers) (Talks *et al.*, 2000; Leek *et al.*, 2002; Stewart *et al.*, 2002). HIF-3α lacks a C-TAD and was described to be a negative regulator rather than an additional transcriptional partner for HIF-1β (Hara *et al.*, 2001; Makino *et al.*, 2001; Makino *et al.*, 2002).

3.3.2 Classical regulation of HIF-1

The presence of HIF-1 has been proposed to be mediated rather exclusively via stability regulation of its α-subunit. Under normoxic conditions, HIF-1α protein is constitutively expressed and synthesized. At the same time it is degraded rapidly, while HIF-1β is constitutively present. HIF-1α degradation has been shown to be mediated by a family of specialized enzymes which are thought to be the oxygen sensor of the cell (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). These enzymes, which are orthologs of *C. elegans* Egl-9, are designated as prolyl hydroxylase domain-containing enzymes (PHD), i.e., prolyl hydroxylases. To date, four isoforms have been characterized, named PHD1, PHD2, PHD3, and PHD4 (Bruick and McKnight, 2001; Epstein *et al.*, 2001; Oehme *et al.*, 2002). The activity of the PHDs depends on the availability of O_2 and the co-factors iron (Fe^{2+}) and 2-oxoglutarate (2-OG) (Hirsila *et al.*, 2003). Thus, if O_2 and the co-factors are present, the PHDs are able to hydroxylate HIF-1α at two distinct proline residues (Pro 402 and Pro 564) (Huang *et al.*, 2002). A very recent report identified a leucine residue (Leu 574) as being essential for PHD recruitment to hydroxylate the proline residues as well (Kageyama *et al.*, 2004). Subsequently, an E3-ubiquitin ligase complex that contains the von Hippel Lindau protein (pVHL), elongin B (ELB), elongin C (ELC), CUL2, and RBX1 forms hydrogen bonds with the hydroxylated proline residues of HIF-1α (Pereira *et al.*, 2003) and ubiquitinates it for degradation. Eventually, poly-ubiquitinated HIF-1α is recognized and degraded by the 26S proteasome (Figure 2A) (Salceda and Caro, 1997; Kallio *et al.*, 1999; Maxwell *et al.*, 1999).

In contrast, O_2 deficiency leads to an inhibition of the PHDs, which prevents pVHL binding and ubiquitination of HIF-1α. Consequently, HIF-1α accumulates due to a decrease in proteasomal degradation (Figure 2B) (Maxwell et al., 1999). A very recent report also indicates that the PHDs are not only inhibited on the activity level, but are also down-regulated on the protein level under hypoxia via the E3-ligase Siah2 (Nakayama *et*

al., 2004). In addition to regulating HIF-1α protein stability, O_2 affects DNA binding and transcriptional activation. This mechanism involves hydroxylation of a critical asparagine residue (Asn 803) within the C-TAD of HIF-1α (Jaakkola *et al.*, 2001; Lando *et al.*, 2002b) and is mediated by a specific asparagine hydroxylase, the factor inhibiting HIF-1 (FIH-1) (Mahon *et al.*, 2001; Hewitson *et al.*, 2002; Lando *et al.*, 2002a). This hydroxylase again requires the presence of O_2 and Fe^{2+}. Under normoxia, hydroxylation at this position leads to steric inhibition of the interaction between the transcriptional co-activator CBP/p300 and HIF-1α (Dames *et al.*, 2002; Freedman *et al.*, 2002). Consequently, HIF-1 transcriptional activity is increased (Sang *et al.*, 2002) under hypoxia, due to the loss of hydroxylation, which allows for formation of the transcriptional complex and subsequent expression of target genes that contain hypoxia responsive element (HRE) sites with the core DNA sequence 5'-RCGTG-3' (Kaelin, 2002; Masson and Ratcliffe, 2003).

It has recently been proposed that prolonged periods of O_2 deficiency activate feed-back mechanisms decreasing HIF-1α protein again (Berra *et al.*, 2001; Berra *et al.*, 2003). This has been ascribed to attenuated protein kinase B (Akt) phoshporylation and subsequent glycogen synthase kinase 3β (GSK3β) (Mottet *et al.*, 2003) or forkhead transcription factor FOXO4 activation (Tang and Lasky, 2003). Alternatively, up-regulation of HIF-1-PHDs may limit HIF-1α protein amount (Epstein *et al.*, 2001; Berra *et al.*, 2003; Cioffi *et al.*, 2003; D'Angelo *et al.*, 2003). As mentioned above, short-term hypoxia, on the contrary, has been reported to down-regulate the PHDs via an E3-ligase (Nakayama *et al.*, 2004).

The above-mentioned mechanisms of regulation provide researchers with multiple possibilities for experimental intervention. Thus, current studies often make use of chemicals mimicking hypoxia, i.e., chemicals accumulating and activating HIF-1 under normoxic conditions. These "chemical hypoxia mimics" include transition metals (e.g., cobalte chloride) (Huang *et al.*, 2003; Yuan *et al.*, 2003), iron chelators (e.g., desferroxamine) (Wang and Semenza, 1993b), as well as substances substituting 2-oxoglutarate (e.g., dimethyloxalglycine) (Mole *et al.*, 2003; Zhao *et al.*, 2004). All of these compounds eventually target the hydroxylases, i.e., they inhibit the activity of FIH-1 as well as the PHDs. In addition, during the last years an increasing number of (patho-) physiological stimuli has been identified as accumulating and/or activating HIF-1 also under normoxic conditions (Stroka *et al.*, 2001). In parallel to the characterization of new HIF-1 inducing agents, a more detailed description of the mechanisms regulating HIF-1 has been established.

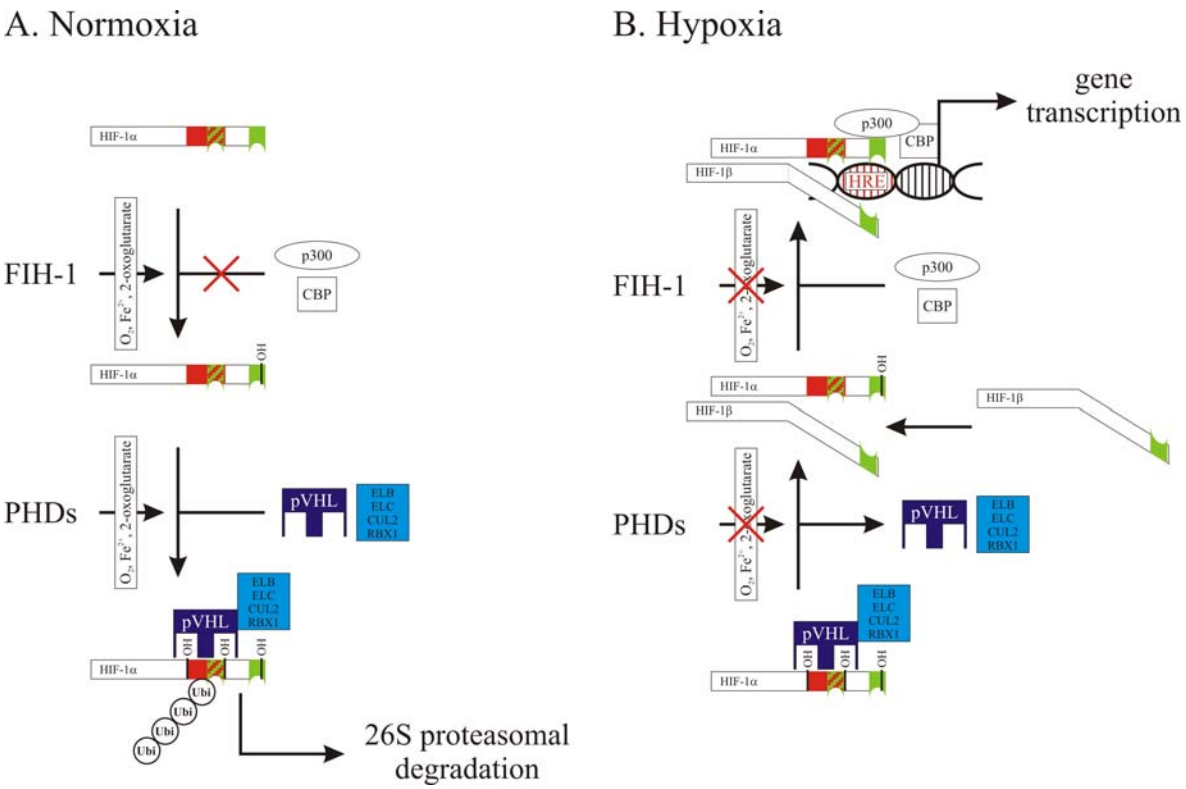

Figure 2: HIF-1α regulation

(A) Under normoxia, i.e., conditions where O_2, Fe^{2+}, and 2-OG are available, the hydroxylases (FIH-1 and PHDs) become active. FIH-1 hydroxylates Asn 803 in the C-TAD of HIF-1α. This modification causes CBP/p300 to dissociate from HIF-1α, thus inactivating HIF-1. The PHDs hydroxylate two proline residues (Pro 402 and Pro 564) within the ODD of HIF-1α marking it for binding of pVHL. pVHL forms a E3-ubiquitin ligase complex with co-factors (ELB, ELC, CUL2, RBX1), which allows for subsequent poly-ubiquitination of HIF-1α. Ubiquitination traditionally marks proteins for degradation by the 26S proteasome. (B) In contrast, if O_2, Fe^{2+}, and/or 2-OG are missing (e.g., under hypoxia), the PHDs become inactive. Thus, HIF-1α accumulates and binds to the β-subunit. Since FIH-1 is inactive under these conditions as well, HIF-1 is able to recruit the transcriptional co-activator CBP/p300 again, which allows for expression of HIF-1 responsive genes, i.e., genes which contain HIF-responsive elements in their promotor region.

Since HIF-1β is equivalent to ARNT, a possibility of competition between hypoxia (HIF-1)- and dioxin-regulated signal transduction (dioxin receptor) has been described lately (Gradin *et al.*, 1996), thus indicating a mechanism which is actually regulated by HIF-1β rather than by HIF-1α availability.

3.3.3 Alternative regulation of HIF-1

Current work supports the hypothesis that HIF-1 regulation might not be quite that straightforward, i.e., other regulatory pathways seem to be involved under certain, if not all conditions. Thus, hypoxia is assumed to induce HIF-1 not exclusively via inhibition of the hydroxylases. For instance, a recent report nicely demonstrates that acetylation of a lysine-residue (Lys 532) within the ODD of HIF-1α by the acetyltransferase ARD1 is critical for proteasomal degradation of HIF-1α (Jeong *et al.*, 2002), i.e., acetylation is shown to increase the interaction between HIF-1α and pVHL.

3.3.3.1 Alternative regulators

Heat shock proteins

Only recently, it has also been shown that the molecular chaperones of the heat shock protein (hsp) family play a role in HIF-1 stabilization. Initially, it was reported that unphosphorylated HIF-1α accumulates in a hsp90-dependent manner under thermal stress (Katschinski *et al.*, 2002). In follow-up studies, hsp90 was described to interact with the PAS domain of HIF-1α, thereby preventing pVHL-independent degradation of HIF-1α (Hur *et al.*, 2002; Isaacs *et al.*, 2002; Mabjeesh *et al.*, 2002). Hsp90- and hsp70-mediated stabilization of HIF-1 requires an active phosphatidylinositol 3-kinase (PI3K) pathway (Zhou *et al.*, 2004). Interestingly enough, binding of hsp70 to HIF-1α was reported to take place in the ODD, though. Both hsps were up-regulated by hypoxic conditions. Similarly, HIF-1β, when overexpressed, was able to prevent pVHL-independent degradation without affecting pVHL-dependent degradation (Isaacs *et al.*, 2004). This effect was attributed to substitution of hsp90 at the PAS domain.

Cytokines

Other important HIF-1 inducers include cytokines. TNF-α, for example, has been described to accumulate and activate HIF-1 (Hellwig-Burgel *et al.*, 1999; Sandau *et al.*, 2001b; Zhou *et al.*, 2003a). HIF-1α was shown to accumulate in an ubiquitinated form under these conditions, i.e., it bound to the pVHL E3-ligase complex, but still localized to the nucleus, where it remained transcriptionally active (Zhou *et al.*, 2003b). This is in accordance with previous reports suggesting that HIF-1 can be active even if ubiquitinated (Hellwig-Burgel *et al.*, 1999; Sandau *et al.*, 2001a). Mechanistically, this has been attributed to a nuclear factor-κB (NF-κB)-dependent increase in HIF-1α translation, rather

than to an inhibition of the degradation (Jung *et al.*, 2003; Zhou *et al.*, 2003b). Other reports proposed reactive oxygen species (ROS) to be involved in TNF-α-induced HIF-1 accumulation (Haddad and Land, 2001), but the exact effect of ROS in stabilizing or destabilizing HIF-1α is still controversially discussed (Albina *et al.*, 2001; Sandau *et al.*, 2001b). The effects of yet another inflammatory mediator, namely nitric oxide (NO), on HIF-1 accumulation and activation is also controversially discussed in the literature. The controversies about this special molecule are due to its ambivalent characteristics with respect to HIF-1. On the one hand it has been described to accumulate and activate HIF-1 under normoxia (Kimura *et al.*, 2000; Sandau *et al.*, 2000; Sandau *et al.*, 2001a; Sandau *et al.*, 2001b; Metzen *et al.*, 2003b; Zhou *et al.*, 2003a), on the other hand hypoxia-induced HIF-1 activation was attenuated by NO treatment (Liu *et al.*, 1998; Sogawa *et al.*, 1998; Huang *et al.*, 1999). NO-induced HIF-1 accumulation was attributed to either inhibition of the PHDs (Wang *et al.*, 2002; Metzen *et al.*, 2003b) and/or to genistein-sensitive PI3K-dependent increase of HIF-1α translation (Kasuno *et al.*, 2004). Thus, even if the exact mechanism awaits further characterization, it potentially is a combination of blocked degradation and increased expression. A more detailed analysis revealed that different NO donors (e.g., GSNO vs. NOC-18) might involve different mechanisms of regulation and, furthermore, that even differences in the concentration of the individual NO-donor result in different physiological outcomes (Palmer *et al.*, 2000). One mechanism which might be of special importance with respect to NO-induced effects on HIF-1 is the formation of reactive nitrogen intermediates (RNI). These RNIs are proposed to be involved in S-nitrosylation of HIF-1α, resulting in its stabilization, but the biological significance of this HIF-1α stabilization remains unclear (Gaston *et al.*, 2003). In addition, NO donors might induce formation of ROS, which again might influence HIF-1 accumulation similarly to hypoxia-induced ROS formation (Genius and Fandrey, 2000). Only very recently, it has been proposed that NO might induce HIF-1α accumulation by affecting its translation via PI3K or mitogen-activated protein kinase (MAPK) pathways (Kasuno *et al.*, 2004; Thomas *et al.*, 2004). Since hypoxia has been shown to stimulate NO synthases (NOSs) (Palmer *et al.*, 1998; Jung *et al.*, 2000), there again seems to be an auto-regulatory mechanism involved, considering that NO appears to inhibit HIF-1 activation under hypoxic conditions. These inhibitory effects have recently been attributed to activation of the PHDs by NO under hypoxic conditions, with subsequent proteasomal degradation of HIF-1α (Hagen *et al.*, 2003; Murphy, 2003).

Growth factors

Other stimuli for HIF-1 activation under normoxia include a large number of growth factors. There are reports demonstrating HIF-1 accumulation in response to factors like the epidermal growth factor (Zhong *et al.*, 2000), the platelet-derived growth factor (Richard *et al.*, 2000; Gorlach *et al.*, 2001), interleukin-1β (Hellwig-Burgel *et al.*, 1999; El Awad *et al.*, 2000; Thornton *et al.*, 2000), the transforming growth factor-β1 (TGF-β1) (Gorlach *et al.*, 2001), insulin-like growth factors (IGFs) 1 and 2 (Feldser *et al.*, 1999; Zundel *et al.*, 2000), and insulin (Feldser *et al.*, 1999; Stiehl *et al.*, 2002; Kietzmann *et al.*, 2003b).

Oncogenes

Similarly, oncogenes can activate HIF-1 under normoxia. This is mainly due to the fact that many proto-oncogenes exert their effects either via phosphorylation pathways also involved in HIF-1 regulation or, as in the case of pVHL, are directly responsible for HIF-1α stability regulation.

For instance, previous reports indicate that gain-of-function mutations of Ras, which are commonly seen in human tumors, result in increased HIF-1α protein levels as well as HIF-1 activation under normoxia (Chen *et al.*, 2001a). Similarly, activation of Src kinase has been reported to increase HIF-1α under normoxia (Jiang *et al.*, 1997). Both Ras as well as Src appear to exert their effect via an inhibition of proline hydroxylation (Chan *et al.*, 2002). Another group showed that Src enhanced the rate of HIF-1α synthesis instead of reducing HIF-1α degradation (Karni *et al.*, 2002).

On the other hand, HIF-1 can also be accumulated under normoxia by the inactivation of tumor suppressors (Harris, 2002). The tumor suppressor PTEN, for example, inhibits the PI3K pathway, thereby causing an inhibition of the proto-oncogene Akt (see 3.3.3.2). Consequently, the loss of PTEN has been shown to facilitate HIF-1-mediated gene expression (Zundel *et al.*, 2000). Similarly, loss-of-function mutations of pVHL have been shown to induce HIF-1 activation by inhibition of 26S proteasomal degradation (see 3.3.2) (Maxwell *et al.*, 1999; Krieg *et al.*, 2000; Hughson *et al.*, 2003).

Another example of the interplay of oncogene and/or tumor suppressor being influenced by hypoxia is presented by the p53-mouse double minute 2 (Mdm2) couple. Since p53 is a major focus of this thesis, effects of or mediated via this pair are presented below (see 3.4).

3.3.3.2 Regulation via phosphorylation

A model putting phosphorylation cascades in a decisive position in response to different stimuli, especially growth factors, has recently been proposed. In this model, HIF-1 induction has been suggested to be activated by downstream targets of certain receptor tyrosin kinases via binding of growth factors to their respective receptors. Such a regulation appears to be mediated mainly by two classes of signaling pathways, i.e., the MAPK and the PI3K pathway (Fukuda *et al.*, 2002; Fukuda *et al.*, 2003).

PI3K

PI3K signaling is largely mediated by the downstream kinase Akt, which has several targets involved in growth, cell cycle, and apoptosis, as well as in translation. One target is the mammalian target of rapamycin (mTOR), which is also named FKBP12/rapamycin-associated protein (FRAP). mTOR, upon phosphorylation, is activated and in turn phosphorylates the 4E-binding protein, thereby releasing the eukaryotic initiation factor-4F (eIF-4F), consequently increasing translation rates. In addition, mTOR activates the p70 ribosomal protein S6 kinase ($p70^{s6k}$), which again enhances translation directly and, at the same time, inhibits GSK3β activity. GSK3β is also inhibited directly by Akt and recently has been proposed to be involved in HIF-1α stability regulation. In this case, GSK3β was suggested to down-regulate HIF-1α protein upon activation (Mottet *et al.*, 2003). There have been other reports, though, which showed that PI3K signaling is not sufficient for HIF-1 activation under hypoxia (Alvarez-Tejado *et al.*, 2002; Arsham *et al.*, 2002). Therefore, the requirement of PI3K as well as the exact mechanisms involved remain elusive. New experiments indicate that PI3K, while not necessarily being involved in HIF-1 stabilization under short-term hypoxia, seems to be required for HIF-1 accumulation under prolonged O_2 depletion when general translation mechanisms are inhibited but HIF-1α is still translated. This seems to be due to PI3K/Akt/GSK3β pathway-mediated translation initiation (Schnitzer, unpublished data).

MAPK

Other important signaling cascades involved in HIF-1 regulation are the members of the MAPK family (Scott *et al.*, 1998). c-Jun NH_2-terminal kinases (JNKs) (Kietzmann *et al.*, 2003a), p38 MAPKs (Conrad *et al.*, 1999; Conrad *et al.*, 2000; Gao *et al.*, 2002), and extracellular signal-regulated kinases (ERKs) have all been shown to regulate HIF-1. ERK 1 and 2 are the most extensively studied MAPKs involved in HIF-1α regulation.

Both have been shown to directly phosphorylate HIF-1α upon activation by upstream molecules (Ras/Raf-1/MEK-1). This phosphorylation was reported to be essential for HIF-1 transactivity (Hofer *et al.*, 2001; Hur *et al.*, 2001) under normoxia as well as under hypoxia, and the exact mechanism seems to involve effects on the interaction between p300 and the C-TAD of HIF-1α (Sang *et al.*, 2003).

3.3.4 Adaptive responses to oxygen depletion

As a consequence of HIF-1 transcriptional activation, an increasing number of genes is transcribed. Figure 3 illustrates the enormous spectrum of HIF-1 targets. Most of the proteins encoded by these genes are involved in adaptive processes counteracting detrimental effects of hypoxia. They play key roles in erythropoiesis, angiogenesis, iron homeostasis, glucose and energy metabolism, as well as cell proliferation and survival.

Erythropoietin (Epo), the protein which actually led to the discovery of HIF-1 in 1992 (Semenza and Wang, 1992), has been described to be up-regulated by hypoxia (Wang and Semenza, 1993c). Epo induces erythropoiesis, thereby increasing the systemic O_2 availability. Similarly, the vascular endothelial growth factor (VEGF) is expressed in a HIF-1-dependent manner and induces formation of new vessels, resulting in a better blood supply of the affected tissue (Forsythe *et al.*, 1996). This is of special importance in tumor development, where angiogenesis poses a big problem for therapy. The same applies to the expression of proteins involved in glycolysis (Wenger, 2000), such as glucokinases (Roth *et al.*, 2004), and glucose transport (e.g., glucose transporter-1 and -3, hexokinase 1 and 2) (Semenza *et al.*, 1994; Iyer *et al.*, 1998a; Wood *et al.*, 1998). These proteins allow the cells to adjust to the conditions of low O_2 tension and, at the same time, to adapt to glucose deprivation, which usually is concomitant with hypoxia. This condition is called ischemia and, in addition, is characterized by acidosis. Consequently, induction of the carbonic anhydrase 9 under these conditions appears only logical (Iyer *et al.*, 1998a; Wykoff *et al.*, 2000; Olive *et al.*, 2001; Watson *et al.*, 2003).

Another important target of HIF-1 transcriptional activation are the PHDs. The PHDs are involved in a possible negative feed-back loop, since they apparently are active to a certain degree even under O_2 deficient conditions and, in addition, contain HIF-responsive elements in their promotor region resulting in increasing PHD levels under prolonged hypoxia (Cioffi *et al.*, 2003; D'Angelo *et al.*, 2003). Similarly the later discussed induction

of natural anti-sense HIF-1α mRNA (asHIF-1α) by HIF-1 activity offers the possibility of limiting HIF-1 activation to a certain period of time (Uchida *et al.*, 2004) (see 3.5).

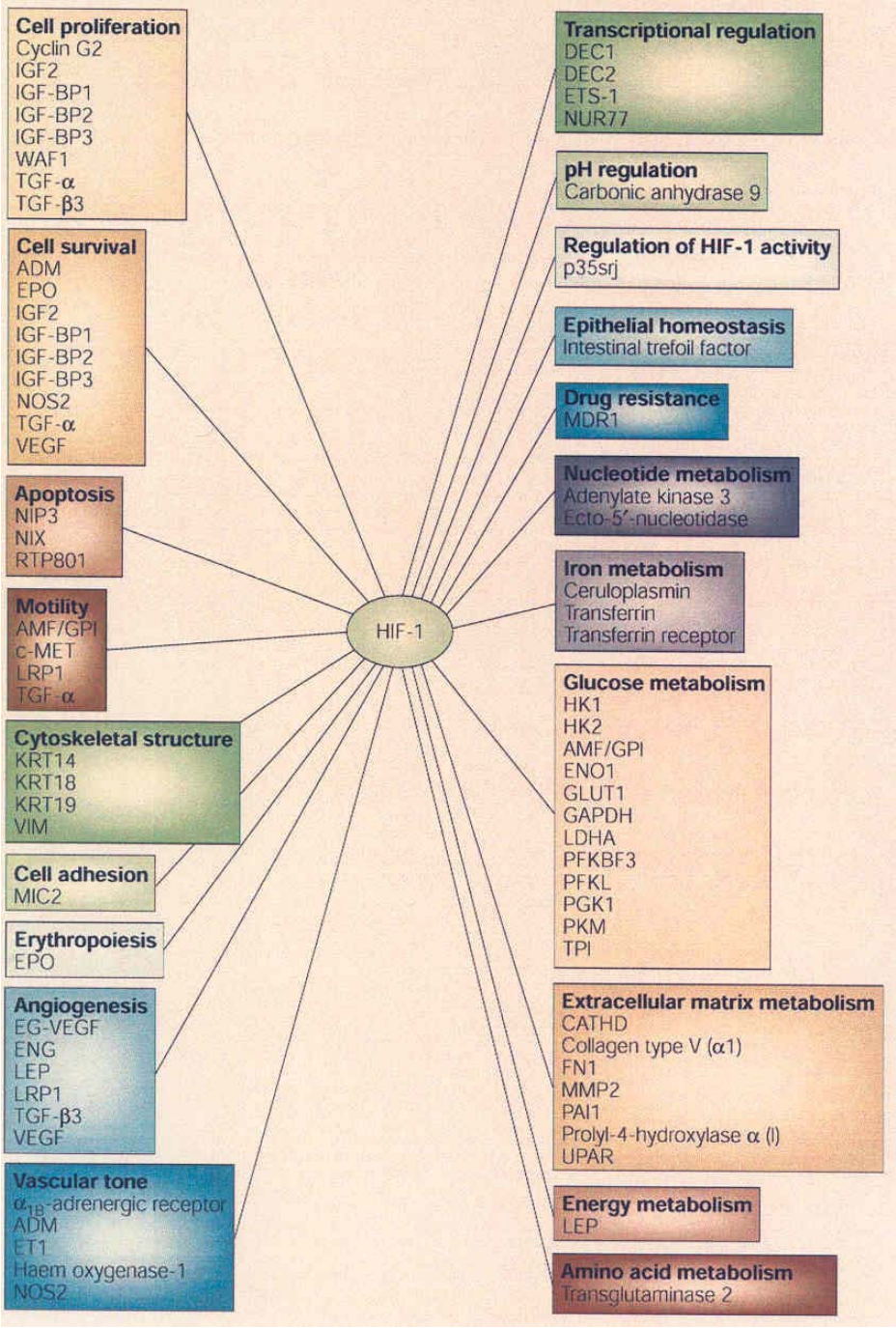

Figure 3: Genes transcriptionally activated by HIF-1

HIF-1 transcriptionally activates genes that are involved in many processes like cell proliferation, cell survival, apoptosis, erythropoiesis, angiogenesis, vascular tone, pH regulation, HIF-1 activity regulation, iron metabolism, glucose metabolism, extracellular matrix metabolism, energy metabolism, amino acid metabolism, and others (figure from Semenza, 2003).

3.3.5 HIF-1 and apoptosis

The term apoptosis was first used by Kerr in 1972 (Kerr *et al.*, 1972) to describe a programmed and orderly way of cell death. In contrast to necrosis, apoptosis appears rather unspectacular, i.e., instead of "exploding", cells are chipped into small particles, which are either taken up by neighboring cells or by nearby phagocytes. Thus, apoptotic events have long been neglected due to their "invisibility". Since apoptosis follows a strict program with respect to morphological and biochemical features, it has been termed "programmed cell death" as well (Hengartner, 2000). Essentially, there are two major death pathways involved in the induction and mediation of apoptosis. Death-receptor-induced apoptosis represents the externally induced pathway to cell death since it requires interaction of ligands with specific receptors (e.g., TNF-receptor and Fas). The death signal is subsequently mediated by formation of an activation complex for pro-caspase-8, which thereby becomes activated, allowing for activation of further down-stream executioner caspases (e.g., caspase-3 and -7) (reviewed in Sartorius *et al.*, 2001). In contrast, the mitochondrial pathway involves the disruption of the mitochondrial membrane potential, with subsequent release of cytochrome C (Cyt C). Upon release, this factor forms a complex with the apoptosis protease activating factor-1 (Apaf-1) and ATP, which allows for recruitment of pro-caspase-9 to this so-called apoptosome. The following activation of caspase-9 again initiates a caspase cascade culminating in the activation of the above-mentioned effector caspases (reviewed in Orrenius, 2004). Eventually, these executioner caspases cleave further cellular targets to execute a controlled and orderly degradation of the individual cell, making it possible for other cells to engulf the resulting apoptotic bodies (Grutter, 2000).

Since most of the reactions induced by HIF-1 promote cell survival, the question arose if HIF-1 can be considered an anti-apoptotic factor. This idea is supported by reports that HIF-1 protects cells from hypoxia-induced apoptosis (Zaman *et al.*, 1999; Akakura *et al.*, 2001; Dai *et al.*, 2003). Considering the mechanisms activated by HIF-1 (as described in 3.3.4), it becomes obvious that this transcription factor might be considered to act towards protection of affected tissue/cells (Baek *et al.*, 2000; Lin *et al.*, 2000; Alvarez-Tejado *et al.*, 2001; Beitner-Johnson *et al.*, 2001; Sirén *et al.*, 2001). In line with this, hypoxia has been found to induce accumulation of anti-apoptotic proteins, e.g., the inhibitor of apoptosis protein-2 (IAP-2) (Dong *et al.*, 2001) and the apoptosis repressor with caspase recruitment domain (ARC) (Neuss *et al.*, 2001). Their regulation was mediated by HIF-1-independent mechanisms, though. In contrast to these observations, there is a bulk of information

indicating apoptosis to be induced by O_2 deprivation (Malhotra *et al.*, 2001). Observations indicated that apoptotic cell death induced by hypoxia and nutrient deprivation was HIF-1-dependent (Carmeliet *et al.*, 1998). Similarly, the pro-apoptotic proteins BNIP3 and NIX have been reported to be up-regulated in response to hypoxia by HIF-1-dependent mechanisms (Bruick, 2000; Guo *et al.*, 2001; Sowter *et al.*, 2001). The induction of cell death mechanisms has also been suggested to involve differential regulation of members of the bcl-2 family (Jung *et al.*, 2001). The exact apoptotic mechanisms involved still await further characterization, but recent results point to an induction of multiple apoptotic pathways by hypoxia, at least for Jurkat cells (Malhotra *et al.*, 2001; Chao *et al.*, 2002; Kim *et al.*, 2003). Essentially, the overall picture concerning hypoxia and apoptosis is rather controversial. Especially the impact of HIF-1 remains elusive with respect to its pro- or anti-apoptotic nature (Piret *et al.*, 2002). Of special interest in this field is the interplay between HIF-1 and the pro-apoptotic transcription factor p53, which is apparently induced by hypoxia as well. This special relationship is discussed in more detail in 3.4.

3.3.6 HIF-1 and diseases

The controversial role of hypoxia/HIF-1 in apoptosis promotion or prevention becomes even more interesting when the involvement of hypoxia in different pathological disease states is considered.

Cerebral and myocardial ischemia

Ischemia, i.e., O_2 and nutrient deficiency, is a common feature of some of the most frequent diseases. The occlusion of blood vessels, for example in artherosclerosis, is a feature of cerebral as well as myocardial infarction, two of the main causes of death in industrialized countries. Under the impact of ischemia, HIF-1 is activated and thought to exert its anti-apoptotic features. Importantly, it induces factors involved in angiogenesis (Lee *et al.*, 2000), consequently counteracting the detrimental conditions present in the areas close to such an occlusion. The ability to respond in this way has been demonstrated to be age-dependently impaired, which at the same time might give some explanation for the increasing damage after ischemic events in elderly people compared to younger people (Rivard *et al.*, 2000).

Cancer

Tumorigenesis is another important pathological state in which hypoxic environments play a decisive role (Dachs and Tozer, 2000). HIF-1α has been described to be overexpressed in many different tumor types, strengthening the importance of HIF-1 with respect to carcinogenesis (Talks *et al.*, 2000; Zhong *et al.*, 2004).

Tumors are characterized by cells which are able to escape the natural cell death program involved in cellular homeostasis for the individual organ. These cells mostly carry several mutations rendering them insensitive to apoptosis-induction and increasing their proliferation. This increase in proliferation eventually puts cells in fast-proliferating areas of the tumor in environments of limited O_2 and nutrient availability. In this case, induction of vessel formation constitutes a negative factor for the organism while being essential for the cells "trapped" within the tumor (Tsuzuki *et al.*, 2000). An intriguing correlation between tumor angiogenesis and HIF-1 levels was reported, suggesting HIF-1 to play a major role under these conditions (Zagzag *et al.*, 2000; Horiuchi *et al.*, 2002; Choi *et al.*, 2003). At the same time, hypoxia was reported to select cells within tumors for resistance to apoptosis (Akakura *et al.*, 2001). HIF-1 has been identified as being predictive for cancer invasiveness (Krishnamachary *et al.*, 2003) and also for malignancy (Brizel *et al.*, 1997). These results are in accordance with reports claiming tumor suppressors like PTEN or pVHL to be negatively correlated to HIF-1α accumulation (Maxwell *et al.*, 1999; Zundel *et al.*, 2000; Harris, 2002). Since HIF-1α has been reported to mediate resistance to chemotherapy and radiation (Aebersold *et al.*, 2001; Unruh *et al.*, 2003), HIF-1α-targeted therapeutic approaches might prove useful for combinatory tumor therapies, since this would allow to essentially cut off the nutrient and O_2 supply for the respective tumorigenic tissue (Lando *et al.*, 2003).

Another important tumor suppressor which has been reported to be mutated in up to 50% of all tumors, namely p53, will be discussed in detail in the following paragraph, since it constitutes a main target of investigation in my work and displays an interesting relation to HIF-1.

3.4 Hypoxia and p53

The tumor suppressor p53 has been shown to accumulate under severe hypoxia, although conflicting reports exist on its transcriptional activity. Therefore, I will initially give a short overview of p53-regulation and its parallels to HIF-1-regulation, before going into more

detail about the current state of research concerning the controversially discussed interactions between HIF-1 and p53.

HIF-1α- vs. p53-regulation – mirrored mechanisms?

HIF-1α and p53 are regulated in a very energy-intensive fashion. Both are constitutively expressed and immediately marked for degradation by specific E3-ubiquitin ligases, pVHL and Mdm2, under non-induced conditions, respectively (Figure 4).

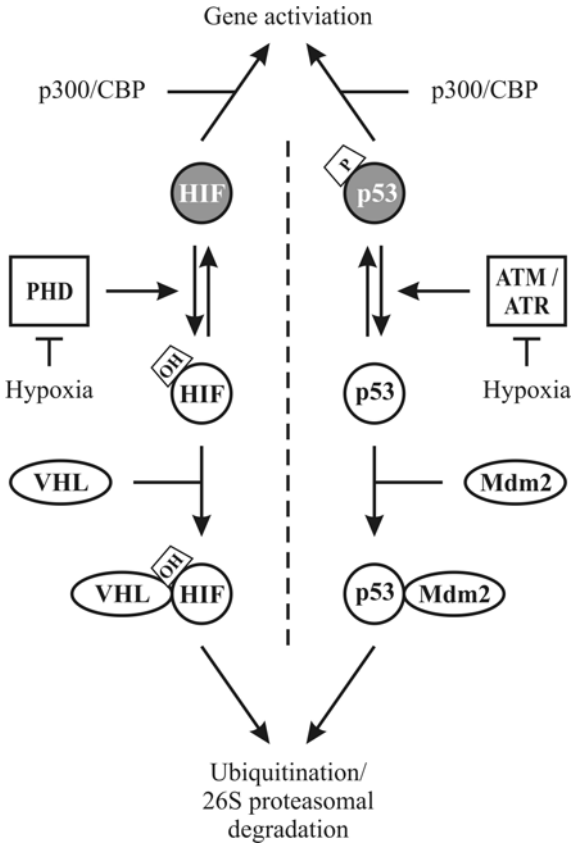

Figure 4: Parallels in the regulation of HIF-1α and p53

HIF-1 is hydroxylated under normoxic conditions by the PHDs, subsequently bound by its specific E3-ubiquitin ligase pVHL, and marked for 26S proteasomal degradation by ubiquitination. Hypoxia inhibits the PHDs, allowing HIF-1 to accumulate and bind p300/CBP, eventually activating HIF-responsive gene expression. In a mirrored fashion, p53, under normoxia, is dephosphorylated by ATM/ATR kinases, thereby marked for binding of its specific E3-ubiquitin ligase Mdm2, and subsequently marked for 26S proteasomal degradation by ubiquitination. Hypoxia again inhibits the relevant enzymes, thus allowing p53 to become/remain phosphorylated, bind p300/CBP, and induce p53 responsive gene expression.

Consequently, HIF-1α and p53 are kept at low levels during normoxia (Blagosklonny, 2001). As described above, HIF-1α is hydroxylated under normoxic conditions, subsequently bound by pVHL and marked for 26S proteasomal degradation by ubiquitination. Under hypoxic conditions, HIF-1α is not modified, thus accumulating, binding to the transcriptional co-factor CBP/p300 and inducing expression of downstream genes. Similarly under normoxia, p53 is bound and targeted for degradation by Mdm2. Under hypoxia, p53 accumulates due to either a direct inhibition or down-regulation of Mdm2 (Alarcon *et al.*, 1999) or phosphorylation of serine residue 15 by the ataxia telangiectasia-mutated (ATM)- and Rad3-related (ATR) kinase, which prevents binding of Mdm2 and therefore allows for accumulation of p53 (Hammond *et al.*, 2003). When accumulated, p53 binds CBP/p300 and becomes transcriptionally active. In addition, ATR-induced phosphorylation was shown to induce replication arrest (Hammond *et al.*, 2002), which is a typical p53-regulated process.

The described regulation via constant, futile synthesis of a protein becomes understandable when the function of both proteins is taken into account. HIF-1 as well as p53 are transcription factors involved in stress responses which require an immediate reaction of the affected cell. In the case of HIF-1, the stressor is O_2 deficiency, eventually endangering the cell's ability to survive. The major stimulus for p53 activation is DNA damage, which again poses a dangerous threat to the cell if not dealt with correctly. Therefore, this mechanism, albeit energy-demanding, pays for the cell and the entire organism in the long run.

HIF-1 – p53-interplay

An *et al.* (1998) claimed that p53 stabilization under hypoxia was HIF-1-dependent. This concept was challenged by others showing that HIF-1-inducing hypoxic conditions were not sufficient to accumulate p53 (Wenger *et al.*, 1998) or that p53, if accumulating under O_2 deficiency, is transcriptionally inactive (Koumenis *et al.*, 2001). One explanation for these differences might be the initially mentioned differences in the definition of hypoxia. p53 accumulation requires severe hypoxia/anoxia for prolonged periods. Thus, other mechanisms besides HIF-1 accumulation might be needed for p53 activation under O_2 depleted conditions. Recent reports indicate that hypoxia alone is not sufficient to accumulate p53 to levels that affect HIF-1α and that additional p53 induction by either DNA-damage (Kaluzova *et al.*, 2004) or acidosis (Pan *et al.*, 2004) is required to allow for HIF-1 repression. This is in accordance with previous data suggesting that hypoxia-

induced cell death requires severe and prolonged hypoxia, at least in cell culture (Halterman *et al.*, 1999). The combination of these data further strengthens the idea that p53 accumulates to a relevant degree only under severe conditions of O_2 depletion, and only under these conditions is it able to target HIF-1α for proteasomal degradation. Similarly, HIF-1α accumulation in response to NO requires intermediate NO concentrations, whereas considerably higher NO levels are needed for p53 accumulation (Thomas *et al.*, 2004). Mechanistically, this has been proposed to be mediated by an interaction between HIF-1α and p53, either directly (Hansson *et al.*, 2002) or via Mdm2 (Chen *et al.*, 2003). Such interactions resulted in p53 stabilization and activation, and concomitantly, HIF-1α degradation. This illustrates that p53 also seems to play a pivotal role in HIF-1α regulation. In line with these observations are reports showing that p53 inhibits HIF-1 activity by targeting HIF-1α for Mdm2-mediated ubiquitination and 26S proteasomal degradation (Ravi *et al.*, 2000). In turn, the loss of p53 has been shown to enhance hypoxia-induced HIF-1α levels and to augment HIF-1-evoked VEGF expression in tumor cells. Besides directly affecting HIF-1α protein level, p53 represses HIF-1-stimulated transcription (Ravi *et al.*, 2000). Interestingly, p53 levels required to affect HIF-1α were higher than those needed for p53-activated gene transcription (Blagosklonny *et al.*, 1998). In combination with results demonstrating the requirement of p53-p300 binding for transrepression of HIF-1 (Blagosklonny *et al.*, 2001), a competition between p53 and HIF-1 for limiting co-factors such as p300 might be proposed, especially since both transcription factors have been described to bind to the same domain of p300 (Freedman *et al.*, 2002). This concept is supported by a report describing a similar mechanism of HIF-1 regulation by CITED2 via competition for p300 (Freedman *et al.*, 2003). In addition, a recent report indicated that HIF-1 can be transcriptionally activated when its binding to p300 is promoted, which supports the concept that p300 is a limiting factor (Datta *et al.*, 2004).

Altogether, the above-mentioned data still draw a rather obscure picture of the effects of p53 on HIF-1. Therefore, one part of the present study attempts to further elucidate the impact of p53 on HIF-1 with a special focus on severe O_2 depletion.

3.5 Hypoxia and transcriptional regulation

Besides regulating HIF-1 transcriptional activity via stabilization of HIF-1α, hypoxia is supposed to transcriptionally regulate the expression of several HIF-1-independent

proteins. For instance, IAP-2 transcription is induced by hypoxia in a HIF-1-independent manner (Dong *et al.*, 2001). Similarly, the cyclin-dependent kinase inhibitor p27 is transcriptionally induced in a HIF-1-independent fashion (Gardner *et al.*, 2001). This demonstrates that hypoxia, apart from regulating pathways involved in post-transcriptional regulation of several proteins (see above), also seems to be involved in the regulation of transcription. Importantly, HIF-1α mRNA levels have also been proposed to be down-regulated by prolonged hypoxia. This represents yet another mechanism of HIF-1 regulation which has been overlooked for a long time.

HIF-1α mRNA regulation

Regulation of HIF-1α protein levels by mRNA alteration has long been neglected since the regulation of this transcription factor has mainly been attributed to inhibition of its degradation in response to hypoxia. In addition, however, an increase of HIF-1α mRNA under hypoxia has been described (Wiener *et al.*, 1996; Roy *et al.*, 2004). A recent publication, in contrast, established a connection between prolonged hypoxia and a decrease in HIF-1α mRNA (Uchida *et al.*, 2004). This regulation has been attributed to a newly identified regulator, namely natural anti-sense HIF-1α mRNA (asHIF-1α) (Rossignol *et al.*, 2002). This factor has been shown to be regulated in a HIF-1-dependent manner and, in turn, to down-regulate HIF-1α mRNA.

Since the regulatory mechanisms of HIF-1α mRNA under O_2 depletion still remain elusive, HIF-1α mRNA regulation under hypoxia/anoxia represents a major target of my experiments.

3.6 Aims of this study

Since its discovery in 1992, HIF-1 has been proposed to be regulated mainly via inhibition of HIF-1α degradation under conditions of low O_2 tension. This paradigm has recently been questioned, due to the discovery of many additional regulatory mechanisms for HIF-1 accumulation and/or activation. Different modes of regulation, such as increased translation, phosphorylation or acetylation, and stabilization by chaperones were described for different stimuli affecting accumulation and activation of HIF-1. Even hypoxia itself, in addition to the degradation pathway, was proposed to affect translation of HIF-1α. At the same time it became apparent that the severity and duration of hypoxia might also prove decisive for HIF-1 stability regulation and activation, especially concerning the decision between preservation of cell viability vs. initiation of cell death.

The first part of my experiments was designed to assess the effects of the severity and duration of O_2 depletion on HIF-1α protein accumulation and HIF-1 transcriptional activity. A special focus was put on the influence of p53 on HIF-1, as this transcription factor has been reported to accumulate in response to O_2 deficiency as well. Therefore, the results should provide a more in-depth characterization of the impact of p53 on the protein level as well as on the transcriptional activity of HIF-1.

In the second part of my study, I intended to further elucidate the effects induced by prolonged periods of low O_2 hypoxia compared to anoxia with respect to alterations in HIF-1α mRNA. Subsequently, the regulatory mechanisms responsible for these changes were characterized, i.e., I determined whether changes were transcriptional or post-transcriptional in nature and if protein synthesis was required.

Thus, this study should allow to further characterize differences attributed to effects of low O_2 vs. no O_2. This will provide new information concerning the impact of p53 on HIF-1, thus improving our understanding of the decision between pro- and anti-apoptotic mechanisms with respect to the severity and duration of hypoxia. As the topic of cell survival vs. cell death mediated by O_2 depletion is controversially discussed in literature, the present study might add another piece of information to this puzzle. In addition, the results of this project might give further insights into a new mechanism proposed for HIF-1 regulation in response to prolonged, severe low O_2 tensions. Again, these considerations regarding HIF-1 regulation could prove useful when prolonged incubations to hypoxic or anoxic conditions are analyzed at the level of HIF-1 stability regulation.

4 Materials and Methods

4.1 Materials

4.1.1 Cells

A human colon carcinoma cell line (RKO) was used for most experiments. This cell line was chosen since it had been described to contain wild-type p53 (Kessis *et al.*, 1993), which was a prerequisite for the first part of the experiments. In addition, the cell line RCC4 was employed, which is a renal clear cell carcinoma cell line. Since these cells are mutated in the gene encoding pVHL, they display elevated HIF-1α levels even under normoxia. The human hepatocyte cell line HepG2 was applied for determination of endogenous erythropoietin.

4.1.2 Bacteria

For preparation of the plasmids, competent *Escherichia coli* DH5*.1α* were used. When high transformation efficiencies were required, XL1-Blue supercompetent cells (Stratagene, Amsterdam, The Netherlands) were used. For expression of GST-fusion proteins *E. coli* BL21 (DE3) were used.

4.1.3 Chemicals and reagents

All chemicals were of the highest grade of purity commercially available and purchased from Sigma-Aldrich (Taufkirchen, Germany), VWR International (Darmstadt, Germany) and Roth (Karlsruhe, Germany). Fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Biochrom (Berlin, Germany), medium and supplements came from PAA (Linz, Austria).

Special reagents and kits are listed in Table 1.

Table 1: Special reagents and kits

Chemical / Kit	Provider
Absolute™ qPCR SYBR® Green Fluorescein Mix	ABgene (Hamburg, Germany)
Advantage® RT-for-PCR-kit	Clontech (Heidelberg, Germany)
Amplify™ Fluorographic Reagent	Amersham Biosciences (Freiburg, Germany)
BD In-Fusion™ PCR Cloning Kit	Clontech (Heidelberg, Germany)
BigDye® Terminator v3.1 Sequencing Reagent	Applied Biosystems (Darmstadt, Germany)
Bio-Rad DC Protein Assay kit	Bio-Rad Laboratories (München, Germany)
Calf Intestine Alkaline phosphorylase	MBI Fermentas (Heidelberg, Germany)
Caspase 3 substrate (DEVD-AMC)	Bachem (Heidelberg, Germany)
DNA marker (Hyperladder™ I and IV)	Bioline (Luckenwalde, Germany)
dNTP mix	Eurogentec (Köln, Germany)
FastPlasmid™ Mini kit	Eppendorf (Hamburg, Germany)
glutathione (GSH-) agarose	Sigma (Steinheim, Germany)
Hind III (including buffer B)	Roche Diagnostics (Mannheim, Germany)
HiSpeed™ Plasmid Maxi Kit	Qiagen (Hilden, Germany)
HotMaster™ Taq DNA Polymerase (including 10x Transcription Buffer)	Eppendorf (Hamburg, Germany)
Hpa I (including buffer A)	Roche Diagnostics (Mannheim, Germany)
Isopropyl-β-D-thiogalacto-pyranoside (IPTG)	Biomol (Hamburg, Germany)

Luminol (3-Aminophthalhydrazide)	Acros Organics (Geel, Belgium)
Nitrocellulose membrane	Amersham Biosciences (Freiburg, Germany)
NucleoSpin® PCR clean-up Gel extraction kit	Macherey-Nagel (Düren, Germany)
peqGOLD RNAPure™	PeqLab Biotechnologie (Erlangen, Germany)
Protease inhibitor mix (PIM)	Roche Diagnostics (Mannheim, Germany)
Protein marker	MBI Fermentas (Heidelberg, Germany)
Pwo-DNA-Polymerase (including 10x Reaction buffer)	PeqLab Biotechnologie (Erlangen, Germany)
SeAP Reporter Gene Assay (Chemiluminescent)	Roche Diagnostics (Mannheim, Germany)
^{35}S-methionine	ICN Biomedicals (Eschwege, Germany)
T_NT SP6/T7-coupled reticulocyte lysate system	Promega (Mannheim, Germany)
Xba I (including buffer Y^+/Tango™)	MBI Fermentas (Heidelberg, Germany)

4.1.4 Antibodies

The antibodies used are listed in Table 2, the dilutions are described in the respective method employed (i.e., Western Blot, Spot membrane).

Table 2: Antibodies

Antibody	Provider
anti-actin (rabbit, polyclonal)	Sigma (Steinheim, Germany)
anti-HA (mouse, monoclonal)	Covance (Richmond, USA)
anti-HIF-1α (mouse, monoclonal)	BD Transduction Laboratories (Heidelberg, Germany)

anti-hsp70 (mouse, monoclonal)	StressGen (Vancouver, Canada)
anti-p53 (FL-393) (rabbit, monoclonal)	Santa Cruz (Heidelberg, Germany)
horseradish peroxidase (HRP)-labelled anti-mouse	Amersham Biosciences (Freiburg, Germany)
horseradish peroxidase (HRP)-labelled anti-rabbit	Amersham Biosciences (Freiburg, Germany)

4.1.5 Plasmids

The plasmids employed are given in Tables 3 and 4, including the characteristics and the providers.

Table 3: Reporter plasmids

Plasmid	Background	Information	Provider
pBKC-SeAP	pBKC	CMV promoter in front of a SeAP encoding gene (Karni *et al.*, 2002)	Dr. A. Levitzki (Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Israel)
Bax-luc		contains the promotor of the bax gene (Lohrum and Scheidtmann, 1996)	Dr. K.H. Scheidtmann (Institute for Genetics, University of Bonn, Germany)
pGLb-UPS	pGL3-basic	contains 5 kb of the 5'-flanking sequence from the human HIF-1α promotor (Iyer *et al.*, 1998b)	Dr. G.L. Semenza (Johns Hopkins University School of Medicine, McKusick-Nathans Institute of Genetic Medicine, Baltimore, USA)
pGL-Epo-HRE-luc	pGL3-promotor	contains 3 HREs of the Epo promotor (Kietzmann *et al.*, 2001)	Dr. T. Kietzmann (Institute of Biochemistry and Molecular Cell Biology, University of Göttingen, Germany)

pGL3-basic		contains a luciferase construct	Promega (Mannheim, Germany)
pGL3-control		contains a luciferase construct	Promega (Mannheim, Germany)

Table 4: Expression plasmids

Plasmid	Background	Information	Provider
pcDNA3			Invitrogen (Karlsruhe, Germany)
pcDNA3-HA-p53	pcDNA3	wildtype p53 supplemented with an HA-tag (Marin *et al.*, 1998)	Dr. K.H. Vousden (Regulation of Cell Growth Laboratory, National Cancer Institute at Frederick, USA)
pcDNA3-HIF-1α (1-826)	pcDNA3	wildtype HIF-1α (Wood *et al.*, 1998)	Dr. P.J. Ratcliffe (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK)
CMVβ-p300-CHA	CMVβ	full-length p300 supplemented with an HA-tag (Hecht *et al.*, 2000)	Dr. A. Hecht (Institute of Molecular Medicine and Cell Research, University of Freiburg, Germany)
pDrive mGAPDH	pDrive	wildtype mouse GAPDH	A. Johann (Department of Cell Biology, University of Kaiserslautern, Germany)
pGex-4T1			Dr. E. Metzen (Institute of Physiology, Medical University of Luebeck, Germany)
GST-p300-CH1-pGex-4T1	pGex-4T1	encodes for aa 300 – 528 of the p300-protein (Lando *et al.*, 2002b)	Dr. E. Metzen

4.1.6 Primers

Primers were purchased from MWG Biotech (Ebersberg, Germany) and metabion (Planegg-Martinsried, Germany) and are described in Table 5.

Table 5: Primers

	forward primer	reverse primer	Product size
HIF-1α	5'- CTC AAA GTC GGA CAG CCT CA -3'	5'- CCC TGC AGT AGG TTT CTG CT -3'	460 bp
Actin	5'- TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA -3'	5'- CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG -3'	660 bp
Epo	5'- TCT GGG AGC CCA GAA GGA AGC CAT –3'	5'- CTG GAG TGT CCA TGG GAC AG -3'	310 bp
3'UTR-HIF-1α (sense) (Infusion)	5'- GCC GTG TAA TTC TAG GCT TTT TCT TAA TTT CAT TCC TTT TTT TG -3'	5'- CCG CCC CGA CTC TAG CCT GGT CCA CAG AAG ATG TTT ATT T -3'	1204 bp
3'UTR-HIF-1α (anti-sense) (Infusion)	5'- GCC GTG TAA TTC TAG CCT GGT CCA CAG AAG ATG TTT ATT TGA TG -3'	5'- CCG CCC CGA CTC TAG TCA TTC CTT TTT TTG GAC ACT GGT GG -3'	1190 bp

4.1.7 Instruments and Software

Table 6: Instruments and software

Instrument/software	Provider
3-gas incubator IG750	Jouan (Unterhaching, Germany)
ABI PRISM® 3100 Genetic Analyzer (16-Capillary-Sequencer with a 50 cm capillary system)	Applied Biosystems (Darmstadt, Germany)
AIDA Image Analyzer (Version 3.11)	Raytest (Straubing, Germany)

DIGAMIX 5KM 402 gas pump	Woesthoff GmbH (Bochum, Germany)
EC120 Mini Vertical Gel System	Thermo Electron (Dreieich, Germany)
Fluoroskan Ascent CF	ThermoLabsystems (Frankfurt, Germany)
IDA gel documentation system	Raytest (Straubing, Germany)
InVivo$_2$ 400 hypoxia working station	Ruskinn (Leicester, UK)
Lumat LB 9507 luminometer	Berthold Technologies (Bad Wildbad, Germany)
Mastercycler®	Eppendorf (Hamburg, Germany)
Multiskan RC plate reader	ThermoLabsystems (Frankfurt, Germany)
MyiQ Single-Color Real-Time PCR Detection System	Bio-Rad Laboratories (München, Germany)
Trans-blot SD blotting chambers	Bio-Rad Laboratories (München, Germany)
Ultraspec 2100 pro photometer	Amersham Biosciences (Freiburg, Germany)

4.2 Methods

4.2.1 Cell biology

4.2.1.1 Cell culture

Human colon carcinoma cells (RKO) and renal carcinoma cells (RCC4) were cultured in Dulbecco's modified eagle medium (DMEM) with 4.5 g/l D-glucose. Human hepatocyte cells (HepG2) were cultured in Earle's minimum essential medium (Earle's MEM). All media were supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. RCC4 medium also contained 1 mM sodium pyruvate and HepG2 medium was supplemented with 1 x non essential amino acids (NEAA). Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C and were transferred twice a week.

4.2.1.2 Hypoxia and anoxia

Hypoxia (0.5% O_2, 5% CO_2, 94.5% N_2) incubations were either performed in a 3-gas incubator IG750 (Jouan, Unterhaching, Germany), or in an $InVivo_2$ 400 hypoxia working station (Ruskinn, Leicester, UK). Anoxia (0% O_2, 5% CO_2, 95% N_2) treatments were carried out in a flow-through manner in plexiglass chambers connected to a DIGAMIX 5KM 402 gas pump (Woesthoff GmbH, Bochum, Germany). The medium was changed directly prior to the experiments.

4.2.1.3 Transient transfection

For Western blot analysis, 1 x 10^6 RCC4 cells were seeded in 6 cm or 1 x 10^6 RKO cells in 10 cm dishes one day prior to transfection. At a rate of approximately 60% confluency, cells were transfected with 4, 5 or 8 µg p53 expression plasmid using PolyFect® transfection reagent (Qiagen, Hilden, Germany), following the manufacturer's handbook. In detail, the required amount of plasmid was diluted in 150 µl (300 µl) serum-free medium, vortexed and spinned down briefly. Then, 15 µl (25 µl) of PolyFect® were added and vortexed. After approximately 10 minutes, the formed complexes were taken up with 1 ml complete medium and added to the cells, which were substituted with 3 ml (7 ml) fresh medium in 6 cm (10 cm) dishes. 16 h after transfection, medium was removed, cells were

washed with PBS and received 2 ml (4 ml) of fresh medium immediately prior to the indicated stimulations.

For luciferase reporter assays, 1 x 10^5 RKO cells were seeded in 6 well plates one day prior to transfection. For p53-mediated effects, they were transfected with 0.005, 0.05, or 0.5 µg p53 expression plasmid and/or co-transfected with 1 µg p300 expression plasmid and either Epo-luc or Bax-luc plasmids (0.5 µg each) using the same protocol. For control experiments, cells were transfected with 0.5 µg SeAP reporter plasmid. Similarly, for mRNA regulation experiments, 1 µg luciferase containing plasmids (pGL3-control, pGL3-control 3'UTR-HIF sense, pGL3-control 3'UTR-HIF anti-sense, pGL3-basic, or pGL3-basic UPS) were used to transfect cells. In contrast to the abovementioned volumes indicated for 6 and 10 cm dishes, the volumes used for transfection were 100 µl of serum-free medium, 15 µl of PolyFect®, 600 µl of complete medium, 1.5 ml of medium initially put on the cells, and 1 ml of medium provided during the experiments for 6-well plates.

For Epo mRNA evaluation, 1 x 10^6 HepG2 cells were seeded in 10 cm dishes one day prior to the transfection with either 5 or 15 µg p53 expression plasmid. Transfection was done according to the calcium phosphate method. In detail, H_2O, $CaCl_2$ (125 mM), and the respective amounts of plasmid were mixed. 2 x HBS (see Appendix I) was added while continuously and vigorously vortexing. The resulting mix was incubated for 15-25 min at room temperature until precipitate appeared. Subsequently, the required amount of mix was added to the cells, gently shaken and incubated at 37°C for 6-16 h. Immediately prior to the experiments, medium was removed, cells were washed with PBS, and 4 ml fresh medium were added to the cells.

4.2.2 Biochemistry

4.2.2.1 Protein determination (Lowry method)

The protein content of cell lysates was determined using the DC Protein Assay kit, which is based on the Lowry method (Lowry *et al.*, 1951). Briefly, a standard dilution series of BSA in H_2O was prepared (0.25 – 2 mg/ml). Samples as well as standards (5 µl) were pipetted into a 96-well plate, 25 µl solution A' (20 µl solution S per ml solution A) were added, and then the colorimetric reaction was started by addition of 200 µl solution B. After incubation for 15 min (room temperature, shaking), extinction was measured at 750 nm using a Multiskan RC plate reader.

4.2.2.2 SDS-PAGE/Western blot analysis

HIF-1α, p53, HA-tagged p53, and actin were quantified by Western blot analysis. In detail, cells were incubated, scraped off, lysed in 150 µl protein lysis buffer A (see Appendix I), and sonicated for 3 sec. Subsequently, lysates were vortexed 3 times for 10 sec and incubated for 10 min on ice each, followed by centrifugation (15000 x g, 4°C, 30 min). The protein content of the lysates was determined using the Lowry method (see 4.2.2.1). 80 µg protein and 10 µl 4 x SDS-PAGE sample buffer (see Appendix I) were mixed, filled up to 40 µl with H_2O, and denatured at 95°C for 5 min. Proteins were resolved on 7.5% SDS-polyacrylamide gels (see Appendix I) using 1 x SDS-running buffer as liquid phase. Subsequently, gels were equilibrated in blotting buffer (see Appendix I) for 5 min, followed by blotting of the proteins onto nitrocellulose by a semi-dry transfer cell. Unspecific binding was blocked with 5% milk/TTBS (see Appendix I) for 1h. Anti-HIF-1α (1:1000 in 5% milk/TTBS), anti-p53 (1:1000 in 5% milk/TTBS), anti-HA (1:1000 in 5% milk/TTBS), or anti-actin antibody (1:500 in 5% milk/TTBS) was added and incubated overnight at 4°C. Afterwards, nitrocellulose membranes were washed 3 times for 5 min each with PBS. For protein detection, blots were incubated with a HRP-labeled goat anti-mouse secondary antibody (1:2000 in 5% milk/TTBS) or HRP-labeled goat anti-rabbit secondary antibody (1:2000 in 5% milk/TTBS) for 1 h, washed 3 times for 5 min each with TTBS and once for 5 min with PBS, followed by 2 min incubation with ECL solution (see Appendix I). Specific proteins were visualized on an x-ray film (Super RX, Fujifilm).
To allow for detection of additional proteins, membranes were stripped, i.e., they were washed 30 min in 0.01 N HCl (pH 3), 3 times shortly in PBS, and finally again 30 min in 0.01 N NaOH (pH 12). After that, the membranes were blocked again as indicated above, and incubation with additional antibodies according to the protocol followed.

4.2.2.3 Coomassie Blue staining

For verification of the molecular weight and purity of specifically prepared proteins, the proteins were separated on 10% SDS-polyacrylamide gels (see Appendix I). Subsequently, the gels were incubated in Coomassie Blue staining solution (see Appendix I) for 30 min. Then, gels were destained in destaining solution for 4-6 h or until the background was strongly reduced to allow for visualization of the protein bands.

4.2.2.4 Expression and preparation of (GST-fusion) proteins in bacteria

pGEX-4T1 (control) and pGEX-4T1 p300-CH1 (p300-CH1) plasmids were transformed into *E. coli* BL21 (DE3) using the heats shock protocol. Following single clone selection, bacteria were grown in 200 ml LB medium with 50 µg/ml ampicillin to an optical density at 600 nm (OD_{600}) of 0.6-0.8, and protein expression was induced with 0.5 mM IPTG for 3 h. After centrifugation, cells were resuspended in 10 ml protein lysis buffer B (see Appendix I), supplemented with lysozyme (1 mg/ml), and incubated for 10 min on ice. 1 x PIM and 1 mM PMSF were added, followed by sonification. After centrifugation (10000 x g, 4°C, 20 min), the protein-containing supernatant was incubated with 300 µl 50% slurry of GSH-agarose in PBS overnight at 4°C. After washing 5 times with PBS containing 1% Triton-X100, the protein bound GSH-agarose was resuspended in 500 µl PBS containing 1% Triton-X100. Proteins bound to GSH-agarose were verified using 10% SDS-polyacrylamide gels, followed by Coomassie Blue staining (see 4.2.2.3).

4.2.2.5 *In vitro* transcription/translation

pcDNA3-HA-p53 and pcDNA3-HIF-1α (1-826) plasmids were used for *in vitro* transcription/translation (IVTT) of p53 and HIF-1α using a $T_NT^®$ SP6/T7-coupled reticulocyte lysate system (Promega) according to the manufacturer's protocol. In detail, 1 µl plasmid (0.5 µg/µl) was gently mixed with 25 µl $T_NT^®$ rabbit reticulocyte lysate, 2 µl $T_NT^®$ reaction buffer, 1 µl $T_NT^®$ T7 RNA polymerase, 1 µl amino acid mixture (minus methionine) (1 mM), 2 µl ^{35}S-methionine (10 µCi/µl), 40 u RNasin® ribonuclease inhibitor, and 16 µl DEPC-treated H_2O. Incubation at 30°C was carried out for 90 min. Then, the resulting proteins were identified on 10% SDS-polyacrylamide gels. The gels were fixed in fixing solution (see Appendix I) (30 min), incubated in Amplify™ Fluorographic Reagent (30 min), dried on a filter paper using a vacuum gel drier for 90 min, and exposed to x-ray films for 8-16 h.

4.2.2.6 Peptide array assay

A cellulose membrane spotted with peptides of HIF-1α with a length of 15 amino acids (aa), an overlap of 12 aa and an offset of 3 aa to the neighboring spots each (Frank, 1992) was employed for this assay. The spot membrane was primed by incubation with 50% ethanol followed by 3 times TTBS for 10 min each. Unspecific binding was blocked using binding buffer (see Appendix I) for 1 h. For detection of binding partners, the membrane

was incubated with target proteins. The incubation with the specific proteins, either *in vitro* transcribed/translated ^{35}S-p53 (100 µl) or bacterially expressed hsp70 (30 µg), was carried out in binding buffer overnight at 4°C. p53-binding to HIF-1α was detected by exposure of the membrane to an x-ray film for 7 days at -70°C. For detection of hsp70-HIF-1α interaction, anti-hsp70 antibody was incubated overnight (1:1000 in binding buffer), followed by 3 washing steps with PBS (1 min each) and subsequent incubation with HRP-labeled goat anti-mouse secondary antibody (1:2000 in binding buffer) for 2 h. After that, the membrane was washed 3 times for 5 min each with PBS, followed by ECL detection.
For re-use purposes, the membrane was stripped, i.e., the membrane was incubated 3 times in stripping mix A (see Appendix I) at 40°C in a sonification bath for 10 min each, followed by the same procedure using stripping mix B (see Appendix I) and a final washing step of 30 min in 50% ethanol.

4.2.2.7 Competition binding assay

10 µl protein bound GSH-agarose (either control or p300-CH1), 40 µl of ^{35}S-HIF-1α and/or ^{35}S-p53 (1 to 100 µl) were mixed in NET-N buffer (see Appendix I). Unprogrammed reticulocyte lysate (URL), i.e., the IVTT mix without addition of a protein-encoding plasmid, was used as a control (10 or 100 µl). In order to increase the visibility of the pellet, 40 µl of GSH-agarose equilibrated with NET-N buffer were added. Incubations were performed for 16 h at 4°C. Following centrifugation (500 x g, 4°C, 5 min), 20 µl of the supernatant were mixed with 20 µl 4 x SDS-PAGE sample buffer and boiled at 95°C for 5 min. The agarose pellet was washed 5 times with NET-N buffer, subsequently mixed with 40 µl 4 x SDS-PAGE sample buffer and boiled at 95°C for 5 min. Protein separation and detection was similar to the procedure described for IVTT. HIF-1α binding to p300-CH1 was evaluated using densitometric analysis and calculated relative to the sample incubated with HIF-1α only.

4.2.3 Molecular Biology

4.2.3.1 Reporter gene analysis

Following incubations, medium was removed, plates were washed with PBS, and the cells were scraped off in 1 x luciferase lysis buffer (see Appendix I). After vortexing for 10 sec, cells were incubated for 10 min on ice and centrifuged (12000 x g, 4°C, 2 min).

Subsequently, 10 µl of the supernatant were mixed with 100 µl of luciferase assay reagent (see Appendix I), and luciferase activity was measured.

SeAP expression was determined according to the manual provided. In detail, following incubations, the medium was centrifuged (12000 x g, 4°C, 2 min). 50 µl of the supernatant were diluted with 150 µl dilution buffer and incubated at 65°C for 30 min. Then, samples were centrifuged (12000 x g, 4°C, 2 min), cooled down on ice, and inactivated by addition of 50 µl inactivation buffer to 50 µl diluted sample with a 5 min incubation at room temperature. Then, 50 µl substrate reagent were added, incubated at room temperature for 10 min with gentle rocking, before the luminescence was measured with a luminometer for 5 sec. Both luciferase and SeAP activity were normalized to controls, i.e., to either normoxia-treated or control-plasmid-transfected cells.

4.2.3.2 Caspase activity assay

Caspase activity was measured using a fluorescence-substrate-based caspase activity assay. For measurement of caspase activity, 2 x 10^6 RKO cells were seeded in 10 cm dishes one day prior to the experiments. After the treatment, cells were scraped off, lysed in 200 µl caspase buffer (see Appendix I), sonicated for 15 sec, followed by centrifugation (15000 x g, 4°C, 10 min). After that, the protein content of the protein containing supernatant was determined following the Lowry method (see 4.2.2.1). 30 µg protein were filled up to 140 µl with caspase buffer (supplemented with 10 mM DTT) and mixed with 10 µl caspase substrate DEVD-AMC (2mM stock solution in DMSO diluted to200 µM in caspase buffer) in 96-well plates. The plates were incubated at 30°C for 1 h and, simultaneously, the cleavage of the caspase substrate was followed on the Fluoroskan Ascent CF instrument (extinction: 360 nm; emission: 465 nm), measurements being performed every 2 min. The caspase activities were calculated as DEVD-AMC cleavage per minute relative to normoxia controls in percent.

4.2.3.3 RNA isolation

For determination of mRNA changes, 2 x 10^6 RKO cells were seeded in 10 cm dishes one day prior to the experiments. Medium was changed directly before starting the indicated treatments. Cycloheximide (10 µM) and/or actinomycin D (5 µg/ml) were added as described for the individual experiment. Changes in mRNA expression were evaluated using RNA extraction, with subsequent reverse transcription, followed by (quantitative) PCR. For this purpose, cells were scraped off, centrifuged (500 x g, 4°C, 5 min), and

subsequently frozen at –70°C. For extraction of total RNA, cells were thawed in 1 ml peqGOLD RNAPure™, resuspended and incubated at room temperature for 5 min. After addition of 0.2 ml chloroform per ml peqGOLD RNAPure™, samples were vortexed for 15 sec, and the incubations were continued for another 15 min. Subsequently, the samples were centrifuged (12000 x g, 4°C, 5 min) to separate the RNA-containing water-phase from the phenol-phase and the intermediate lipid layer. For precipitation of the RNA, 0.5 ml isopropanol were added to the collected water-phase, and the mixture was incubated at room temperature for 10 min, followed by centrifugation (12000 x g, 4°C, 10 min). Then, the pellet was washed twice with 1 ml 75% ethanol in DEPC-treated H_2O (see Appendix I) and the supernatant was removed by centrifugation (12000 x g, 4°C, 10 min). After discarding the last supernatant, the pellet was dried and finally resuspended in 50 µl DEPC-treated H_2O by incubation at 55°C for 5-15 min. The RNA content was determined using the optical density (OD) at 260 nm. An OD_{260} of 1 is equivalent to 40 µg/ml RNA. Quality was checked by the ratios of OD_{260}/OD_{280} and OD_{260}/OD_{230}.

4.2.3.4 Reverse transcription (RT)

RT was performed according to the provided manual using the Advantage® RT-for-PCR kit. In brief, 2000 ng RNA in 12.5 µl DEPC-treated H_2O were mixed with 1 µl random hexamers and incubated at 70°C for 2 min. Then, 4 µl 5 x reaction buffer, 1 µl dNTP-mix (10 mM), 0.5 µl recombinant RNase inhibitors, and 1 µl MMLV reverse transcriptase were added on ice. Subsequently, the mix was incubated at 42°C for 1 h, and at the end the MMLV reverse transcriptase was inactivated at 95°C for 5 min. The resulting cDNA was diluted 1:5 before being used for further analyses.

4.2.3.5 Polymerase chain reaction (PCR)

For qualitative analysis, cDNA samples were used for conventional PCR. For this purpose, 5 µl of cDNA were mixed with 10 µl (= 10 pmol) forward and reverse primers each for HIF-1α, actin, or Epo (see 4.1.6), 5 µl 10 x transcription buffer, 2 µl dNTPs (20 mM), 17 µl destilled H_2O, and 1 µl HotMaster™ Taq DNA Polymerase. The PCRs were performed according to the following profiles:

HIF-1α / actin:

Initial denaturation		95°C	30 sec
Cycles:	Denaturation	95°C	30 sec
(HIF-1α: 24x)	Annealing	55°C	30 sec
(actin: 19x)	Elongation	68°C	1 min
Final extension		68°C	10 min

Epo:

Initial denaturation		95°C	30 sec
Cycles:	Denaturation	95°C	30 sec
(Epo: 30x)	Annealing	60°C	30 sec
	Elongation	68°C	1 min
Final extension		68°C	10 min

Subsequently, the PCR products were separated using 2% agarose gels in 0.5 x TBE, stained in ethidium bromide solution (0.5 mg/l) for 20 min, destained in H_2O, and visualized by UV excitation.

4.2.3.6 Quantitative PCR

For quantitative analysis, cDNA samples were subjected to quantitative real-time PCR. For this purpose, cDNA (4 µl) was mixed with 0.4 µl forward and reverse primers each for HIF-1α or actin (10 pmol/µl), distilled H_2O (5.2 µl), and 10 µl Absolute™ qPCR SYBR® Green Fluorescein Mix in 96-well PCR plates. The mix was briefly spinned down and the plates were sealed with optical tape.

The PCR was performed using the MyiQ Single-Color Real-Time PCR Detection System according to the following profile:

Activation I		50°C	2 min
Activation II		95°C	15 min
Cycles: (45 x)	Denaturation	95°C	15 sec
	Annealing	55°C	30 sec
	Elongation	72°C	30 sec
Final denaturation		95°C	1 min
Final renaturation		55°C	1 min
Melting curve	(80x)	+ 0.5°C	10 sec

Measurement of fluorescence was carried out during the elongation phase (mean value) for quantification and during the melting curve (with measurements being performed for each temperature) for analysis of the product identity.

For quantification purposes, one cDNA sample was used as a standard sample in concentrations of either 10%, 50%, or 100%, to allow for relative comparison in between the samples.

Initially, the primers (HIF-1α and actin) were validated for use in the quantitative system by serial dilution of a sample (1, 0.1, 0.01, 0.001, 0.0001) using the above-mentioned protocol. Both HIF-1α (Figure 5) and actin primers (data not shown) proved to be useful for quantitative real time PCR, since both amplified one product only and remained linear over several orders of magnitude.

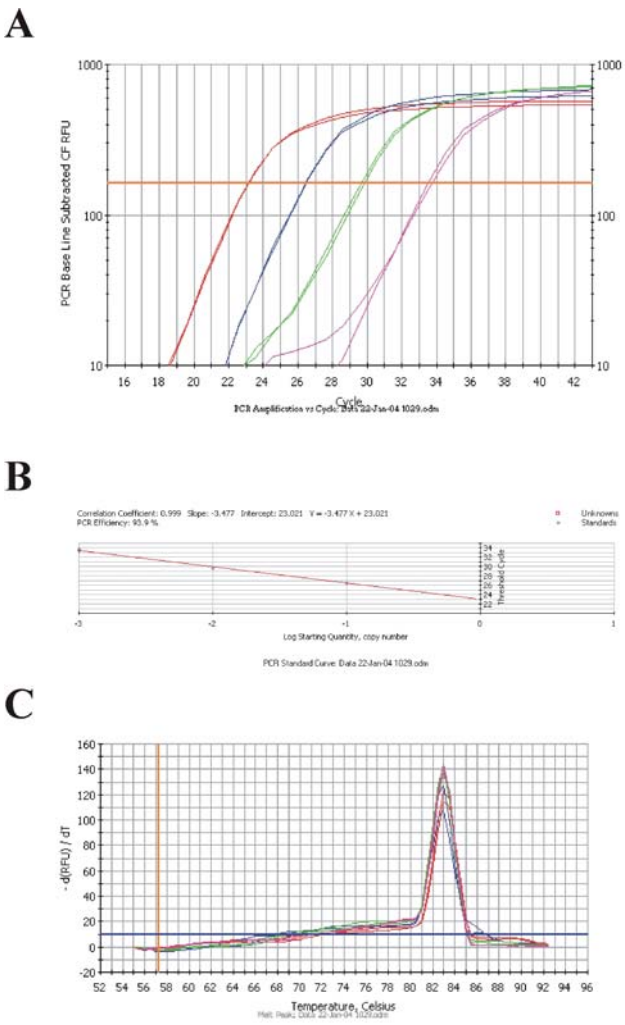

Figure 5: Validation of the HIF-1α primers for quantitative real-time PCR

A random cDNA sample was serially diluted (1 to 0.001) and subjected to quantitative real-time PCR. (A) The fluorescence increase resulted in threshold cycles which showed a clear concentration dependency. (B) This allowed for subsequent quantification. (C) The melting curve nicely showed that only one PCR product, identical in all samples, was amplified.

4.2.3.7 Preparation of 3'UTR-HIF reporter plasmids

To assess its impact on mRNA stability, the 3'UTR of HIF-1α (see Appendix III) was cloned into a pGL3-control plasmid (see Appendix III) showing constitutively high luciferase activity. For that, PCR products containing HIF-1α 3'UTR either in sense or anti-sense orientation were introduced behind the luciferase encoding region of the pGL3-control vector using the BD In-Fusion™ PCR Cloning Kit. This method is based on recombination processes performed by the BD InFusion enzyme.

Construction of the insert

For preparation of the inserts, forward and reverse primers against HIF-1α 3'UTR for sense and anti-sense, respectively, were designed (see 4.1.6). These primers contain 15 bps homologous to the region next to the Xba I restriction site (position: 1943 bp) of the pGL3-control vector. These 15mer sequences enable the recombination into the pGL3-control plasmid and result in sense 3'UTR-HIF-1α or anti-sense 3'UTR-HIF-1α integration, respectively.

For the PCR reaction, 5 µl of cDNA was mixed with 10 µl (= 10 pmol) forward and reverse primers each, 5 µl 10 x reaction buffer, 1 µl dNTPs (20 mM), and 2.5 u Pwo-DNA-polymerase, and filled up to 50 µl with distilled H_2O. The PCR was performed according to the following profiles:

Initial denaturation		95°C	2 min
Initiation cycle	Denaturation	95°C	30 sec
	Annealing	56°C	30 sec
	Elongation	72°C	4 min
Cycles: (3'UTR-HIF-1α: 35x)	Denaturation	95°C	30 sec
	Annealing	62°C	30 sec
	Elongation	72°C	4 min
Final extension		72°C	10 min

Subsequently, the PCR products were separated using 1% agarose gels in 0.5 x TBE, stained in ethidium bromide solution (0.5 mg/l) for 20 min, destained in H_2O, and visualized by UV excitation. The respective bands were excised from the agarose gel with a clean scalpel and the products were extracted out of the agarose using the NucleoSpin® PCR clean-up Gel extraction kit according to the manual. Briefly, the excised gel was lysed at 50°C until complete solubilization in 300 µl NT1 buffer per 100 mg gel. Then, the samples were loaded on NucleoSpin® Extract columns and centrifuged (8000 x g, 1 min). The binding of the DNA to the columns was followed by washing. For the first washing,

NT2 buffer (500 µl) was added to the column, then the column was centrifuged (11000 x g, 1 min), and the flow-through was discarded. The following washing steps were performed using NT3 buffer. First, 600 µl NT3 buffer were applied to the columns, followed by centrifugation (11000 x g, 1 min) and discarding of the supernatant. Then, 200 µl NT3 buffer were applied and the silica membrane was dried by centrifugation at 11000 x g for 2 min. Finally, the DNA was eluted by incubating the columns for 1 min at room temperature with the elution volume of 25 µl pre-heated (70°C) distilled H_2O, followed by a final centrifugation (11000 x g, 1 min).

Insertion of the PCR-fragment into a recipient vector (InFusion)

pGL3-control vector was digested using the Xba I restriction enzyme. In detail, pGL3-control vector (1 µg) was mixed with Buffer Y^+/Tango™ (2 µl) and Xba I (2 µl) and filled up to 20 µl with distilled H_2O. Then the mixture was incubated at 37°C for 2 h. For inactivation of the restriction endonuclease, incubation was continued at 65°C for 20 min. In order to minimize religation of the vector, the linearized vector was dephosphorylated by incubation at 37°C for 30 min with calf intestine alkaline phosphorylase (1 u). Subsequently, linearization of the vector was controlled by agarose gel separation (0.8% in 0.5 x TBE), followed by staining in ethidium bromide solution (0.5 mg/l) for 20 min, destaining in H_2O, and visualization by UV light.

Then, the linearized vector was used to receive the above-mentioned HIF-1α 3'UTR insert. The insertion was performed following the manufacturers guidelines for the BD In-Fusion™ PCR Cloning Kit. Briefly, equal amounts of insert and vector (100 ng each) were mixed with BD InFusion reaction buffer, BSA (50 µg/ml), and BD InFusion enzyme (20 u). The reaction mix was filled to 20 µl with sterile H_2O and incubated for 30 min at room temperature. Then, the reaction was stopped on ice and the resulting product was transformed into different competent *E. coli* strains according to the below-described protocol (see 4.2.4.2).

Verification of correct insertion by agarose gel electrophoresis

To verify the insertion of the HIF-1α 3'UTR into the pGL3-control vector, clones of the transformed *E. coli* were picked, transferred into 5 ml LB (containing 100 µg/ml ampicillin), and grown for 16 h at 37°C. Plasmid was isolated out of 2 ml of the resulting suspension culture using a FastPlasmid™ Mini kit according to the manufacturer's manual. Briefly, after centrifugation (12000 x g, 1 min), the medium was decanted, and 400 µl ice-

cold complete lysis solution were thoroughly mixed with the pellet by constant vortexing for 30 sec. The resulting suspension was incubated at room-temperature for 3 min before transfer to a spin column assembly. Thereafter, the assembly was centrifuged (12000 x g, 1 min) and the supernatant was discarded. Then, the column was washed with diluted wash buffer, i.e., 400 µl were added to the column, the assembly was centrifuged (12000 x g, 1 min), the supernatant was discarded, and the assembly was centrifuged again (12000 x g, 1 min) for drying purposes. The DNA was eluted using 50 µl sterile H_2O and centrifugation (12000 x g, 1 min).

Subsequently, 20 µl of plasmid were digested using the Xba I restriction enzyme (protocol see above), and the resulting fragments were separated on a 0.8% agarose gel in 0.5 x TBE. Finally, the gels were stained in ethidium bromide solution (0.5 mg/l) for 20 min, destained in H_2O, and visualized by UV excitation.

Verification of correct insertion by sequencing

To eventually prove that HIF-1α 3'UTR sense or anti-sense were actually inserted and also to demonstrate that the constructs did not receive mutations in the course of the different experimental steps, the plasmids were sequenced using the BigDye® Terminator v3.1 Sequencing protocol. In detail, pGL3-control HIF-1α 3'UTR (sense) or (anti-sense) plasmid (5 µl) were diluted with dilution buffer (1 µl). Then, 1 µl primer (10 pmol/µl), 3'UTR-HIF-1α (sense) (Infusion) or 3'UTR-HIF-1α (anti-sense) (Infusion), respectively, and 2 µl BigDye® Terminator mix were added, and the mixture was filled up to 10 µl with distilled H_2O. Subsequently, the mix was put to the following program:

Initial denaturation		96°C	1 min
Cycles:	Denaturation	95°C	10 sec
(25x)	Annealing	50°C	5 sec
	Elongation	60°C	4 min

The resulting products were precipitated by adding 90 µl distilled H_2O, 10 µl Na-acetate (3 M, pH 5.2), and 250 µl 99.8% ethanol to the reaction mix, followed by incubation at -80°C for 30 min. Then, the precipitate was pelleted (13000 x g, 4°C, 30 min), washed with 250 µl 70% ethanol (centrifugation: 13000 x g, 4°C, 10 min), and dried (in the dark).

The dried sequencing reaction product was then subjected to an ABI PRISM® 3100 16-Capillary-Sequencer with a 50 cm capillary system.
The resulting sequences were subsequently aligned to the predicted sequences of either the 3'UTR-HIF-1α pGL3-control (sense) or (anti-sense) plasmids (see Appendix IV). Further experiments were performed with a verified clone of the 3'UTR-HIF-1α pGL3-control (sense) or (anti-sense) plasmids each.

4.2.4 Microbiology

4.2.4.1 Preparation of competent *E. coli*

E. coli DH*5.1α* were rendered competent for transformation using the heat shock method. Bacteria were plated on LB agar (see Appendix I) and incubated overnight at 37°C. A single colony was picked and inoculated in 5 ml LB medium (see Appendix I) overnight at 37°C.
The bacteria were then added to 100 ml prewarmed medium and inoculated on a shaker at 37°C, until an OD_{600} of 0.5 was reached. Then, the culture was cooled on ice for 5 min, centrifuged (4000 x g, 4°C, 5 min), and the supernatant was gently discarded (on ice). The cells were resuspended in 30 ml cold TFB1 buffer (see Appendix I) and incubated on ice for an additional 90 min. Then, the cells were collected by centrifugation (4000 x g, 4°C, 5 min). The supernatant was discarded again (on ice), and the cells were resuspended in 4 ml ice-cold TFB2 buffer (see Appendix I). Thereafter, 100 µl aliquots were frozen in liquid nitrogen and stored at –80°C.

4.2.4.2 Transformation of bacterial cells by the heat shock protocol

E. coli DH*5.1α* were transformed using the heat shock protocol. For this purpose, bacteria were thawed on ice, and 100 ng of plasmid DNA were added. After gentle mixing by pipetting up and down, cells were incubated on ice for 30 min. After a heat shock (45 sec, 42°C), they were kept on ice for 2 min. Then, 400 µl of LB medium were added to the cells, followed by an incubation for 60 min at 37°C with continuous shaking.
Similarly, XL1-blue supercompetent cells, after being thawed on ice, received 1.7 µl β-mercaptoethanol and were maintained on ice for 10 min (gently swirling every 2 min). The following heat shock procedure was equivalent to the DH*5.1α* transformation

described above. Instead of adding LB medium, though, 400 µl SOC medium (see Appendix I) were added for initial growth at 37 °C (60 min).
To select transformed bacteria, 200 µl of the cultures were inoculated on ampicillin-containing (100 µg/ml) LB agar plates. A single clone was picked, cultured overnight at 37°C, and subsequently used for different purposes (e.g., for plasmid preparation or protein expression).

4.2.4.3 Bacterial culture and plasmid preparation

For preparation of plasmids, 2 ml LB medium with bacteria containing the required plasmid (e.g., 3'UTR-HIF-1α pGL3-control (sense) or (anti-sense) plasmids) were transferred to 400 ml LB medium containing 100 µg/ml ampicillin and grown at 37°C overnight. The isolation of the plasmid out of the bacteria was performed according to the manufacturer's manual using a HiSpeed™ Plasmid Maxi Kit. In detail, after pelleting the bacteria by centrifugation (600 x g, 4°C, 15 min), the pellets were resuspended in 10 ml buffer P1. Then, buffer P2 (10 ml) was added and the suspension was mixed gently but thoroughly by inverting 5 times, followed by 5 min incubation at room temperature. Thereafter, 10 ml chilled buffer P3 were added, immediately mixed and the lysate was transferred to the QIAfilter cartridge, where it was incubated for 10 min at room temperature. Subsequently, the lysate was filtered into a previously equilibrated (with 10 ml buffer QBT) HiSpeed Maxi Tip and the filters were washed using 60 ml buffer QC. Then, the DNA was eluted using 15 ml buffer QF and precipitated by addition of 10.5 ml isopropanol (5 min, room temperature). The eluate/isopropanol mix was filtered through a QIAprecipitator Maxi Module using constant pressure. After washing the bound DNA with 2 ml 70% ethanol, it was dried by quickly pressing air through the QIAprecipitator repeatedly. Finally, the DNA was eluted using 1 ml H_2O, and the DNA content was measured on an Ultraspec 2100 pro photometer.

5 Results

5.1 p53-mediated regulation of HIF-1

HIF-1 is known to be regulated at multiple levels. Among the factors described to be involved in regulatory mechanisms, p53 was shown to interact with HIF-1α, though many details of this interaction remain obscure. The purpose of the following experiments was to assess the influence of p53 on HIF-1. For that, I examined the effects of p53 on HIF-1α protein, as well as HIF-1 transcriptional activity.

5.1.1 HIF-1α protein accumulation under hypoxia/anoxia

It is widely accepted that HIF-1α protein accumulates under conditions of low oxygen tension. To test if this prerequisite also applies to my system, I exposed RKO cells to either hypoxia (0.5% O_2) or anoxia (0% O_2) for different periods of time.

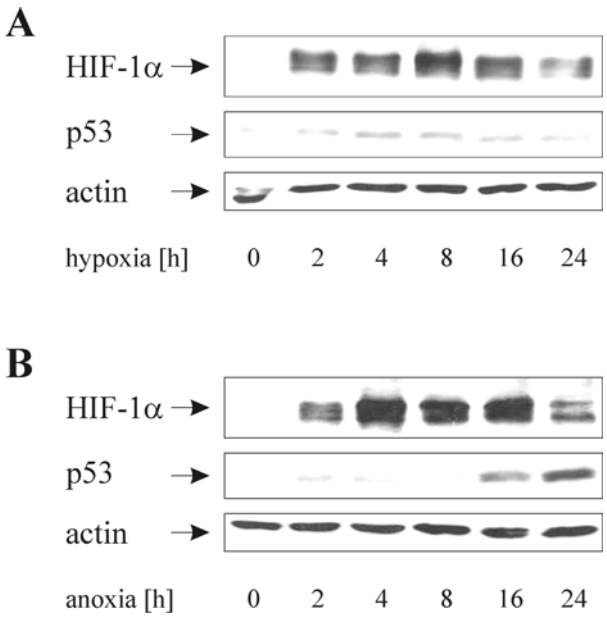

Figure 6: Expression of HIF-1α and p53 under hypoxia/anoxia.

HIF-1α and p53 protein accumulation were determined in RKO cells following exposure to increasing periods of hypoxia (A) or anoxia (B). HIF-1α, p53, and actin protein levels were determined by Western analysis. Blots are representative for at least 3 independent experiments.

Under these experimental conditions, HIF-1α accumulated similarly under both hypoxic (Figure 6A) and anoxic (Figure 6B) conditions to a maximum at 8-16 h, thereafter decreasing again towards 24 h. In addition, prolonged incubations under anoxia, in contrast to hypoxia, caused an accumulation of p53. First traces of p53 were detectable at 16 h, with a further increase at 24 h of anoxic incubations.

These results gave first indications that down-regulatory mechanisms might become active after prolonged periods of oxygen depletion, an involvement of p53 remaining uncertain, though.

5.1.2 Impact of p53 on HIF-1α protein accumulation

As the impact of p53 on HIF-1α has been controversially discussed in recent literature, I intended to further elucidate the impact of p53 protein on HIF-1α protein. For this purpose, I transiently transfected RKO cells with a p53 expression plasmid, resulting in constitutively elevated p53 protein levels. Overexpressed p53, which contained an HA-tag, was slightly larger than the endogenous p53, thus making a differentiation between endogenous and exogenous p53 possible (Figure 7).

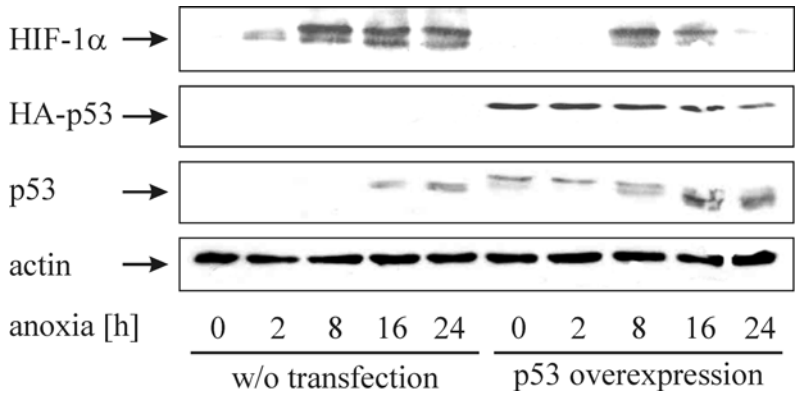

Figure 7: Expression of HIF-1α under anoxia in combination with p53 overexpression.

HIF-1α protein accumulation was determined in parent (w/o transfection) or p53 overexpressing RKO cells, following exposure to increasing periods of anoxia. HIF-1α, p53, HA-p53, and actin protein levels were determined by Western analysis. Blots are representative for at least 3 independent experiments.

Transient overexpression of p53 in RKO cells caused a marked decrease in HIF-1α protein levels mainly at incubation periods longer than 16 h, indicating that the initial

accumulation of HIF-1α remained largely unaffected and only the following down-regulation phase under enduring anoxia was enforced. I attributed these observations to the fact that p53 (endogenous plus exogenous) was sufficient to show distinct effects on HIF-1α protein at these later time-points.

In order to verify that the observed effects could indeed be attributed to p53, I transiently transfected different amounts of p53 plasmid into RCC4 cells. These cells constitutively accumulate HIF-1α even under normoxia due to a defect of pVHL, which consequently leads to a disturbed HIF-1α degradation.

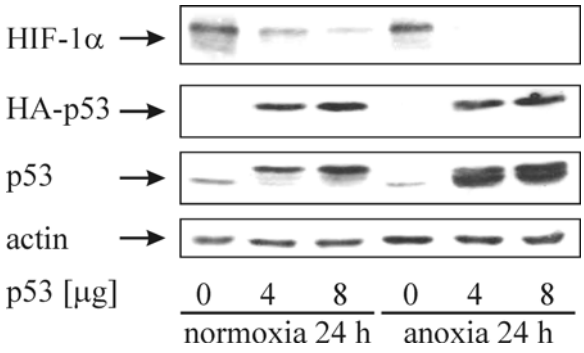

Figure 8: HIF-1α stability under anoxia in combination with p53 overexpression in RCC4 cells.

HIF-1α, HA-p53, endogenous plus exogenous p53 (p53), and actin were determined by Western analysis, following 24 h of normoxia or anoxia in RCC4 cells. Cells remained untransfected or were transfected with 4 µg vs. 8 µg of p53 expression plasmid. Blots are representative for at least 3 independent experiments.

RCC4 cells displayed strongly reduced HIF-1α protein levels in response to transient overexpression of p53 (Figure 8). This effect depended on the amount of plasmid introduced into the cells and became more pronounced under anoxic conditions. Under the latter conditions, additional accumulation of endogenous p53 seemed to be stimulated by exogenous p53 expression, thus resulting in an increase in the total p53 protein load. The defect of pVHL in these cells also indicates that the observed HIF-1α degradation is pVHL-independent.

Based on these results, I suggest that high p53 protein levels in the cell are able to cause down-regulation of HIF-1α protein via a pVHL-independent pathway.

5.1.3 Apoptotic events under anoxia and p53 overexpression

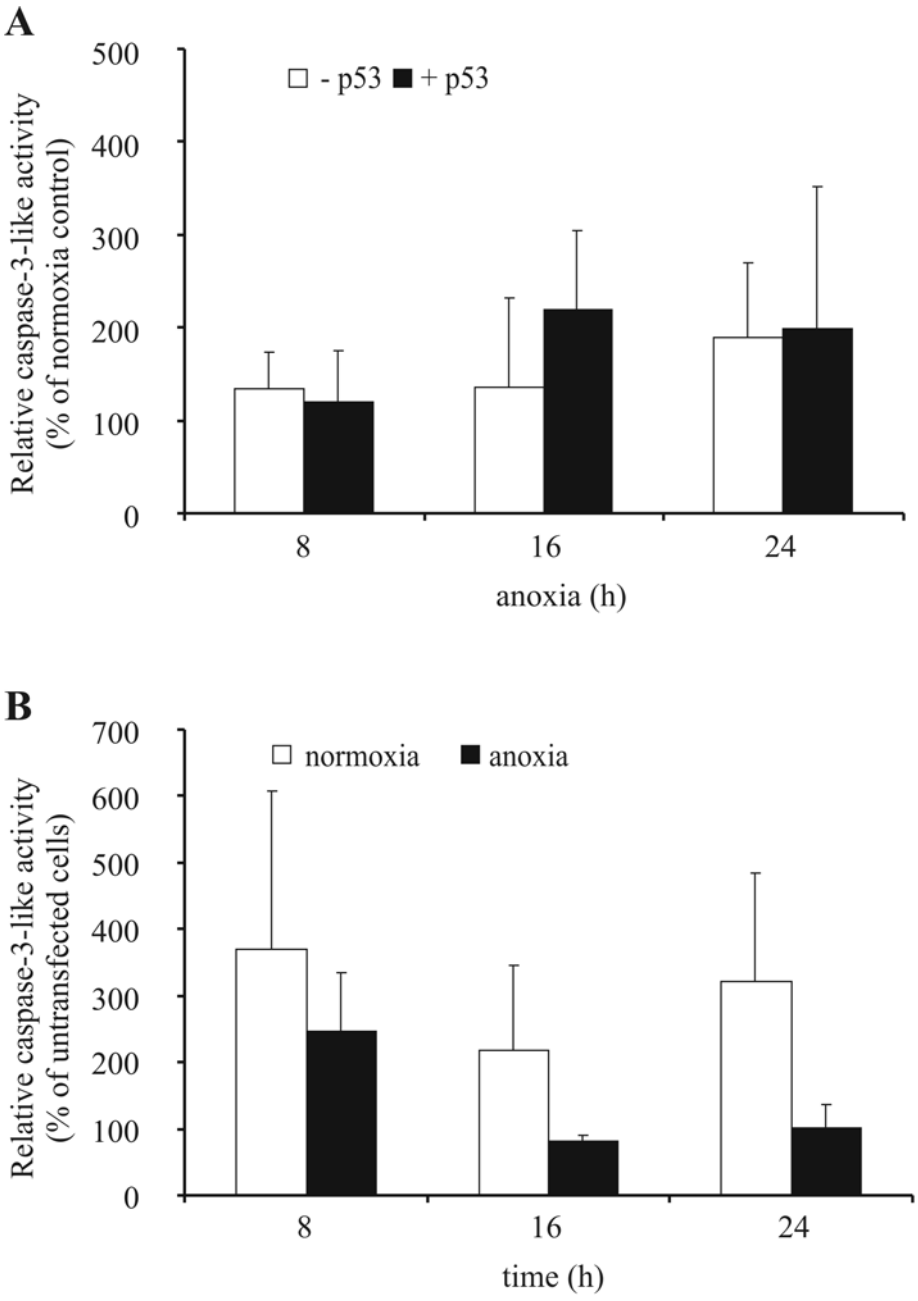

Figure 9: Caspase-3-like activity under anoxia and/or p53 overexpression.

Caspase-3-like activity was determined using fluorometric measurement of cleaved DEVD-AMC in lysates of RKO cells. Cells were either untransfected or transfected with p53 expression plasmid (5 µg) and exposed to normoxia or anoxia for 8, 16 or 24 h. Caspase-3-like activity was normalized to (A) normoxia-treated cells or (B) non-transfected cells. Data are mean values ± SD, $n \geq 3$.

To ensure that the observed changes in HIF-1α protein were not due to cell death, RKO cells were transiently transfected with a p53 overexpression plasmid and exposed to normoxia or anoxia. Subsequently, caspase-3-like activity was measured.

Anoxia did not induce apoptosis to any significant degree in RKO cells with or without p53 overexpression compared to normoxia-treated cells (Figure 9A). A slight increase at 24 h (independent of the p53 overexpression status) was not significant.

Similarly, no significant caspase activity was observed under p53-overexpressing conditions (Figure 9B). The fact that no correlation between caspase-3-like activity and duration of the treatment could be established further strengthened the point that neither anoxia nor p53 overexpression caused cell-death.

Consequently, I excluded the possibility that the observed changes in HIF-1α protein were due to cell death artifacts, and, therefore, concluded that they indeed represent changes on the protein level.

5.1.4 Impact of anoxia and p53 overexpression on p53 transcriptional activity

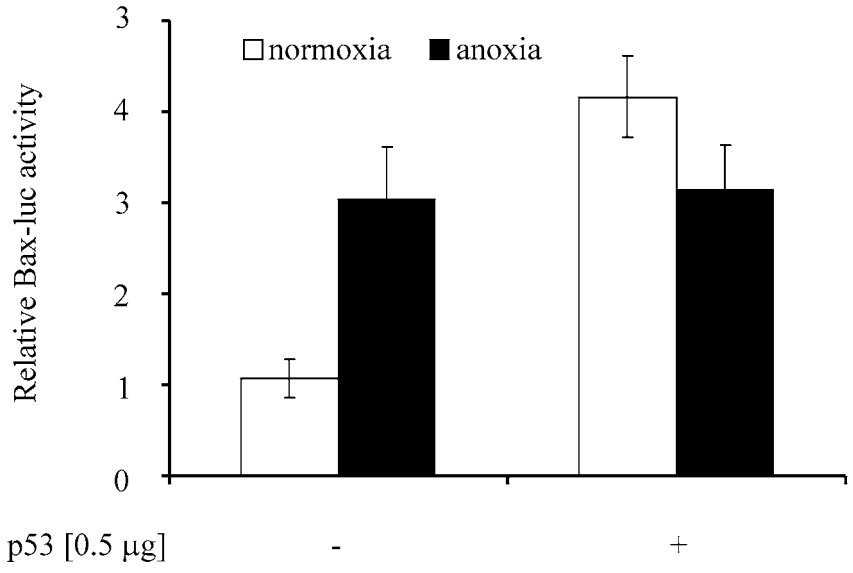

Figure 10: p53 transcriptional activity during anoxia and/or p53 expression.

RKO cells transfected with 0.5 µg Bax-luciferase plasmid were exposed to either normoxia or anoxia for 24 h under conditions of co-transfection with or without 0.5 µg control p53 expression plasmid. Relative Bax-luciferase activity is normalized to cells incubated under normoxia for 24 h. Data are mean values ± SD, $n \geq 3$.

As contradicting reports exist concerning the transcriptional status of p53 under oxygen-depleted conditions, I designed the following experiments to assess the transcriptional activity of endogenous p53 under anoxia, as well as that of p53 introduced into the cells via transient overexpression. For that purpose, I transfected RKO cells with a Bax-luciferase reporter plasmid containing p53 response elements (Lohrum and Scheidtmann, 1996) and then exposed them to anoxia and/or co-transfection with plasmids forcing p53 expression.
Overexpression of p53 caused a 4.2 ± 0.5 fold increase in Bax-luciferase activity compared to cells transfected with Bax-reporter alone. Anoxia (0% O_2, 24 h) evoked p53 stabilization and induced Bax-luciferase activity 3.0 ± 0.6 fold compared to normoxia-treated cells, while anoxia did not induce further Bax-luciferase activity under p53-overexpressing conditions (Figure 10). These results demonstrate that both endogenous p53 accumulating under anoxia and transiently overexpressed p53 are transcriptionally active in this system.

5.1.5 Impact of p53 on HIF-1 transcriptional activity

The observation that p53 actually is transcriptionally active under these conditions led me to speculate that the impact of p53 on HIF-1 may not only be seen on the protein level. Thus, I went on to investigate HIF-1 transactivation. I co-transfected RKO cells with the pGL-Epo-HRE plasmid (Epo-luc), which harbors three copies of the erythropoietin (Epo) hypoxia-responsive element (HRE) in front of a luciferase construct, as described previously (Kietzmann *et al.*, 2001), with either a p53 expression plasmid or a pcDNA3 control plasmid. Luciferase activity consequently represents a measure for HIF-1 transactivation.
Since hypoxia (0.5% O_2, 24 h), in contrast to anoxia (0% O_2, 24 h), did not induce endogenous p53 expression (Figure 6A) (Wenger *et al.*, 1998), experiments were performed under hypoxic conditions in order to ensure that modulation of HIF-1 transactivation resulted exclusively from exogenous p53. Hypoxia caused a 2.5 ± 0.6 fold induction of Epo-luciferase activity compared to normoxia (Figure 11A). Transient overexpression of p53 attenuated HIF-1 transcriptional activity by roughly 75% under normoxic conditions, i.e., p53 blocked basal reporter activity. Importantly, under hypoxia, expression of p53 substantially lowered Epo-luciferase activity. p53 reduced HIF-1 transactivation below control, i.e., normoxia, values. Differences reached statistical significance (ANOVA: $F = 64.205$, $P < 0.0001$, Fisher's PLSD: $P \leq 0.0001$).

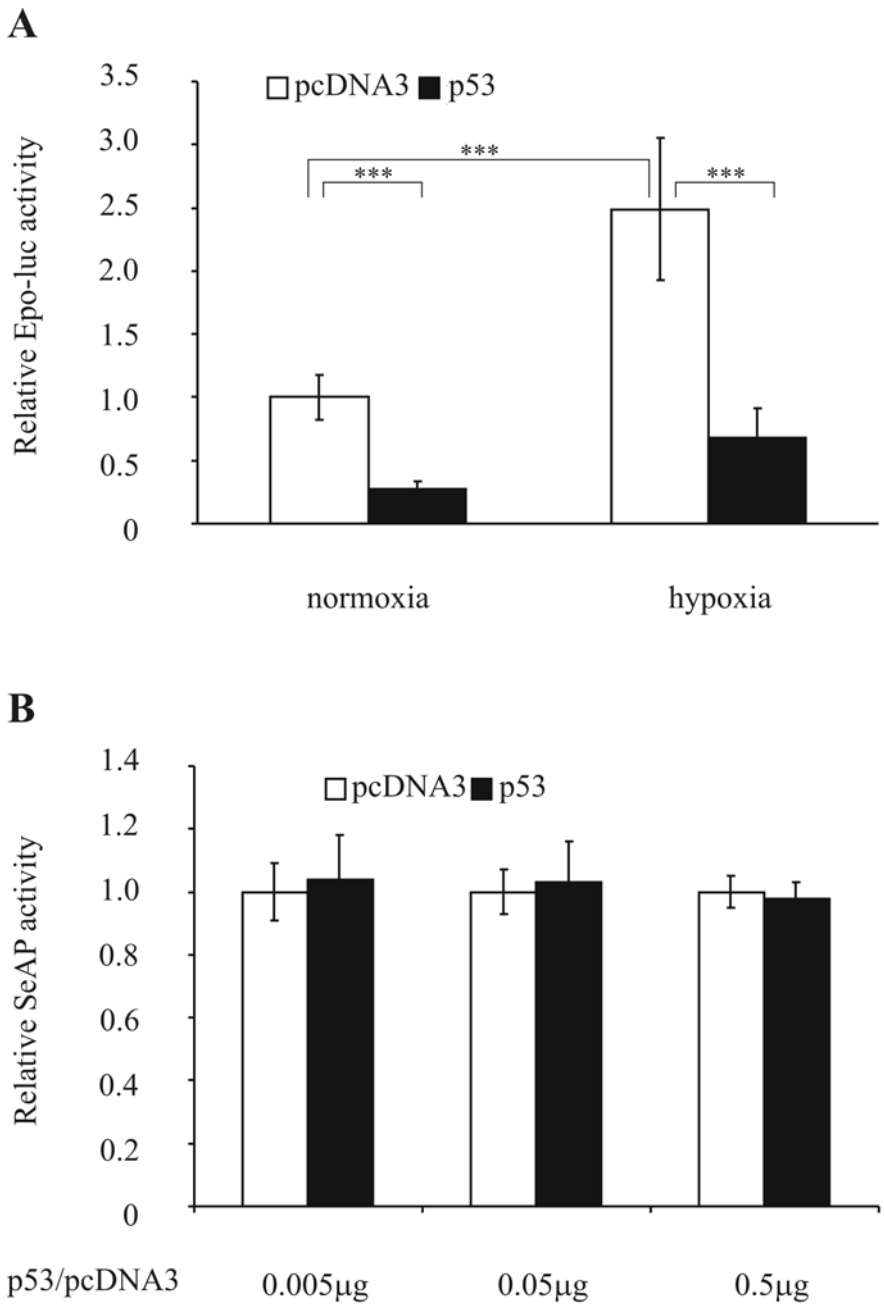

Figure 11: HIF-1 transcriptional activity during hypoxia and p53 expression.

(A) RKO cells transfected with 0.5 µg Epo-luciferase plasmid were exposed to either normoxia or hypoxia for 24 h under conditions of co-transfection with 0.5 µg control plasmid (pcDNA3, open columns) or 0.5 µg p53 expression plasmid (p53, filled columns). Relative Epo-luciferase activity is normalized to control plasmid transfected cells incubated under normoxia for 24 h. Data are mean values ± SD, $n \geq 4$ (ANOVA: $F = 64.205$, $P < 0.0001$, Fisher's PLSD test: ***: $P \leq 0.0001$). (B) RKO cells transfected with 0.5 µg SeAP reporter plasmid were exposed to hypoxia for 24 h under conditions of co-transfection with 0.5 µg control plasmid (pcDNA3, open columns) or 0.5 µg p53 expression plasmid (p53, filled columns). Relative SeAP activity is normalized to cells transfected with corresponding amounts of control plasmid.

In order to exclude unspecific effects of p53, I co-transfected a constitutive active SeAP reporter plasmid with either a p53 expression plasmid or the pcDNA3 control plasmid. p53 did not inhibit SeAP activity compared to the control plasmid (Figure 11B). Apparently, p53, besides affecting HIF-1α stability under anoxia, also reduced HIF-1 transcriptional activity under hypoxic conditions. An interference of p53 with the transcriptional activity of HIF-1 may result from competition for limiting co-activators such as p300.

To verify that the observed effects did not result from transfection artifacts, the expression of endogenous erythropoietin was measured using RT-PCR. Since RKO cells did not show measurable Epo-mRNA levels, experiments were conducted in a hepatocyte cell line (HepG2). Overexpression of p53 decreased Epo-mRNA expression considerably when 15 µg of p53 were transfected (Figure 12). Thus, it can be assumed that p53 is able to suppress HIF-1 transcriptional activity under physiological conditions.

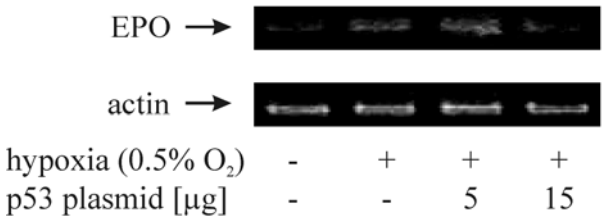

Figure 12: Epo mRNA during hypoxia and p53 expression.

HepG2 cells transfected with 5 µg or 15 µg p53 expression plasmid were exposed to hypoxia for 24 h. Epo-mRNA content was assessed using RT-PCR.

5.1.6 In vitro binding of p53 to HIF-1α

Since one possible reason for the inhibition of HIF-1 transactivity by p53 is a direct binding of p53 to HIF-1α, I incubated *in vitro* transcribed/translated ^{35}S-p53 protein (100 µl) with a spot membrane containing overlapping peptide fragments of HIF-1α protein each 15 amino acids in length (Frank, 1992). Detection was carried out at -70°C for 7 days. For reasons of comparability, binding of bacterially expressed hsp70 (30 µg) was performed in parallel.

p53 did not show binding to any part of HIF-1α (Figure 13A). The control protein hsp70 bound to the peptide fragments containing the amino acids 403 to 417 of HIF-1α (Figure 13B.I). The strong binding observed at amino acids 682-699 (Figure 13B.II) stemmed from HIF-1α antibody, which had been tested in a previous experiment and

showed an extremely strong binding measurable even after multiple stripping steps. The control binding underlines the sensitivity of this system, thus even strengthening the observation that a direct interaction between these two transcription factors is highly unlikely.

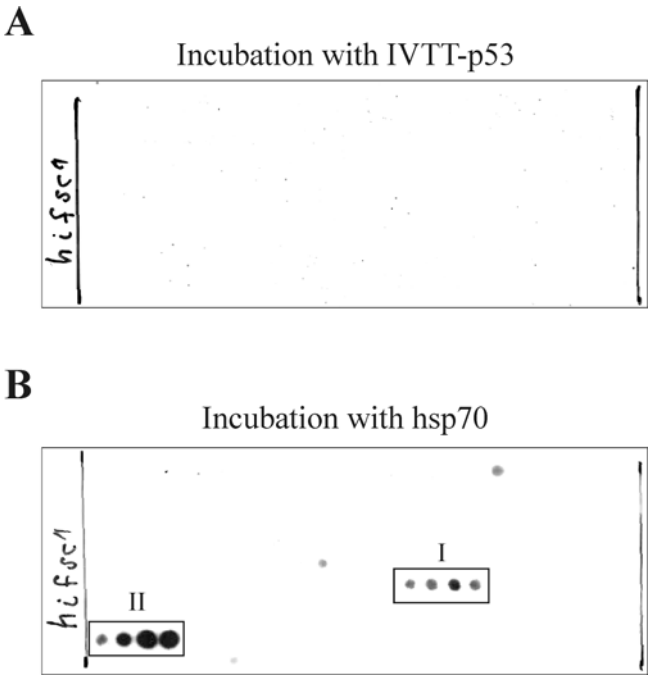

Figure 13: Binding of IVTT-^{35}S-p53 to a HIF-1α peptide spot membrane.

A peptide-spot membrane containing overlapping peptide fragments of the HIF-1α protein (15 aa each) was incubated overnight at 4°C with (A) IVTT-generated ^{35}S-p53 (100 µl) or (B) bacterially expressed hsp70 (30 µg). Detection of radiolabelled p53 was carried out at –70°C for 7 days.

Therefore, I considered the competition for co-factors required for the activity of both transcription factors as another mechanism behind the transcriptional repression of HIF-1 by p53.

5.1.7 Impact of p300 on p53-mediated HIF-1 transcriptional repression

One protein which is required for full transcriptional activity of both HIF-1 and p53 is the transcriptional co-factor p300. To test whether limiting amounts of p300 explain impaired HIF-1 transactivation under the influence of p53, I co-transfected RKO cells with a p300 expression plasmid (1 µg) and increasing amounts of either a p53 or a pcDNA3 control plasmid, in addition to the Epo-luciferase reporter plasmid (0.5 µg). Hypoxia (0.5% O_2,

24 h)-evoked HIF-1 transcriptional activity decreased with co-transfection of increasing amounts of p53 expression plasmid (Figure 14A).

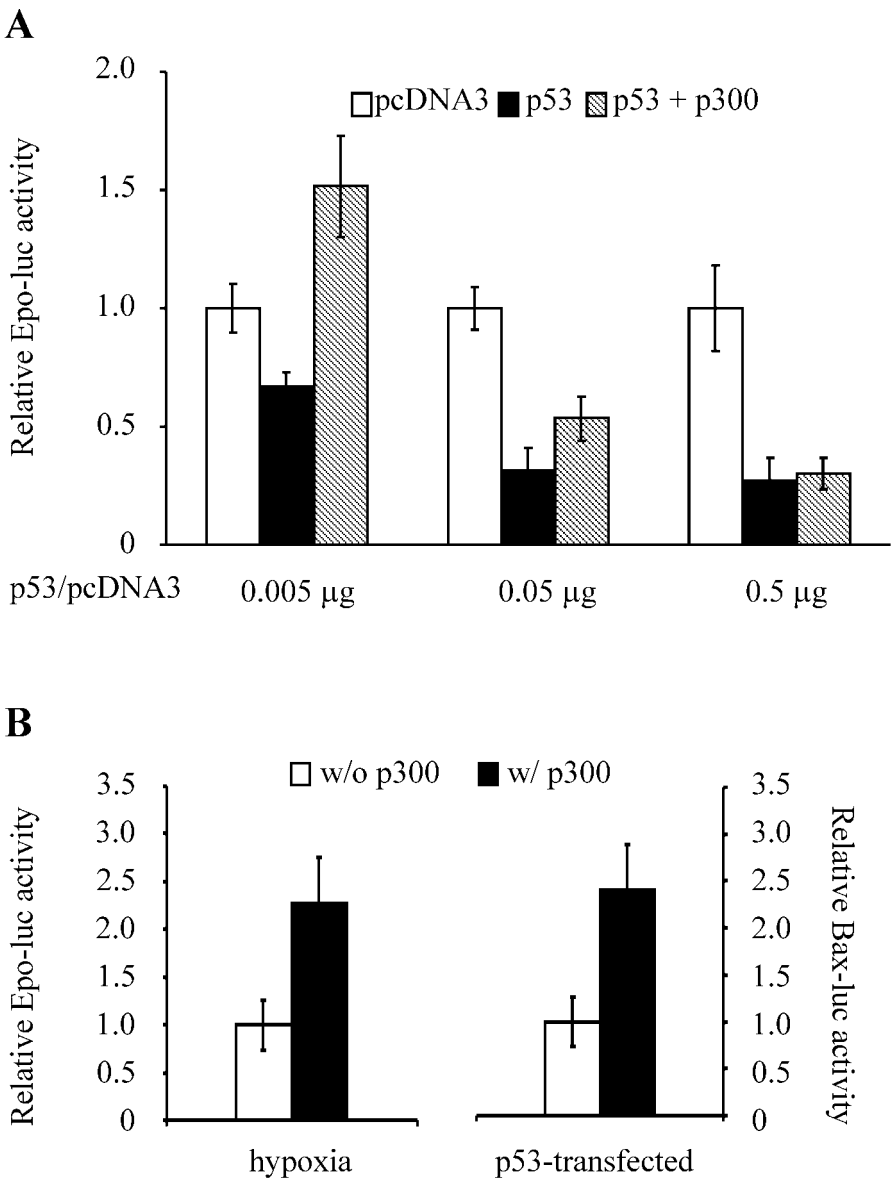

Figure 14: HIF-1 transcriptional activity during hypoxia in the presence of p300 and p53.

(A) RKO cells were exposed to hypoxia for 24 h under conditions of transfection with indicated amounts of control plasmid (pcDNA3, open columns), p53 expression plasmid (p53, filled columns), and p53/p300 (1 µg) co-expression (p53+p300, hatched columns). Relative Epo-luciferase activity is normalized to control plasmid transfected cells exposed to hypoxia for 24 h. (B) RKO cells were exposed to hypoxia for 24 h with or without co-transfection of 1 µg p300 expression plasmid. Relative Epo-luciferase activity is normalized to cells without p300 transfection. Alternatively, cells were transfected with plasmids allowing for expression of p53 (0.5 µg), Bax-luciferase and/or p300 (1 µg). Relative Bax-luciferase activity is normalized to cells transfected with p53 only. Data are mean values ± SD, $n \geq 4$ (ANOVA: $F = 48.726$, $P < 0.0001$).

Co-transfection of p300 altered Epo-luciferase activity significantly, partially reversing the inhibition seen with p53. Co-transfection of 0.005 µg p53 and p300 provoked 1.52 ± 0.21 fold stimulation of luciferase activity compared to the inhibition seen with p53 alone, the difference being significant ($P < 0.0001$). Co-transfection of p300 in the presence of 0.05 µg p53 plasmid significantly reversed the inhibition seen with p53 alone ($P = 0.0114$). Importantly, inhibition by p53 was around 70%, while co-transfection of p300 reduced this value to roughly 50%. Increasing the amount of p53 expression plasmid to 0.5 µg eliminated the action of p300 in reversing inhibition. Therefore, I conclude that p53-mediated HIF-1 inhibition can be antagonized by p300, though at low p53 expression only.

To ensure that p300 is indeed a limiting factor for both HIF-1 and p53 transcriptional activity, RKO cells were either co-transfected with an Epo-luciferase plasmid (0.5 µg) and a p300 expression plasmid (1 µg) and incubated under hypoxic conditions for 24 h, or co-transfected with plasmids expressing Bax-luciferase (0.5 µg), p53 (0.5 µg), and p300 (1 µg). Both HIF-1 and p53 reporter activity significantly increased upon co-expression of p300 (Figure 14B).

These results indicate that p300 is limiting for both p53 and HIF-1 transcriptional activity and that there is a direct competition between p53 and HIF-1α for binding of p300, eventually limiting HIF-1 transcriptional activity.

5.1.8 Competitive binding of HIF-1α and p53 to p300

In order to exclude that the described p300-mediated reversal of p53-evoked HIF-1 transactivity inhibition was a stimulatory effect of p300 on HIF-1 transactivity only, and not a direct competition as proposed, an *in vitro* competition binding assay was employed. For this purpose, I used *in vitro* transcription/translation in the presence of ^{35}S-methionine to generate ^{35}S-p53 and ^{35}S-HIF-1α proteins. Subsequently, these proteins were incubated with bacterially expressed p300-CH1 bound to GSH-agarose. Only the CH1-domain of p300 was used, since this region has been identified as containing a binding site for both p53 and HIF-1α (Freedman *et al.*, 2002; Dial *et al.*, 2003).

Increasing concentrations of p53 reduced binding of HIF-1α to p300-CH1. Relative densitometric analysis of HIF-1α bound to p300 showed a decrease from starting values of 100% to values around 30% to 40% (Figure 15A). To exclude unspecific effects, I replaced p53 with unprogrammed reticulocyte lysate (URL). URL displayed no such effect on HIF-1α binding (Figure 15B), supporting the specificity of the observed effects.

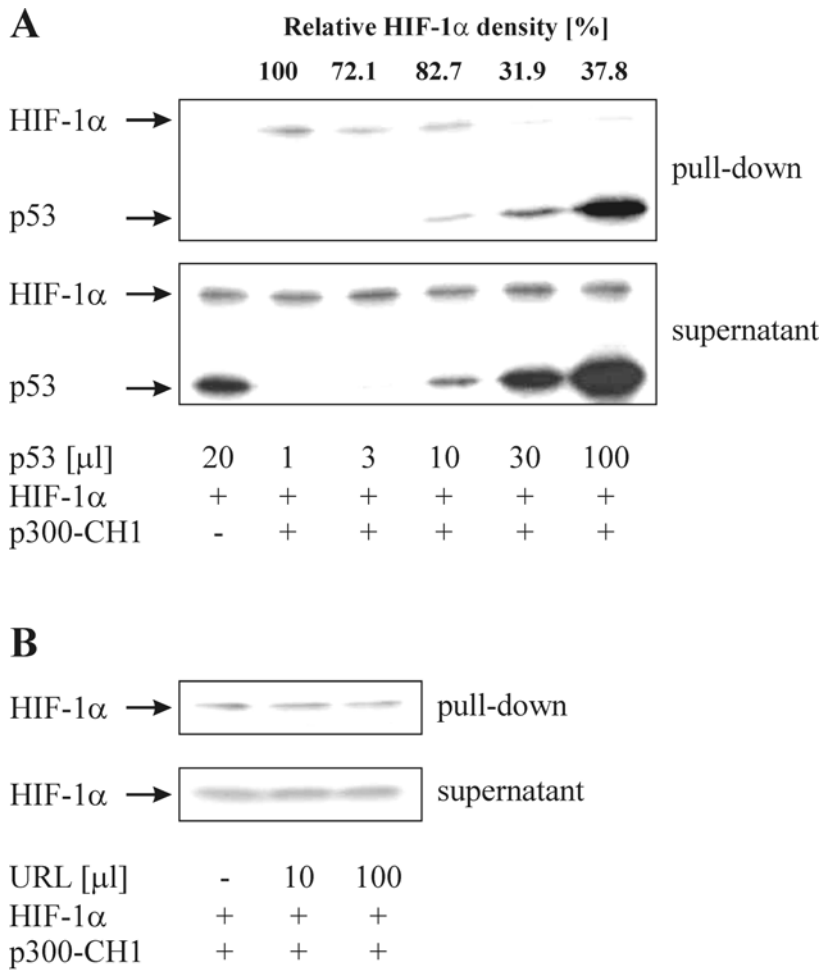

Figure 15: Competitive binding of HIF-1α and p53 to p300.

(A) IVTT-generated ^{35}S-p53 and/or ^{35}S-HIF-1α, (B) ^{35}S-HIF-1α and/or unprogrammed reticulocyte lysate (URL) were incubated at different ratios with p300-CH1-bound GSH-agarose. Subsequently, the pull-down and the supernatant, as loading control, were analyzed for bound p53 or HIF-1α by autoradiography. HIF-1α pulled down by p300-CH1-bound GSH-agarose was determined by densitometry.

5.1.9 HIF-1 regulatory mechanisms under hypoxia and anoxia

The first part of the experiments was carried out to study the regulation of HIF-1 by p53, a protein accumulating only under conditions of prolonged anoxia, but not under hypoxia. As this already demonstrates that different regulatory mechanisms might be involved under hypoxia vs. anoxia, the second part of the experiments was designed to further characterize the differences between hypoxic vs. anoxic regulation of HIF-1. Therefore, HIF-1α mRNA regulation was chosen as a another target of investigation.

5.2 Regulation of HIF-1α mRNA

The second part of the experiments was intended to characterize mechanisms regulating HIF-1α mRNA levels. Therefore, I determined HIF-1α mRNA content and half-life under hypoxia/anoxia and, furthermore, I tried to relate possible changes in HIF-1α mRNA to transcriptional or post-transcriptional events.

5.2.1 Impact of hypoxia and anoxia on HIF-1α mRNA

It has long since been a dogma that HIF-1α regulation under oxygen depletion occurs mainly, if not entirely, on the protein synthesis and/or degradation level. Only recently has it been reported that negative regulation might also occur on the mRNA level (Uchida *et al.*, 2004).

In order to test whether changes in HIF-1α mRNA expression occur, RKO cells were incubated under hypoxia (0.5% O_2) or anoxia (0% O_2) from 4 to 24 h, and, subsequently, changes in HIF-1α mRNA relative to actin mRNA were determined using reverse transcription and quantitative PCR with respect to normoxia-treated controls. To verify the identity of the amplified fragments, the same cDNA samples were analyzed using conventional PCR.

Hypoxia did not show an influence on HIF-1α mRNA (Figure 16A). The relative HIF-1α mRNA level remained between 92% and 120% of the normoxia values, the changes not being significant at any time. In contrast to hypoxia, anoxia significantly decreased HIF-1α mRNA. Relative HIF-1α levels decreased from 88% at 4 h via 78% at 8 h and 55% at 16 h to 53% at 24 h of anoxia. These data suggest that prolonged and complete depletion of oxygen causes down-regulation of HIF-1α mRNA. Since hypoxia did not show any effects on HIF-1α mRNA even though the protein strongly accumulated (Figure 6), these results are partially in contrast to a recent report in which down-regulation of HIF-1α mRNA was proposed to be HIF-1 regulated (Uchida *et al.*, 2004).

The agarose gel separation verified the identity of the amplified products, the HIF-1α PCR product being 460 bp and the actin PCR fragment being 660 bp long, as predicted by sequence analysis (Figure 16B). Moreover, these data supported the results of the quantitative analyses, with HIF-1α mRNA levels strongly decreasing at 16 h and 24 h of anoxia while the actin mRNA content remained constant.

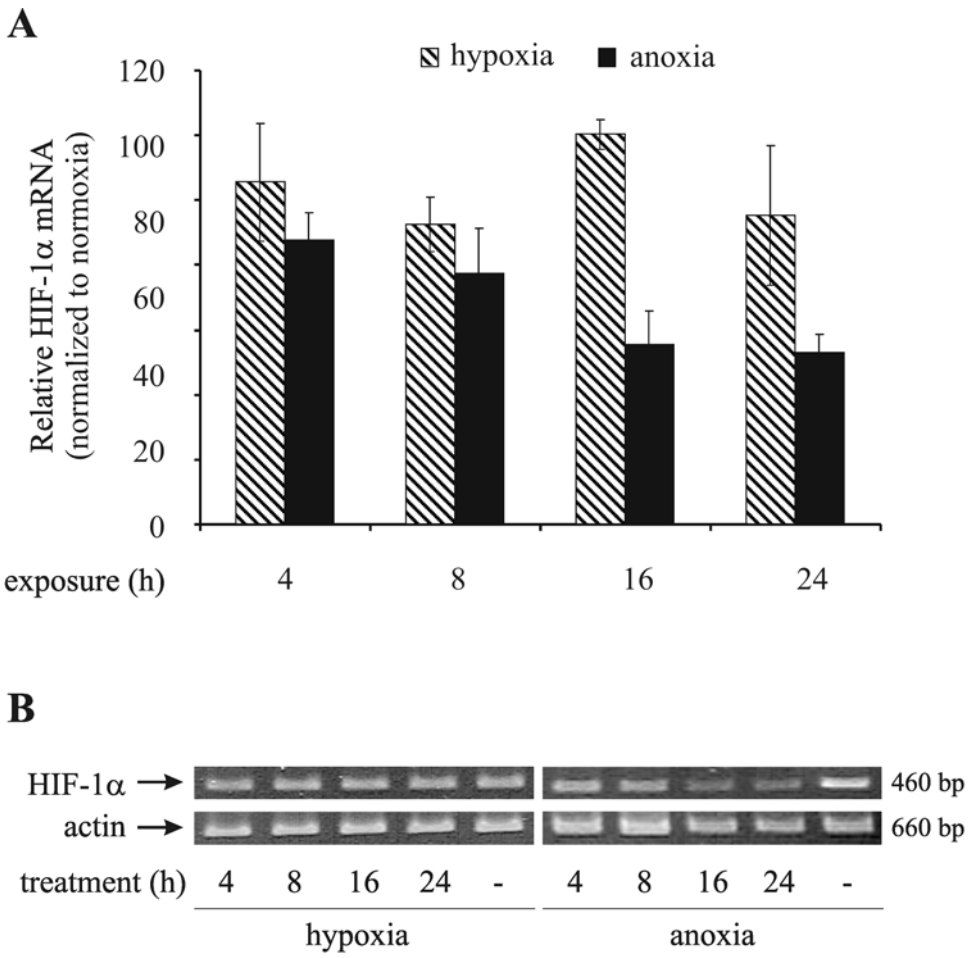

Figure 16: HIF-1α mRNA levels under hypoxia and anoxia.

(A) RKO cells were incubated to hypoxia (hatched columns) or anoxia (filled columns) for increasing periods of time. Thereafter, HIF-1α mRNA (relative to actin mRNA) was measured using reverse transcription and subsequent quantitative real-time PCR. Data are normalized to normoxia values and are mean values ± SD, n ≥ 4. (B) The same cDNA samples were analyzed using conventional PCR, agarose gel electrophoresis, and subsequent ethidium bromide staining to verify the identity of the amplified HIF-1α and actin fragments. Gels are representative for at least 3 independent experiments.

5.2.2 Impact of hypoxia and anoxia on HIF-1α transcription

To test whether the down-regulation of HIF-1α mRNA results from transcriptional changes, a reporter construct containing 5 kb of the HIF-1α promotor in front of a luciferase construct (Iyer *et al.*, 1998b) was transfected into RKO cells, prior to incubation to either normoxia, hypoxia or anoxia for 24 h.

Hypoxia as well as anoxia caused an approximately 2.5-fold increase of HIF-1α promotor activity (Figure 17), which is in accordance with previously published data (Iyer *et al.*, 1998b). The fact that HIF-1α promotor activity was increased rather than decreased under

the tested conditions suggests that HIF-1α transcription is not affected by oxygen depletion, at least not negatively. Thus, I assumed that HIF-1α mRNA down-regulation under prolonged anoxia is most likely due to post-transcriptional regulation.

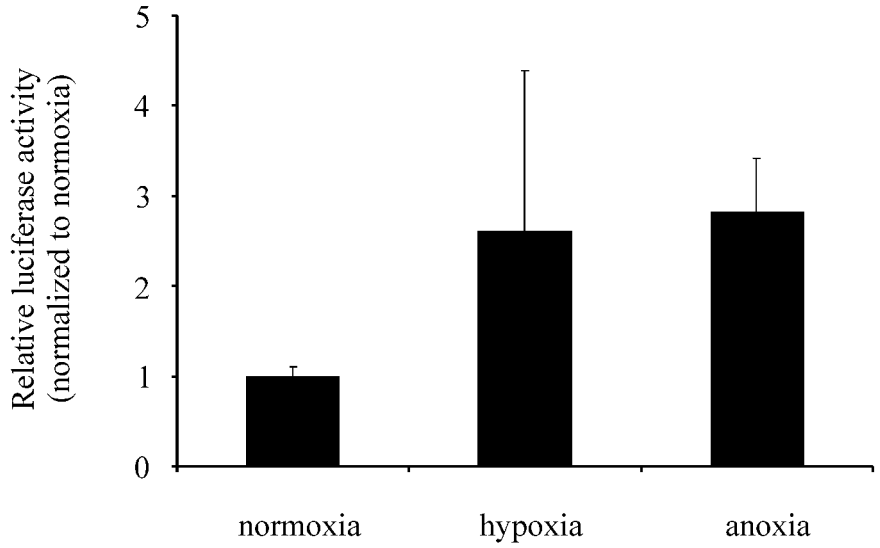

Figure 17: Impact of hypoxia and anoxia on HIF-1α promotor activity.

RKO cells were transfected with 1 µg pGL3-basic plasmid containing 5 kb of the HIF-1α promotor, followed by incubation to normoxia, hypoxia or anoxia for 24 h. Luciferase activity was normalized to normoxia-treated cells. Data are mean values ± SD, n ≥ 3.

5.2.3 Impact of protein translation inhibition on HIF-1α mRNA under anoxia

To further characterize the mechanisms involved, I tried to determine if general protein synthesis is required for HIF-1α mRNA regulation by incubating RKO cells under anoxia with co-treatment of the translation inhibitor cycloheximide (CHX) [10 µM]. CHX was added either at the beginning of the exposure or in the middle of the treatment.

As figure 18 nicely illustrates, 12 h of anoxic treatment caused a pronounced decrease in HIF-1α mRNA to 37% of normoxia levels. Co-treatment with CHX completely restored HIF-1α mRNA levels to normoxia values. Differences were measurable neither if CHX was added for 6 h (104%) nor for 12 h (87%). These results indicate that protein synthesis is essential for the down-regulation of HIF-1α mRNA under prolonged anoxia, which is in accordance with data published by Uchida *et al.* (2004).

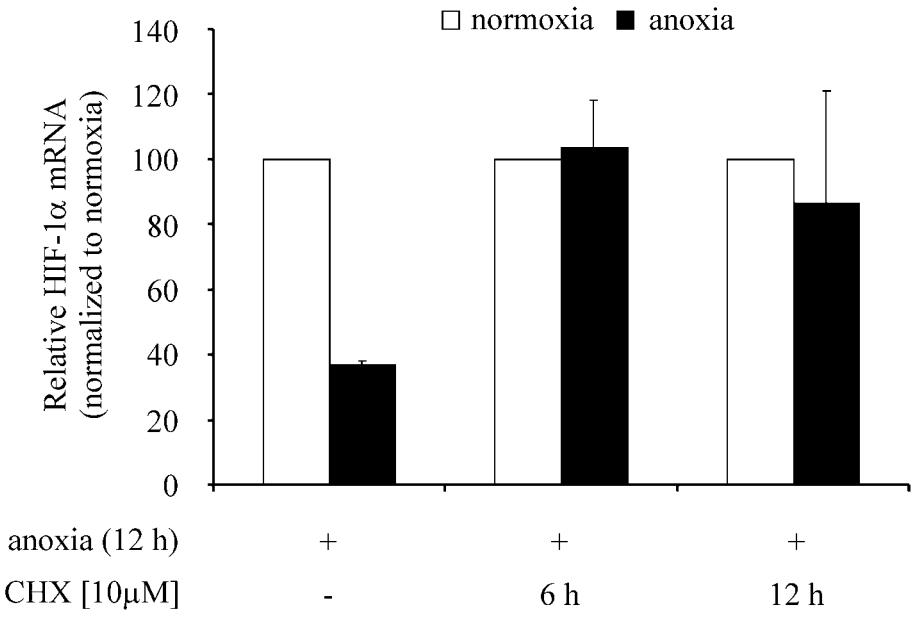

Figure 18: Impact of CHX on HIF-1α mRNA levels under anoxia.

RKO cells were incubated under normoxic (open columns) or anoxic (filled columns) conditions for 12 h. CHX [10 μM] was added either after 6 h of the respective treatment, with subsequent continuation of normoxia/anoxia for 6 h, or at the beginning of the incubations. Thereafter, HIF-1α mRNA (relative to actin mRNA) was measured using reverse transcription and subsequent quantitative real-time PCR. Data are normalized to normoxia values and are mean values ± SD, $n \geq 3$.

To further characterize the temporal pattern of events, I exposed RKO cells to anoxia for increasing periods of time, applying CHX after 6 h.

Anoxia caused a time-dependent decrease in relative HIF-1α mRNA from 70% at 8 h to 36% and 37% at 12 h and 16 h, respectively. At 8 h of anoxia with 2 h of CHX co-treatment, no effects of CHX were measurable (mRNA levels reaching 65%). 6 h and 10 h of CHX co-treatment, i.e., 12 h and 16 h of anoxia, restored HIF-1α mRNA levels (89% and 103%, respectively) (Figure 19). The fact that, after 8 h of anoxia, no restoration of HIF-1α mRNA could be achieved by translation inhibition either indicates that the required protein is synthesized at anoxic exposures longer than 8 h, or that 2 h of CHX treatment were not sufficient to eliminate the synthesis of the candidate protein. This further strengthens the hypothesis that, in contrast to the above-mentioned report, other mechanisms than HIF-1-mediated events might be involved in the observed regulatory processes, since HIF-1α protein is already present after only 4 h of anoxic treatment (Figure 6B).

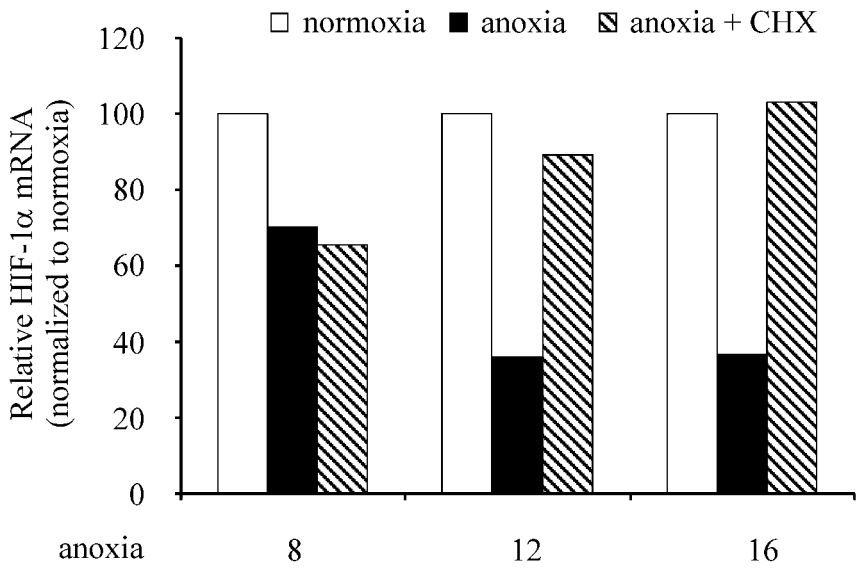

Figure 19: Impact of CHX on HIF-1α mRNA levels under anoxia (temporal validation).
RKO cells were incubated for increasing periods to normoxia (open columns), anoxia (filled columns) or anoxia with CHX (hatched columns). CHX [10 μM] was added after 6 h, and anoxic incubation was continued until completion of the entire anoxia treatment. Thereafter, HIF-1α mRNA (relative to actin mRNA) was measured using reverse transcription and subsequent quantitative real-time PCR. Data are normalized to normoxia values.

As the results presented do not yet clearly identify the mechanisms potentially involved in HIF-1α mRNA (down-) regulation, I tried to determine HIF-1α mRNA half-life, in order to further characterize these processes.

5.2.4 Impact of hypoxia and anoxia in combination with protein translation inhibition on HIF-1α mRNA half-life

To study HIF-1α mRNA half-life, RKO cells were pre-incubated under hypoxia or anoxia for 8 h before actinomycin D [5 μg/ml], a transcription inhibitor, was added. Then incubations were continued for up to 8 h.

Hypoxia did not cause any changes to HIF-1α mRNA half-life as compared to normoxia, mRNA remaining between 83% and 96% (Figure 20). Calculations indicated that HIF-1α mRNA did not change under hypoxia. Thus, I assumed that the half-life remained unaltered under hypoxia when compared to normoxia. Anoxia caused HIF-1α mRNA levels to decrease rather rapidly to levels between 20% and 35% at 4 h to 8 h post

actinomycin D treatment. Exponential calculation of the changes resulted in a half-life of 3.78 h.

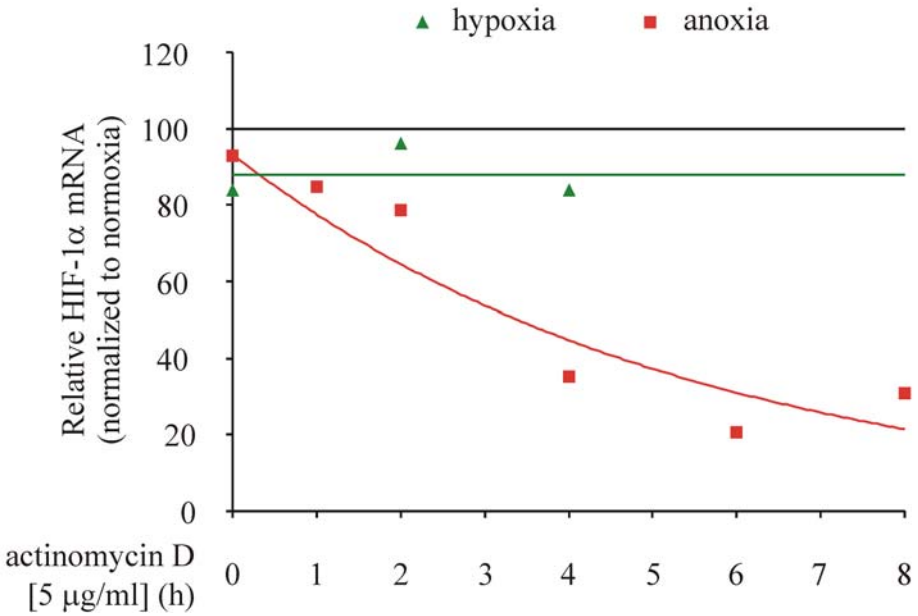

Figure 20: HIF-1α mRNA half-life under hypoxia and anoxia.

RKO cells were pre-incubated for 8 h under either hypoxia (green Δ) or anoxia (red □). Then, actinomycin D [5 µg/ml] was added to inhibit transcription, and the HIF-1α mRNA content (relative to actin mRNA) was measured using an RT with subsequent quantitative real-time PCR. Data are normalized to normoxia values (black line) and exponential trend lines have been calculated.

This again demonstrates the differences between hypoxia and anoxia. The requirement of a complete depletion of oxygen also rules out the suggestion of an entirely HIF-1-mediated mechanism. In combination with the lack of changes in HIF-1α transcription (Figure 17), these data suggest post-transcriptional changes in HIF-1α mRNA stability.

In order to test if inhibition of translation shows an effect on HIF-1α mRNA half-life under anoxia, cells were pre-exposed to anoxia for 8 h (CHX being added after 4 h to ensure its effects) before actinomycin D was added and exposure continued for up to 8 h.

Translation inhibition restored HIF-1α mRNA half-life completely to normoxic values (Figure 21). Again, calculation of the half-life indicated that no changes occurred as compared to normoxia.

These results underscore that HIF-1α mRNA down-regulation under prolonged anoxia was dependent on protein synthesis. They further suggest the regulation to be post-transcriptional.

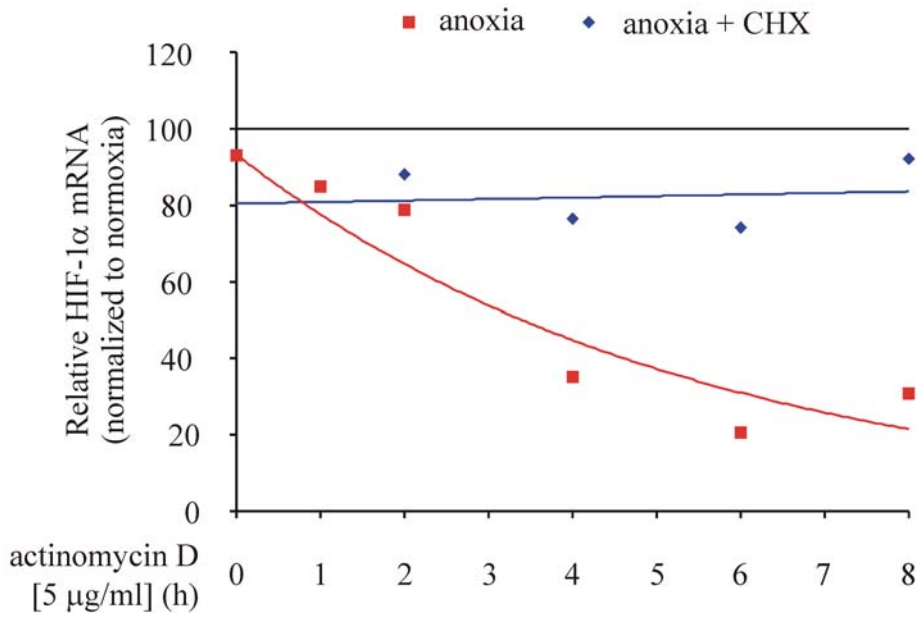

Figure 21: HIF-1α mRNA half-life under anoxia with or without co-treatment with CHX.
RKO cells were pre-incubated for 8 h under anoxia (red □). In the CHX treated cells (blue ◇), CHX [10 µM] was added after 4 h of pre-treatment. Then, actinomycin D [5 µg/ml] was added to inhibit transcription, and the HIF-1α mRNA content (relative to actin mRNA) was measured using RT with subsequent quantitative real-time PCR. Data are normalized to normoxia values (black line) and exponential trend lines have been calculated.

5.2.5 Impact of anoxia on HIF-1α 3'UTR mediated mRNA stability regulation

During the last few years, it has been appreciated that 3' untranslated regions (3'UTRs) of mRNA which contain adenylate- and uridylate-rich (AU-rich) sites are often involved in mRNA stability regulation. Protein factors involved in this process bind to these sequences and either stabilize or destabilize mRNA. The presence of AU-rich elements in the 3'UTR of HIF-1α mRNA potentially allows for the involvement of such mechanisms in HIF-1α mRNA regulation.

To test if HIF-1α mRNA might be regulated via the 3'UTR, the 3' UTR was introduced in a pGL3-control vector behind the luciferase encoding region. The resulting plasmids

(pGL3-control 3'UTR-HIF sense or pGL3-control; 0.5 µg) were transfected into RKO cells. Exposure to anoxia was carried out for 24 h.

HIF-1α 3'UTR (sense) caused luciferase activity to decrease to 83 ± 5% of control plasmid transfected cells when incubated under anoxia. At the same time, HIF-1α 3'UTR (sense) caused a decrease in normoxia values to 94 ± 5% (Figure 22).

This indicates that HIF-1α 3'UTR actually has a destabilizing effect on mRNA (in this case luciferase mRNA).

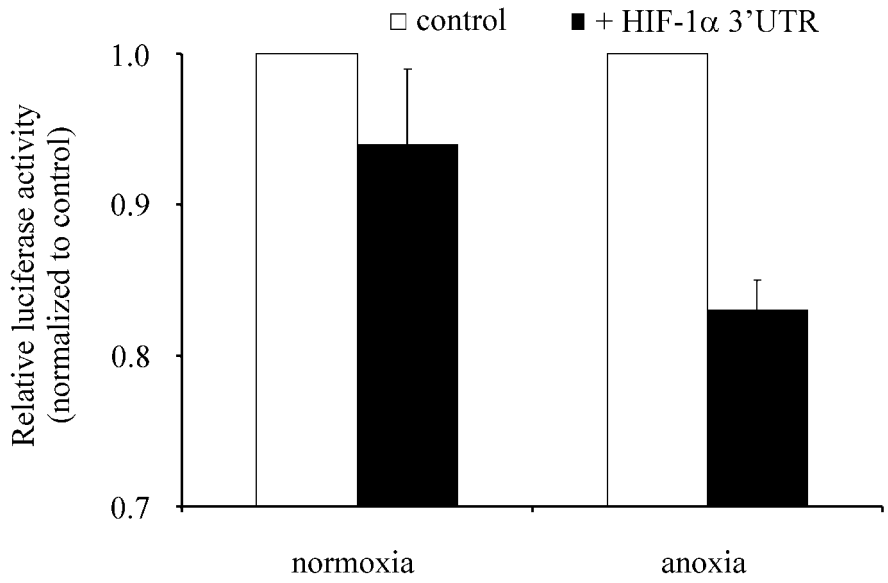

Figure 22: Impact of HIF-1α 3'UTR on mRNA stability.

RKO cells were transfected with either 0.5 µg pGL3-control plasmid (open columns) or 0.5 µg pGL3-control plasmid containing the HIF-1α 3'UTR (sense) behind the luciferase encoding region (filled columns). Transfected cells were incubated to normoxia or anoxia for 24 h. Luciferase activity was normalized to control plasmid transfected cells. Data are mean values ± SD, $n \geq 3$.

The fact that the observed effect was rather small may be due to the extremely high activity of the used vector system. The pGL3-control vector contains in addition to the SV40 promotor, an SV40 enhancer element 3' to the poly-A signal. Thus, the absolute values were very high, and the activity might possibly "over-run" the system, thus making a negative regulation only detectable to a low degree.

5.2.6 Impact of anti-sense HIF-1α 3'UTR on mRNA stability regulation

As Uchida *et al.* (2004) reported that the regulation of HIF-1α mRNA stability might be controlled by natural anti-sense HIF-1α mRNA (asHIF-1α), I tried to assess the impact of anti-sense orientated HIF-1α 3'UTR on mRNA stability by introducing HIF-1α 3'UTR into the pGL3-control vector in anti-sense orientation, subsequently transfecting RKO cells with HIF-1α 3'UTR (sense and anti-sense) containing pGL3-control plasmid (0.5 µg) or native pGL3-control plasmid, and exposing them to either normoxia or anoxia.

HIF-1α 3'UTR (anti-sense) caused a massive decrease in luciferase activity to approximately 20% of the control plasmid transfected cells under both normoxia and anoxia (Figure 23). This inhibition was much more pronounced than the inhibition induced by the sense orientated HIF-1α 3'UTR.

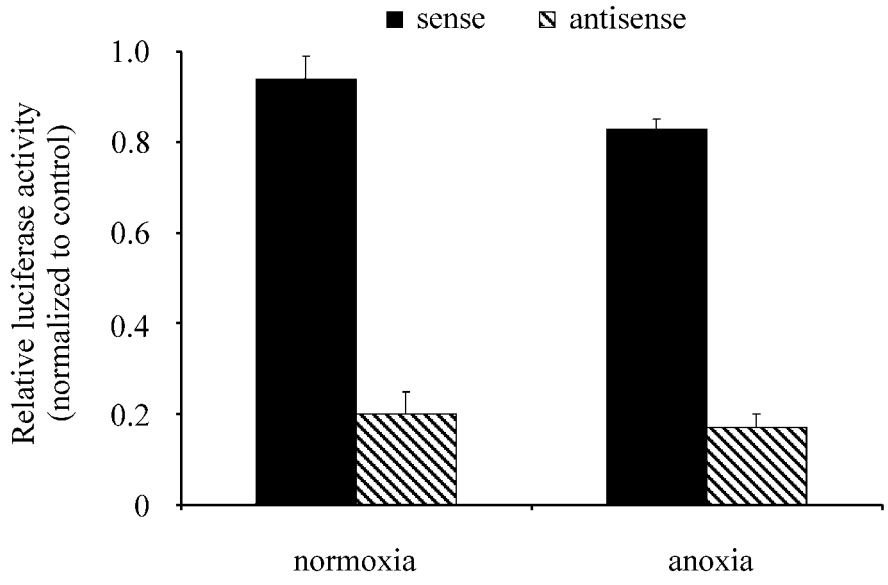

Figure 23: Impact of the orientation of HIF-1α 3'UTR on mRNA stability.

RKO cells were transfected with 0.5 µg pGL3-control plasmid containing HIF-1α 3'UTR either in sense (filled columns) or anti-sense (hatched columns) orientation behind the luciferase encoding region. Transfected cells were incubated to normoxia or anoxia for 24 h. Luciferase activity was normalized to control plasmid transfected cells. Data are mean values ± SD, $n \geq 3$.

Therefore, I considered the proposed destabilizing factor to bind to anti-sense instead of sense 3'UTR. Although the anti-sense construct used only represents a part of the natural asHIF-1α, it was sufficient to account for the regulatory function. Since the regulation

apparently did not require the binding of the anti-sense 3'UTR to the regulated mRNA, I concluded that binding of regulating factors (most likely destabilizing in nature) does not occur directly to HIF-1α 3'UTR, which would be considered a *cis*-effect, but rather indirectly via binding to asHIF-1α, thus being a *trans*-effect. This hypothesis is different from the current model, which suggests that binding of natural asHIF-1α allows for better presentation of AU-rich elements in the 3'UTR of HIF-1α mRNA.

6 Discussion

HIF-1α accumulation and HIF-1 transactivation constitute the master regulatory system for coping with conditions of reduced oxygen availability (Wenger, 2002). The regulatory mechanisms of HIF-1 activation under oxygen depletion have largely been attributed to the orchestrated action of specific oxygen-dependent hydroxylases, which mediate either inhibition of HIF-1 activity, i.e., FIH-1 (Mahon *et al.*, 2001; Lando *et al.*, 2002a; Linke *et al.*, 2004), or degradation of HIF-1α via ubiquitination, i.e., the PHDs (Maxwell and Ratcliffe, 2002; Berra *et al.*, 2003; Metzen *et al.*, 2003a; Metzen and Ratcliffe, 2004). In addition, the HIF-1 system is known to be activated by other factors, such as growth factors, hormones, or nitric oxide, as well (Semenza, 2002; Stiehl *et al.*, 2002; Brune and Zhou, 2003; Huang and Bunn, 2003; Pugh and Ratcliffe, 2003). The exclusive attribution of HIF-1 regulation to hydroxylases has increasingly been challenged by recent reports, indicating that oxygen depletion as well as other HIF-1-regulating stimuli require more than just an intact hydroxylase system. For instance, HIF-1 regulation is influenced by phosphorylation or acetylation (Jeong *et al.*, 2002) and stabilization by chaperones (Minet *et al.*, 1999; Mabjeesh *et al.*, 2002; Isaacs *et al.*, 2004; Zhou *et al.*, 2004). An increase of HIF-1 translation compared to the general reduction of translation that is observed under hypoxia has also been reported (Gorlach *et al.*, 2000; Lang *et al.*, 2002). On the other hand, it became obvious that under prolonged and severe hypoxic stimulations, HIF-1 can be down-regulated again, allowing the affected cells either to be eliminated, if the stimulus proves to be too severe or too long, or to terminate the HIF-1 response in order to return to the normal state after adaptation to the new environment. Again, after an initial attenuation of PHD levels in response to oxygen depletion (Nakayama *et al.*, 2004), the regulatory mechanisms under prolonged conditions of hypoxia were attributed mainly to increased accumulation/induction of the PHDs (Cioffi *et al.*, 2003; D'Angelo *et al.*, 2003). This pattern of regulation apparently depends on HIF-1 activity, thus providing a negative feed-back self-regulatory mechanism (Cioffi *et al.*, 2003). It became apparent that the severity and duration of hypoxia might also prove decisive for HIF-1 stability regulation and activation, especially concerning the decision between preservation of cell viability vs. initiation of cell death (Halterman *et al.*, 1999; Piret *et al.*, 2002).

Therefore, the aims of my study were to investigate the differences induced by conditions of low O_2 vs. no O_2 with respect to the impact on the HIF-1 response. More specifically, I focused on two recently identified regulatory mechanisms:

(1) the impact of p53 on HIF-1 under prolonged hypoxia/anoxia, which might give further insights regarding the decision between pro-survival vs. pro-cell-death signals, and

(2) the impact of prolonged hypoxia/anoxia on HIF-1α mRNA, which might allow for characterization of a completely new regulatory principle for HIF-1.

6.1 Impact of p53 on HIF-1 under prolonged hypoxia/anoxia

In the first part of the experiments, I showed that HIF-1α accumulated similarly under hypoxia and anoxia, reaching maximum levels after 8 h and decreasing thereafter. Accumulation and activation of p53 only occurred under prolonged anoxic treatments, though. Overexpression of transcriptionally active p53 resulted in a remarkable down-regulation of HIF-1α in RKO cells under anoxia. The fact that the same response was observed in pVHL-deficient RCC4 cells demonstrates that pVHL-independent mechanisms are responsible for p53-mediated attenuation of the HIF-1 accumulation. At the same time, hypoxic transactivation of HIF-1 was attenuated by enforced expression of p53, an effect which was reversed by overexpression of p300, thus indicating that a limitation of p300 might be involved. Additional *in vitro* binding assays substantiated the idea of a competition between HIF-1α and p53 for binding to limited amounts of the shared co-activator p300 as the mechanistic explanation. This conclusion was further strengthened by the exclusion (i) of a direct binding of p53 to HIF-1α via a spot membrane binding assay and (ii) of apoptosis by use of caspase activity assays.

The issue of p53-mediated regulation of HIF-1 is controversially discussed in recent literature. There is unquestionable evidence that p53 is a target under conditions of severe/prolonged hypoxia, although conflicting reports exist on the exact conditions required. Some groups reported that 6 h of anoxia were sufficient to accumulate p53 (An *et al.*, 1998; Hammond *et al.*, 2002), while others claimed that 48 h were required (Suzuki *et al.*, 2001). Reports concerning transactivation of p53 also revealed discrepancies. Some studies suggest transactivation of p53 by anoxia (Zhu *et al.*, 2002), whereas others showed a stabilized but transcriptionally inactive p53 protein (Koumenis *et al.*, 2001). Recently, p53 accumulation due to oxygen depletion alone has been questioned entirely by Pan and co-workers, who showed that p53 accumulation depended on acidosis, rather than oxygen depletion, in several cell lines (Pan *et al.*, 2004). Although this group used oxygen

concentrations as low as 0.02% O_2, it remains unclear whether anoxia might be the trigger for additional responses, such as p53 accumulation, and, thus, the O_2 concentrations used were still too high. As shown in this study, prolonged periods of anoxia beyond 16 h were required to cause accumulation of transcriptionally active p53 in RKO cells, as determined by a Bax-luciferase reporter assay (Figure 6B and Figure 10). In contrast, hypoxia (0.5% O_2) neither stabilized p53 nor induced its transcriptional activation (Figure 6A). Previously, p53 has been shown to down-regulate HIF-1α protein by reducing its half-life (Ravi *et al.*, 2000). In contrast to a report claiming a direct interaction between the core domain of p53 and HIF-1α as the mechanistic background (Hansson *et al.*, 2002), I ruled out this possibility by showing that wild-type p53 did not bind a HIF-1α spot membrane (Figure 13). This is in line with more recent evidence suggesting that Mdm2 is required to bridge the two proteins, thereby facilitating HIF-1α degradation (Chen *et al.*, 2003). Destruction of HIF-1α by p53 is corroborated by results showing that transient overexpression of p53 markedly reduced HIF-1α protein. Anoxia accelerated this process, most likely due to activation of endogenous p53, as seen in RCC4 cells (Figure 8). It is of interest that overexpression of p53 reduced, but did not abolish, the initial phase of HIF-1α accumulation in RKO cells under anoxia (Figure 7), while stabilization of endogenous p53 further enhanced the degradation of HIF-1α. Subsequent experiments in RCC4 cells provided unequivocal proof that p53 uses a pVHL-independent mechanism to destabilize HIF-1α, which may support the concept of the E3-ligase Mdm2 being capable of ubiquitinating and marking HIF-1α for degradation (Ravi *et al.*, 2000). The HIF-1α–Mdm2–p53 system can be considered to be part of an increasingly complex feed-back system for limiting HIF-1 responses under prolonged hypoxia/anoxia (Berra *et al.*, 2001; Berra *et al.*, 2003; Mottet *et al.*, 2003).

Besides affecting HIF-1α protein and, thus, indirectly hindering HIF-1α–HIF-1β-complex formation as well as target gene activation, direct inhibition of HIF-1 transcriptional activity by p53 constitutes an additional level of regulation. Attenuated HIF-1 transactivity by p53 has been suggested previously (Blagosklonny *et al.*, 1998; Ravi *et al.*, 2000) and was confirmed by my observations in RKO cells (Figure 11 and Figure 12). Since I excluded direct binding of p53 to HIF-1α by the absence of p53 binding to a HIF-1α spot-membrane (Figure 13), the limitation of the transcriptional co-activator p300 offers a mechanistic explanation (Arany *et al.*, 1996; Avantaggiati *et al.*, 1997; Lill *et al.*, 1997; Sang *et al.*, 2003). This hypothesis is further supported by findings that the binding site at

p300 for both p53 and HIF-1α is the CH1 domain, although p53 additionally complexes with the CH3 domain (Freedman *et al.*, 2002). My experiments revealed that p53 transactivity as well as HIF-1 transactivity is enhanced by co-transfection of p300, supporting the notion that p300 is limiting for both transcription factors (Figure 14B). At the same time, co-transfection of p300 reversed the inhibition of HIF-1 responsive reporter activity facilitated by low-level p53 expression (Figure 14A). As experiments *in vitro* showed competition of p53 and HIF-1α for binding to p300, I presumed that the same mechanism operates in cells (Figure 15). However, p300 did not antagonize inhibition of HIF-1 reporter activity if p53 overexpression was high enough to affect HIF-1α protein level. This implies that, depending on the p53 expression level, distinct mechanisms suppress HIF-1.

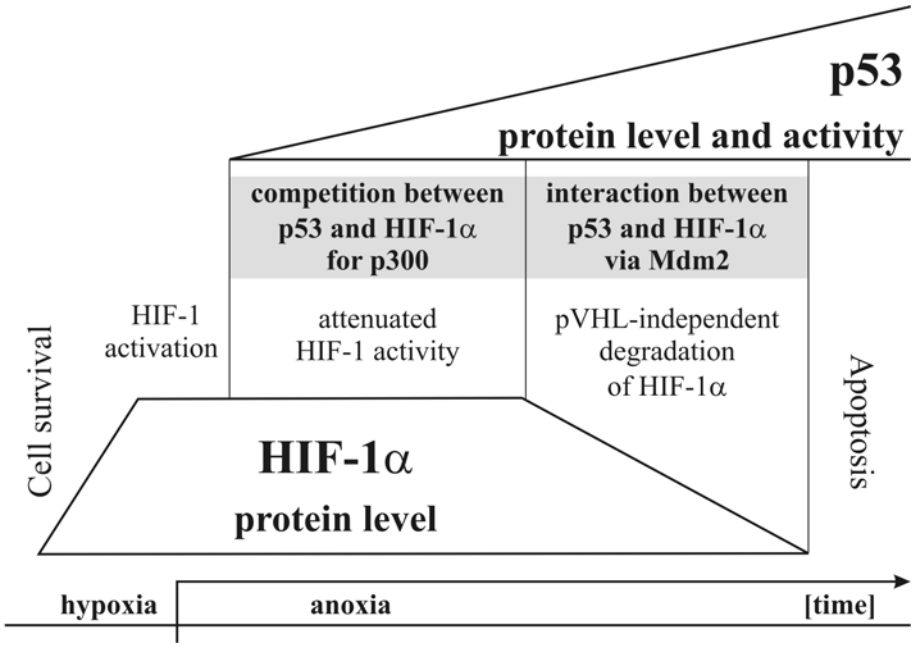

Figure 24: Proposed model of HIF-1 – p53 interactions

Hypoxic induction of HIF-1 transactivation is repressed under conditions of prolonged anoxia due to p53 accumulation and competition for p300. Under prolonged anoxia, p53 protein levels further increase and promote pVHL-independent HIF-1α destruction.

This allows for the proposal of the following model of HIF-1 – p53 interactions (Figure 24). Thus, hypoxic conditions cause HIF-1α accumulation and HIF-1 transactivation. Under more severe conditions, i.e., anoxia, p53 accumulates and competes

with HIF-1α for binding to p300, which results in HIF-1 transcriptional repression. With prolonged treatment, amounts of p53 increase, which in turn facilitates HIF-1α degradation.

This model is supported by findings that p53 mutated in its p300-binding domain failed to block HIF-1 transactivation (Blagosklonny *et al.*, 2001). In contrast to the present study, the same group could did not achieve reversal of p53-induced HIF-1 transactivation inhibition by p300 overexpression (Blagosklonny *et al.*, 1998). These differences might be explained by the amount of p53 transfected, as p300 releases HIF-1 inhibition only if p53 is expressed at low levels. These results provide further evidence for the importance of p53 in modulating HIF-1 responses. Moreover, this might have implications for oxygen depletion-induced cell death, i.e., the induction of HIF-1 transcriptional activity could be necessary for hypoxia-induced cell protection in the early phase, while the inhibition of HIF-1 and induction of p53 activity in response to more severe conditions could eventually result in the initiation of cell death processes in the late phase.

As these results demonstrate that different regulatory mechanisms might be involved in hypoxia vs. anoxia, the second part of the experiments was designed to further characterize the differences between hypoxic vs. anoxic regulation of HIF-1.

6.2 Impact of prolonged hypoxia/anoxia on HIF-1α mRNA levels

In a recent publication, Uchida and co-workers suggested that mRNA regulation, in contrast to current concepts, might be involved in HIF-1α regulation after all. The observed regulatory mechanisms became apparent under prolonged hypoxic treatments and involved down-regulation of HIF-1α mRNA stability (Uchida *et al.*, 2004). Therefore, the intention behind the second part of the experiments was to further elucidate the mechanisms involved, with a special focus on possible differences between hypoxia and anoxia.

6.2.1 mRNA regulation – general mechanisms

Regulation of mRNA has long been attributed mainly to transcriptional mechanisms. Furthermore, recent reports indicated that mRNA may be regulated, at least in part, at post-transcriptional levels, i.e., via alterations of the stability. mRNA degradation appears to be tightly regulated, among others by the so far most widespread and efficient determinants of

RNA stability, i.e., adenylate- and uridylate-rich (AU-rich) elements (AREs) found in the 3'untranslated regions (3'UTRs) of many unstable mammalian mRNAs. Such sequences were first described for the 3'UTR of TNF-α mRNA (Caput *et al.*, 1986), with a minimal sequence suggested to be UUAUUUAUU (Zubiaga *et al.*, 1995). This minimal sequence, however, has been questioned since and still awaits complete characterization. These AREs appear to control the half-life of the respective mRNA. Stability regulation mediated by these AREs often requires the binding of specific AU-binding proteins (AUBPs). The exact mechanisms involved in changes of mRNA stability via binding of these proteins still remain elusive, though. Speculations connect the AUBP-mediated stability regulation either with an interaction to a deadenylase (Gao *et al.*, 2001), with changes in the accessibility of the polyA-tail for polyA ribonucleases (Wilusz *et al.*, 2001), or with recruitment of the exosome (Chen *et al.*, 2001b). The latter offers an explanation for opposing effects of stabilizing vs. destabilizing factors potentially binding similar sequences. Thus, stabilizing AUBPs might protect AREs from an interaction with the exosome, whereas destabilizing factors might actually recruit the exosome to the RNA in question for degradation (van Hoof and Parker, 1999; van Hoof and Parker, 2002) (Figure 25).

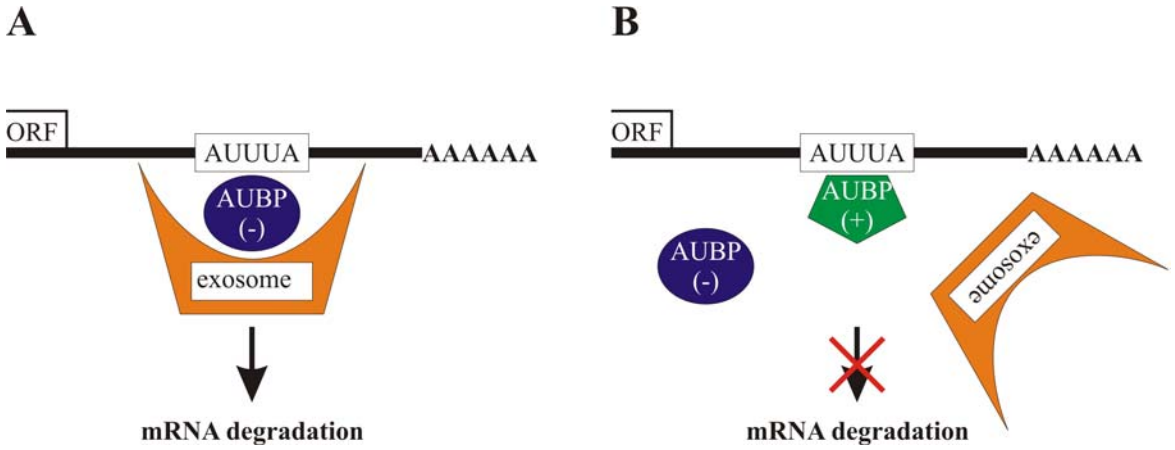

Figure 25: Regulation of mRNA stability by AUBPs

(A) Under mRNA destabilizing conditions, destabilizing factors (AUBP (-)) bind to the AREs of the 3'UTR of respective mRNAs. This allows for recruitment of the exosomal degradation machinery and subsequent mRNA degradation. (B) mRNA stabilizing AUBPs (AUBP (+)) might replace destabilizing AUBPs, thereby preventing exosome recruitment. Alternatively, stabilizing factors might bind simultaneously with the destabilizing factors, only affecting exosome recruitment (adapted from Bevilacqua *et al.*, 2003).

An increasing number of AUBPs has been identified in recent years. Among the best characterized factors are the stabilizing factor HuR, which is a member of the ELAV-like protein family (Myer *et al.*, 1997; Sakai *et al.*, 1999) and the destabilizing AUF-1 (AU-rich element/poly(U)-binding/degradation factor-1) (Wilson and Brewer, 1999) and tristetraproline (TTP) (Thompson *et al.*, 1996).

6.2.2 HIF-1α mRNA regulation

My results indicate that, in RKO cells, prolonged hypoxia (0.5% O_2) and anoxia (0% O_2) differently influence HIF-1α mRNA levels. Anoxia, but not hypoxia, reduced HIF-1α mRNA to approximately 50% of the normoxia values. Since no down-regulation of HIF-1α promotor activity was detectable at the same time, the mRNA changes were attributed to changes in mRNA stability. This was supported by the observation that HIF-1α mRNA half-life remained the same for normoxia and for hypoxia, while anoxia caused a decrease of the half-life. Furthermore, the changes in HIF-1α mRNA stability depended on translational activity, suggesting that the regulation involves the synthesis of destabilizing factors, rather than the down-regulation of the expression of stabilizing factors. The inhibition of the translation showed relevant effects only when the treatment exceeded 8 h, which indicates that prolonged anoxic treatments seem to be required. The further characterization of the mechanistic background supported the involvement of the 3'UTR of HIF-1α, since HIF-1α 3'UTR was able to attenuate a luciferase signal by approximately 15% under anoxia. The fact that anti-sense HIF-1α 3'UTR evoked an even more pronounced inhibition allows for speculations of *trans*-acting events being involved.

The regulation of HIF-1α mRNA has long been ignored, since the regulation of this transcription factor has mainly been attributed to inhibition of its degradation in response to hypoxia. A minor induction of HIF-1α mRNA has been reported to occur under hypoxia (Wiener *et al.*, 1996; Roy *et al.*, 2004). Only recently, contrasting results have been described for prolonged hypoxia (Uchida *et al.*, 2004), which is partly in accordance with my observation that HIF-1α mRNA decreases under prolonged anoxia, but not hypoxia (Figure 16) due to a strong reduction of the half-life (Figure 20). HIF-1α transcription remained largely unaltered under these conditions, and, if at all, it seemed to be enhanced to a small degree (Figure 17), thus excluding transcriptional effects. Uchida and co-workers attributed the down-regulation of HIF-1α mRNA to natural antisense HIF-1α mRNA (asHIF-1α) (Rossignol *et al.*, 2002), which they described to be regulated by HIF-1

via an HRE in its promotor region. Consequently, upon HIF-1 dependent transcription, asHIF-1α is assumed to bind to the 3'UTR of HIF-1α mRNA, resulting in enhanced degradation of HIF-1α mRNA. This model offers yet another feed-back mechanism for the termination of the HIF-1 response under oxygen depletion. Mechanistically, the down-regulation has been attributed to a better availability of the AU-rich elements in the HIF-1α 3'UTR after binding of asHIF-1α. In line with this, the down-regulatory effects in my settings appeared to depend on an intact protein translation (Figure 18 and Figure 21). On the other hand, the proposed dependency on HIF-1 activity dashes with my findings that hypoxia was neither sufficient to down-regulate HIF-1α mRNA levels (Figure 16) nor to decrease HIF-1α mRNA half-life (Figure 20). Thus, I assumed that, in addition to asHIF-1α, other factors are necessary to decrease HIF-1α mRNA levels. The proposed involvement of the 3'UTR in HIF-1α mRNA regulation led me to consider previously described mechanisms for general mRNA stability regulation as a possible mechanistic explanation (see 6.2.1). Therefore, I introduced the 3'UTR of HIF-1α into a constitutively active luciferase construct (pGL3-control). The observed decrease in luciferase activity in response to the introduction of HIF-1α 3'UTR under normoxia (approximately 5%) indicates that HIF-1α 3'UTR alone might already exert minor effects. This effect was significantly more pronounced under anoxia (approximately 20%) (Figure 22), supporting the proposed involvement of additional factors, which are induced by prolonged incubations under anoxia. The fact that inhibition of luciferase activity relative to normoxia values (approximately 15%) was rather weak when compared to the described down-regulation of HIF-1α mRNA levels to approximately 50% of the normoxia values might be explained in part by the vector used. The pGL3-control vector, in addition to an SV40 promotor, contains an SV40 enhancer element behind the polyA-signal (Appendix III), resulting in extremely high activities under all conditions. Thus, the inhibitory effects induced by mRNA destabilization might be not strong enough to overrule the high transcription rates. Consequently, the rather mild inhibition might be the result of inhibitory events which are more or less attenuated by an extremely efficient transcription due to the vector characteristics.

Most interestingly, the pGL3-control vector containing HIF-1α 3'UTR in anti-sense orientation, which was used as a specificity control for the sense HIF-1α 3'UTR, resulted in a very strong inhibition of luciferase activity under both normoxia and anoxia (Figure 23).

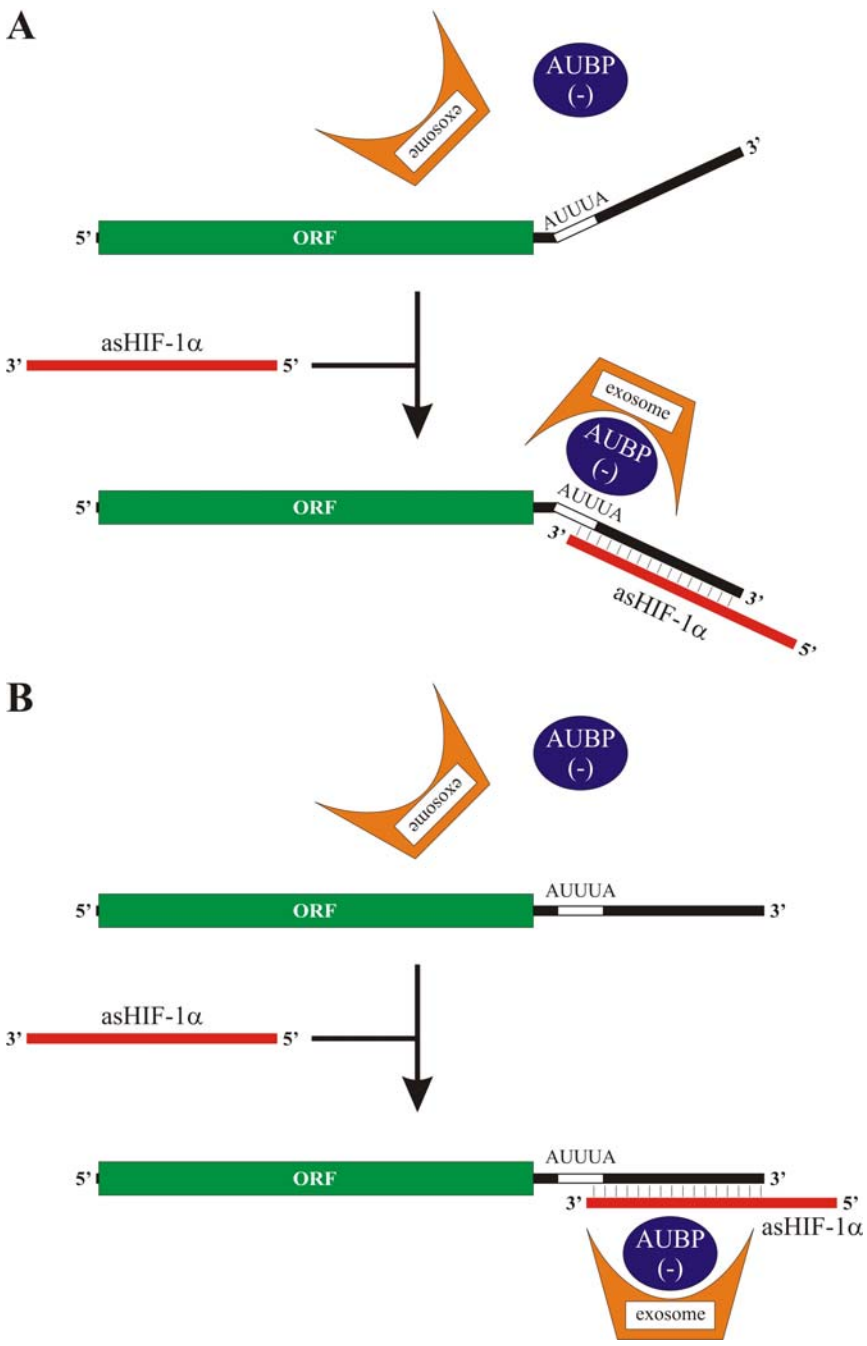

Figure 26: Proposed models for HIF-1α 3'UTR-mediated regulation of HIF-1α mRNA levels.
(A) A potential *cis*-mechanism might involve binding of asHIF-1α to HIF-1α mRNA in the 3'UTR, resulting in an increased accessibility of the AREs (AUUUA) for binding of destabilizing AUBPs (AUBP (-)), eventually allowing for exosomal degradation. (B) In contrast, a *trans*-mediated degradation of HIF-1α mRNA might be induced by binding of the exosomal machinery to asHIF-1α. Thus, HIF-1α mRNA degradation might not be direct, but rather mediated by asHIF-1α.

Since toxic effects of remnant of the plasmid preparation were ruled out by additional cleaning of the plasmid, I assumed these effects to be specific for the anti-sense HIF-1α

3'UTR. As the anti-sense HIF-1α 3'UTR fragment represents a major part of the asHIF-1α, the involvement of *trans*-effects might provide a mechanistic explanation. More precisely, in contrast to the previously suggested mechanism (Uchida *et al.*, 2004), asHIF-1α (or the anti-sense HIF-1α 3'UTR fragment) might not be necessary after all to increase the accessibility of the AU-rich elements in the 3'UTR of HIF-1α mRNA for binding of destabilizing AUBPs. Thus, instead of a direct *cis*-effect, destabilizing AUBPs might bind to asHIF-1α to mark it for exosomal degradation. The fact that asHIF-1α binds HIF-1α mRNA, at the same time, would then allow for concomitant degradation of this indirect target, thus constituting a *trans*-effect (Figure 26), i.e., direct effects would be considered to act in a *cis*-fashion, while *trans*-effects would be indirect via a mediator.

To completely elucidate the aforementioned models, further experiments are required. Thus, an alternative vector with lower activities might be used for assessing the impact of HIF-1α 3'UTR on mRNA stability. This would avoid the possibility of overrunning the system with extremely high transcription rates, thus allowing for a better characterization of the effects specifically induced by the 3'UTR. For a final determination of the mechanisms involved, the use of cross-linking experiments would be necessary. These assays would make it possible to determine the interaction of proteins (e.g., mRNA destabilizing factors) with specific mRNA sequences. As a consequence, a differentiation between *cis*- and *trans*-mediated effects might be possible.

6.3 New aspects of HIF-1 regulation under prolonged hypoxia/anoxia

The results of my experiments present new evidence for the presence of hydroxylase-independent mechanisms of HIF-1 regulation. In addition to PHD-/FIH-1-induced inhibition of HIF-1 accumulation and transactivation under normoxic conditions, other mechanisms appear to limit the HIF-1 response under prolonged and severe hypoxia. As a summary, I want to present a more elaborate picture of HIF-1 regulation according to previous work, supplemented by my recent data (see Figure 27).

Both subunits of the HIF-1 heterodimer, HIF-1α as well as HIF-1β, are constitutively expressed under normoxic conditions. While the β-subunit remains present, HIF-1α is permanently marked for proteasomal degradation by specific hydroxylases, i.e., the PHDs. Under hypoxia and anoxia, the PHDs as well as FIH-1, which inhibits HIF-1 transactivity under normoxic conditions, are inhibited. This is due to the fact that they require the presence of oxygen. In addition, the PHDs appear to be down-regulated on the protein

level as well (Nakayama *et al.*, 2004). Consequently, HIF-1α rapidly accumulates and HIF-1 transcriptional activity is induced, resulting in the transcription of HIF-1 responsive genes. After the initial accumulation and activation of HIF-1, i.e., under prolonged hypoxia and anoxia, the levels of PHDs increase again in a HIF-1-dependent manner. Their constitutive, minimal activity allows for the restriction of the HIF-1 response to a certain period of time. At the same time, prolonged anoxia (but not hypoxia) induces p53 accumulation and activation. Upon p53 induction, the initially low p53 levels are sufficient to suppress HIF-1 transactivity by competition for the transcriptional co-factor p300. This facilitates the attenuation of HIF-1-dependent gene transcription in the cell even if the HIF-1 complex is still present. Under prolonged anoxic conditions, p53 levels further increase, eventually allowing for formation of complexes between p53, Mdm2, and HIF-1α. As a consequence, HIF-1α is degraded in a pVHL-independent manner, thus completely terminating the HIF-1 response. Apart from the effects on HIF-1α protein and HIF-1 transcriptional activity, anoxia (but not hypoxia) also affects HIF-1α mRNA by induction of asHIF-1α in a HIF-1 responsive fashion. This mRNA construct apparently causes degradation of the otherwise constitutively present HIF-1α mRNA. A mechanistic explanation might be the increased availability of AU-rich elements present in the 3'UTR of HIF-1α mRNA. These elements have been described to be responsible for mRNA stability regulation of various mRNAs (e.g., TNF-α, Bcl-2). Alternatively, asHIF-1α might be the target of certain mRNA-destabilizing proteins itself. Since asHIF-1α obviously binds to HIF-1α mRNA, the latter might be co-degraded in the degradation process of asHIF-1α. Thus, HIF-1α mRNA degradation would be an indirect or *trans*-effect.

As indicated above, both HIF-1α mRNA destabilization as well as p53-mediated HIF-1 inhibition and HIF-1α degradation only occur under prolonged anoxia. One might speculate that the described processes constitute an alternative mechanism for limiting HIF-1 responses under prolonged complete oxygen depletion, apart from the above-mentioned PHD induction. Keeping in mind that the oxygen-dependency possibly limits the effects of regained PHD activity to hypoxia, these alternative mechanisms for limiting a HIF-1 response might also play an important role. Thus, the proposed regulatory mechanisms for terminating HIF-1-mediated events under anoxia, as well as PHD induction under hypoxia offer different pathways to eliminate the potentially cell-protective HIF-1 signal. Cells eventually require a back door for apoptosis induction under prolonged conditions of low oxygen tension if the hypoxic stimulus persists and becomes

too severe, and, thus, might prove deleterious for the cell otherwise. In order to prevent further damage to the surrounding tissue, the cells might, therefore, be eliminated by inhibition of protective signals, consequently allowing for programmed degradation (i.e., apoptosis). This saves neighboring cells from harm done by necrotic events.

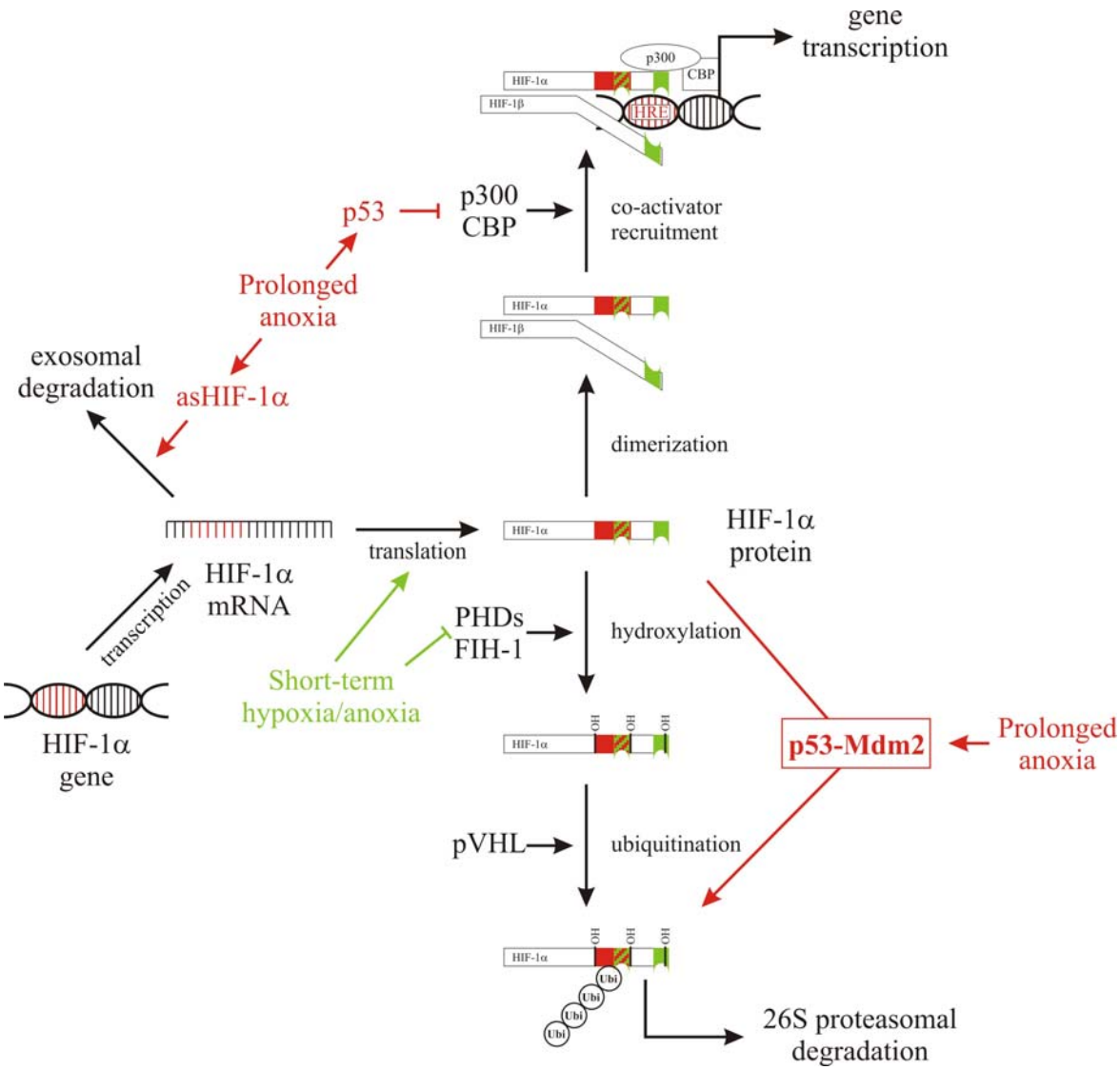

Figure 27: HIF-1 regulation in response to hypoxia/anoxia

Short-term hypoxia/anoxia (green) further enhances HIF-1α translation. Moreover, these conditions inhibit the hydroxylases either via depletion of the essential oxygen (PHDs and FIH-1) and/or via a down-regulation of the protein (PHDs). Thus, HIF-1α accumulates and HIF-1 becomes transcriptionally active, resulting in transcription of HIF-responsive genes. Prolonged anoxia (but not hypoxia) (red), on the other hand, causes degradation of HIF-1α mRNA by an asHIF-1α-mediated mechanism. Furthermore, p53 is induced by prolonged anoxia, limiting HIF-1 transactivity by out-competing HIF-1α for binding to p300 and by degrading HIF-1α via a pVHL-independent mechanism. Thus, under prolonged anoxia, the HIF-1 response is eliminated.

In summary, the presented data provide new information concerning the impact of p53 on HIF-1, which might be of importance for the decision between pro- and anti-apoptotic mechanisms depending upon the severity and duration of hypoxia. Furthermore, the results of this project give further insights into a novel mechanism of HIF-1 regulation, namely mRNA down-regulation under prolonged anoxic incubations. These mechanisms appear to be activated only in response to prolonged anoxia, but not to hypoxia. These considerations regarding HIF-1 regulation should be taken into account when prolonged incubations to hypoxic or anoxic conditions are analyzed at the level of HIF-1 stability regulation.

Moreover, as, to our knowledge, *trans*-mediated mRNA stability regulation processes have not yet been described for any known gene of interest, the proposed mechanism might represent an entirely new variant of mRNA regulation.

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8 Appendices

8.1 Appendix I: Buffers and solutions

Blotting Buffer

Tris/HCl	25 mM
Glycine	192 mM
Methanol	20%

→ Check pH to be 8.3

Binding buffer

Tris/HCl	50 mM
NaCl	150 mM
EDTA	5 mM
Sodium fluoride	1 mM
Sodium-ortho-vanadate	1 mM

→ Adjust pH to 7.0

Nonidet P-40	0.5%
Glycerin	20%

<u>To be added freshly prior to use:</u>

Protease inhibitor mix	1 x
PMSF	1 mM
BSA	1%

Caspase buffer

Hepes	100 mM
Sucrose	10%
CHAPS	0.1%
EDTA	1 mM

→ Adjust pH to 7.5

Coomassie Blue staining solution

Methanol	40%
Acetic acid	10%
Distilled H_2O	50%
Coomassie Brilliant Blue R250	500 mg/l

Coomassie Blue destaining solution

Methanol	40%
Acetic acid	10%
Distilled H_2O	50%

DEPC-treated water

Diethylpyrocarbonate (DEPC)	1 ml
H_2O	999 ml

→ stir overnight

→ autoclave

Enhanced chemoluminescence (ECL) solution

Solution A

Tris/HCl	100 mM
Luminol	250 mg/l

→ Adjust pH to 8.6

Solution B

Coumaric acid	1.1 mg/ml (in DMSO)

Complete (to be prepared fresh before use):

Solution A	3 ml
Solution B	30 µl
H_2O_2	1 µl

Fixing solution

Methanol	50%
Acetic acid	10%
H_2O	40%

2 x HBS (Hepes buffered saline)

Hepes	50 mM
NaCl	280 mM
Na_2HPO_4	1.5 mM

→ Adjust pH to 7.05

LB agar (Luria Bertani)

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
Agaragar	15 g/l

→ autoclave directly

To be added when solution has cooled to below 50°C:

Ampicillin	100 µg/l

LB broth (Luria Bertani)

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l

→ autoclave directly

Lower Tris Buffer

Tris/HCl	1.5 M

→ Adjust pH to 8.8

Luciferase lysis buffer

Tris/H_3PO_4	25 mM
DTT	2 mM
Triton X-100	1%
Glycerol	10%

→ Check pH to be 7.8

Luciferase assay reagent

Tricine	20 mM
Magnesium carbonate hydroxide	1.07 mM
Magnesium sulfate 7-hydrate	2.67 mM
EDTA-K^+	100 µM
DTT	33.3 mM
ATP	530 µM
Coenzyme A lithium	0.213 mg/ml
D-luciferine	470 mM

→ Check pH to be 7.8

NET-N buffer

Tris/HCl	20 mM
NaCl	100 mM
EDTA	1 mM

→ Adjust pH to 8.0

Nonidet P-40	0.5%

<u>To be added freshly prior to use:</u>

Protease inhibitor mix	1 x
PMSF	1 mM

PBS

NaCl	140 mM
KCl	2.7 mM
NaH_2PO_4	8.1 mM
KH_2PO_4	1.5 mM

Protein lysis buffer A

Tris/HCl	50 mM
NaCl	150 mM
EDTA	5 mM
→ Adjust pH to 7.5	
Nonidet P-40	0.5%

<u>To be added freshly prior to use:</u>

Protease inhibitor mix	1 x
PMSF	1 mM

Protein lysis buffer B

Triton-X100	1%
N-laurylsarcosine	1.5%
Triethanolamine	25 mM
EDTA	1 mM
→ in PBS	

4 x Sample Buffer

Tris/HCl	125 mM
SDS	2%
Glycerine	20%
Bromophenol Blue	0.002%
→ Adjust pH to 6.9	

<u>To be added freshly prior to use:</u>

DTT	5 mM

SDS-Running Buffer

Tris/HCl	25 mM
Glycine	190 mM
SDS	1%

→ Check pH to be 8.3

SOC medium

Tryptone	20 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l

→ autoclave directly

Filter-sterilized components to be added freshly prior to use:

$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	2 mM

Sodium dodecyl sulfate (SDS)-polyacrylamide gels

	Separating gels		**Stacking gel**
	7.5%	**10%**	**4%**
H_2O distilled	5.525 ml	4.9 ml	6.4 ml
Lower Tris buffer	2.5 ml	2.5 ml	-
Upper Tris buffer	-	-	2.5 ml
40% Acrylamide/Bis-acrylamide (37.5% : 1.0% w/v)	1.875 ml	2.5 ml	1 ml
10% (w/v) SDS	100 µl		
10% (w/v) ammonium persulfate	50 µl		
TEMED	5 µl		

Stripping mix A

Urea	8 M
SDS	1%

→ in PBS (pH 7.0)

To be added freshly prior to use:

β-mercaptoethanol	0.5%

→ Adjust pH to 7.0 (using acetic acid)

Stripping mix B

Acetic acid	10%
Ethanol	50%
H_2O	40%

TBE (Tris-Borat-EDTA) buffer

Tris/Borate	90 mM
EDTA	1 mM

→ Adjust pH to 8.0

TBS (Tris buffered saline)

Tris/HCl	50 mM
NaCl	140 mM

→ Check pH to be 7.4

TFB1

RbCl	100 mM
$MnCl_2$	50 mM
Potassium acetate	30 mM
$CaCl_2$	10 mM
Glycerol	15%

→ Adjust pH to 5.8

→ Sterilize by filtration

TFB2

MOPS	10 mM
RbCl	10 mM
$CaCl_2$	75 mM
Glycerol	15%

→ Adjust pH to 6.8 with KOH

→ Sterilize by filtration

TTBS

Tween-20	0.05%

→ in TBS

Solution B	30 µl
H_2O_2	1 µl

Upper Tris Buffer

Tris/HCl	0.5 M

→ Adjust pH to 6.8

8.2 Appendix II: HIF-1α spot membrane (spot sequences)

Spot		Sequence		Spot		Sequence	
1	1	MEGAGGANDKKKISS	15	51	151	RNGLVKKGKEQNTQR	165
2	4	AGGANDKKKISSERR	18	52	154	LVKKGKEQNTQRSFF	168
3	7	ANDKKKISSERRKEK	21	53	157	KGKEQNTQRSFFLRM	171
4	10	KKKISSERRKEKSRD	24	54	160	EQNTQRSFFLRMKCT	174
5	13	ISSERRKEKSRDAAR	27	55	163	TQRSFFLRMKCTLTS	177
6	16	ERRKEKSRDAARSRR	30	56	166	SFFLRMKCTLTSRGR	180
7	19	KEKSRDAARSRRSKE	33	57	169	LRMKCTLTSRGRTMN	183
8	22	SRDAARSRRSKESEV	36	58	172	KCTLTSRGRTMNIKS	186
9	25	AARSRRSKESEVFYE	39	59	175	LTSRGRTMNIKSATW	189
10	28	SRRSKESEVFYELAH	42	60	178	RGRTMNIKSATWKVL	192
11	31	SKESEVFYELAHQLP	45	61	181	TMNIKSATWKVLHCT	195
12	34	SEVFYELAHQLPLPH	48	62	184	IKSATWKVLHCTGHI	198
13	37	FYELAHQLPLPHNVS	51	63	187	ATWKVLHCTGHIHVY	201
14	40	LAHQLPLPHNVSSHL	54	64	190	KVLHCTGHIHVYDTN	204
15	43	QLPLPHNVSSHLDKA	57	65	193	HCTGHIHVYDTNSNQ	207
16	46	LPHNVSSHLDKASVM	60	66	196	GHIHVYDTNSNQPQC	210
17	49	NVSSHLDKASVMRLT	63	67	199	HVYDTNSNQPQCGYK	213
18	52	SHLDKASVMRLTISY	66	68	202	DTNSNQPQCGYKKPP	216
19	55	DKASVMRLTISYLRV	69	69	205	SNQPQCGYKKPPMTC	219
20	58	SVMRLTISYLRVRKL	72	70	208	PQCGYKKPPMTCLVL	222
21	61	RLTISYLRVRKLLDA	75	71	211	GYKKPPMTCLVLICE	225
22	64	ISYLRVRKLLDAGDL	78	72	214	KPPMTCLVLICEPIP	228
23	67	LRVRKLLDAGDLDIE	81	73	217	MTCLVLICEPIPHPS	231
24	70	RKLLDAGDLDIEDDM	84	74	220	LVLICEPIPHPSNIE	234
25	73	LDAGDLDIEDDMKAQ	87	75	223	ICEPIPHPSNIEIPL	237
26	76	GDLDIEDDMKAQMNC	90	76	226	PIPHPSNIEIPLDSK	240
27	79	DIEDDMKAQMNCFYL	93	77	229	HPSNIEIPLDSKTFL	243
28	82	DDMKAQMNCFYLKAL	96	78	232	NIEIPLDSKTFLSRH	246
29	85	KAQMNCFYLKALDGF	99	79	235	IPLDSKTFLSRHSLD	249
30	88	MNCFYLKALDGFVMV	102	80	238	DSKTFLSRHSLDMKF	252
31	91	FYLKALDGFVMVLTD	105	81	241	TFLSRHSLDMKFSYC	255
32	94	KALDGFVMVLTDDGD	108	82	244	SRHSLDMKFSYCDER	258
33	97	DGFVMVLTDDGDMIY	111	83	247	SLDMKFSYCDERITE	261
34	100	VMVLTDDGDMIYISD	114	84	250	MKFSYCDERITELMG	264
35	103	LTDDGDMIYISDNVN	117	85	253	SYCDERITELMGYEP	267
36	106	DGDMIYISDNVNKYM	120	86	256	DERITELMGYEPEEL	270
37	109	MIYISDNVNKYMGLT	123	87	259	ITELMGYEPEELLGR	273
38	112	ISDNVNKYMGLTQFE	126	88	262	LMGYEPEELLGRSIY	276
39	115	NVNKYMGLTQFELTG	129	89	265	YEPEELLGRSIYEYY	279
40	118	KYMGLTQFELTGHSV	132	90	268	EELLGRSIYEYYHAL	282
41	121	GLTQFELTGHSVFDF	135	91	271	LGRSIYEYYHALDSD	285
42	124	QFELTGHSVFDFTHP	138	92	274	SIYEYYHALDSDHLT	288
43	127	LTGHSVFDFTHPCDH	141	93	277	EYYHALDSDHLTKTH	291
44	130	HSVFDFTHPCDHEEM	144	94	280	HALDSDHLTKTHHDM	294
45	133	FDFTHPCDHEEMREM	147	95	283	DSDHLTKTHHDMFTK	297
46	136	THPCDHEEMREMLTH	150	96	286	HLTKTHHDMFTKGQV	300
47	139	CDHEEMREMLTHRNG	153	97	289	KTHHDMFTKGQVTTG	303
48	142	EEMREMLTHRNGLVK	156	98	292	HDMFTKGQVTTGQYR	306
49	145	REMLTHRNGLVKKGK	159	99	295	FTKGQVTTGQYRMLA	309
50	148	LTHRNGLVKKGKEQN	162	100	298	GQVTTGQYRMLAKRG	312

Spot		Sequence	
101	301	TTGQYRMLAKRGGYV	315
102	304	QYRMLAKRGGYVWVE	318
103	307	MLAKRGGYVWVETQA	321
104	310	KRGGYVWVETQATVI	324
105	313	GYVWVETQATVIYNT	327
106	316	WVETQATVIYNTKNS	330
107	319	TQATVIYNTKNSQPQ	333
108	322	TVIYNTKNSQPQCIV	336
109	325	YNTKNSQPQCIVCVN	339
110	328	KNSQPQCIVCVNYVV	342
111	331	QPQCIVCVNYVVSGI	345
112	334	CIVCVNYVVSGIIQH	348
113	337	CVNYVVSGIIQHDLI	351
114	340	YVVSGIIQHDLIFSL	354
115	343	SGIIQHDLIFSLQQT	357
116	346	IQHDLIFSLQQTECV	360
117	349	DLIFSLQQTECVLKP	363
118	352	FSLQQTECVLKPVES	366
119	355	QQTECVLKPVESSDM	369
120	358	ECVLKPVESSDMKMT	372
121	361	LKPVESSDMKMTQLF	375
122	364	VESSDMKMTQLFTKV	378
123	367	SDMKMTQLFTKVESE	381
124	370	KMTQLFTKVESEDTS	384
125	373	QLFTKVESEDTSSLF	387
126	376	TKVESEDTSSLFDKL	390
127	379	ESEDTSSLFDKLKKE	393
128	382	DTSSLFDKLKKEPDA	396
129	385	SLFDKLKKEPDALTL	399
130	388	DKLKKEPDALTLLAP	402
131	391	KKEPDALTLLAPAAG	405
132	394	PDALTLLAPAAGDTI	408
133	397	LTLLAPAAGDTIISL	411
134	400	LAPAAGDTIISLDFG	414
135	403	AAGDTIISLDFGSND	417
136	406	DTIISLDFGSNDTET	420
137	409	ISLDFGSNDTETDDQ	423
138	412	DFGSNDTETDDQQLE	426
139	415	SNDTETDDQQLEEVP	429
140	418	TETDDQQLEEVPLYN	432
141	421	DDQQLEEVPLYNDVM	435
142	424	QLEEVPLYNDVMLPS	438
143	427	EVPLYNDVMLPSPNE	441
144	430	LYNDVMLPSPNEKLQ	444
145	433	DVMLPSPNEKLQNIN	447
146	436	LPSPNEKLQNINLAM	450
147	439	PNEKLQNINLAMSPL	453
148	442	KLQNINLAMSPLPTA	456
149	445	NINLAMSPLPTAETP	459
150	448	LAMSPLPTAETPKPL	462

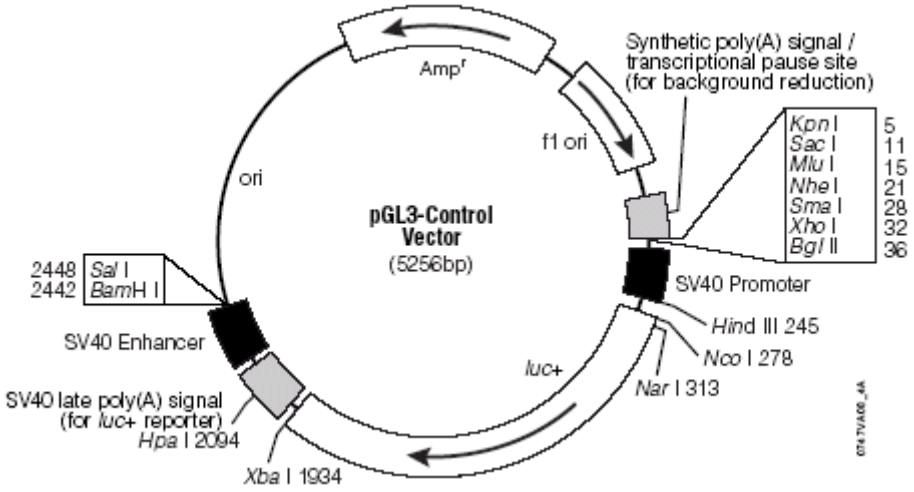
Spot		Sequence	
151	451	SPLPTAETPKPLRSS	465
152	454	PTAETPKPLRSSADP	468
153	457	ETPKPLRSSADPALN	471
154	460	KPLRSSADPALNQEV	474
155	463	RSSADPALNQEVALK	477
156	466	ADPALNQEVALKLEP	480
157	469	ALNQEVALKLEPNPE	483
158	472	QEVALKLEPNPESLE	486
159	475	ALKLEPNPESLELSF	489
160	478	LEPNPESLELSFTMP	492
161	481	NPESLELSFTMPQIQ	495
162	484	SLELSFTMPQIQDQT	498
163	487	LSFTMPQIQDQTPSP	501
164	490	TMPQIQDQTPSPSDG	504
165	493	QIQDQTPSPSDGSTR	507
166	496	DQTPSPSDGSTRQSS	510
167	499	PSPSDGSTRQSSPEP	513
168	502	SDGSTRQSSPEPNSP	516
169	505	STRQSSPEPNSPSEY	519
170	508	QSSPEPNSPSEYCFY	522
171	511	PEPNSPSEYCFYVDS	525
172	514	NSPSEYCFYVDSDMV	528
173	517	SEYCFYVDSDMVNEF	531
174	520	CFYVDSDMVNEFKLE	534
175	523	VDSDMVNEFKLELVE	537
176	526	DMVNEFKLELVEKLF	540
177	529	NEFKLELVEKLFAED	543
178	532	KLELVEKLFAEDTEA	546
179	535	LVEKLFAEDTEAKNP	549
180	538	KLFAEDTEAKNPFST	552
181	541	AEDTEAKNPFSTQDT	555
182	544	TEAKNPFSTQDTDLD	558
183	547	KNPFSTQDTDLDLEM	561
184	550	FSTQDTDLDLEMLAP	564
185	553	QDTDLDLEMLAPYIP	567
186	556	DLDLEMLAPYIPMDD	570
187	559	LEMLAPYIPMDDDFQ	573
188	562	LAPYIPMDDDFQLRS	576
189	565	YIPMDDDFQLRSFDQ	579
190	568	MDDDFQLRSFDQLSP	582
191	571	DFQLRSFDQLSPLES	585
192	574	LRSFDQLSPLESSSA	588
193	577	FDQLSPLESSSASPE	591
194	580	LSPLESSSASPESAS	594
195	583	LESSSASPESASPQS	597
196	586	SSASPESASPQSTVT	600
197	589	SPESASPQSTVTVFQ	603
198	592	SASPQSTVTVFQQTQ	606
199	595	PQSTVTVFQQTQIQE	609
200	598	TVTVFQQTQIQEPTA	612

Spot		Sequence	
201	601	VFQQTQIQEPTANAT	615
202	604	QTQIQEPTANATTTT	618
203	607	IQEPTANATTTTATT	621
204	610	PTANATTTTATTDEL	624
205	613	NATTTTATTDELKTV	627
206	616	TTTATTDELKTVTKD	630
207	619	ATTDELKTVTKDRME	633
208	622	DELKTVTKDRMEDIK	636
209	625	KTVTKDRMEDIKILI	639
210	628	TKDRMEDIKILIASP	642
211	631	RMEDIKILIASPSPT	645
212	634	DIKILIASPSPTHIH	648
213	637	ILIASPSPTHIHKET	651
214	640	ASPSPTHIHKETTSA	654
215	643	SPTHIHKETTSATSS	657
216	646	HIHKETTSATSSPYR	660
217	649	KETTSATSSPYRDTQ	663
218	652	TSATSSPYRDTQSRT	666
219	655	TSSPYRDTQSRTASP	669
220	658	PYRDTQSRTASPNRA	672
221	661	DTQSRTASPNRAGKG	675
222	664	SRTASPNRAGKGVIE	678
223	667	ASPNRAGKGVIEQTE	681
224	670	NRAGKGVIEQTEKSH	684
225	673	GKGVIEQTEKSHPRS	687
226	676	VIEQTEKSHPRSPNV	690
227	679	QTEKSHPRSPNVLSV	693
228	682	KSHPRSPNVLSVALS	696
229	685	PRSPNVLSVALSQRT	699
230	688	PNVLSVALSQRTTVP	702
231	691	LSVALSQRTTVPEEE	705
232	694	ALSQRTTVPEEELNP	708
233	697	QRTTVPEEELNPKIL	711
234	700	TVPEEELNPKILALQ	714
235	703	EEELNPKILALQNAQ	717
236	706	LNPKILALQNAQRKR	720
237	709	KILALQNAQRKRKME	723
238	712	ALQNAQRKRKMEHDG	726
239	715	NAQRKRKMEHDGSLF	729
240	718	RKRKMEHDGSLFQAV	732
241	721	KMEHDGSLFQAVGIG	735
242	724	HDGSLFQAVGIGTLL	738
243	727	SLFQAVGIGTLLQQP	741
244	730	QAVGIGTLLQQPDDH	744
245	733	GIGTLLQQPDDHAAT	747
246	736	TLLQQPDDHAATTSL	750
247	739	QQPDDHAATTSLSWK	753
248	742	DDHAATTSLSWKRVK	756
249	745	AATTSLSWKRVKGCK	759
250	748	TSLSWKRVKGCKSSE	762

Spot		Sequence	
251	751	SWKRVKGCKSSEQNG	765
252	754	RVKGCKSSEQNGMEQ	768
253	757	GCKSSEQNGMEQKTI	771
254	760	SSEQNGMEQKTIILI	774
255	763	QNGMEQKTIILIPSD	777
256	766	MEQKTIILIPSDLAC	780
257	769	KTIILIPSDLACRLL	783
258	772	ILIPSDLACRLLGQS	786
259	775	PSDLACRLLGQSMDE	789
260	778	LACRLLGQSMDESGL	792
261	781	RLLGQSMDESGLPQL	795
262	784	GQSMDESGLPQLTSY	798
263	787	MDESGLPQLTSYDCE	801
264	790	SGLPQLTSYDCEVNA	804
265	793	PQLTSYDCEVNAPIQ	807
266	796	TSYDCEVNAPIQGSR	810
267	799	DCEVNAPIQGSRNLL	813
268	802	VNAPIQGSRNLLQGE	816
269	805	PIQGSRNLLQGEELL	819
270	808	GSRNLLQGEELLRAL	822
271	811	NLLQGEELLRALDQV	825
272	814	QGEELLRALDQVN	828

8.3 Appendix III: Vectors

pGL3-control vector:



HIF-1α 3'UTR was introduced at the Xba I restriction site (bp 1934) in sense or anti-sense orientation resulting in 3'UTR-HIF-1α pGL3-control (sense) or (anti-sense) plasmids, respectively

3'UTR of HIF-1α cDNA:

2765

GCTTTTTCTT	AATTTCATTC	CTTTTTTGG	ACACTGGTGG	CTCACTACCT
AAAGCAGTCT	ATTTATATTT	TCTACATCTA	ATTTTAGAAG	CCTGGCTACA
ATACTGCACA	AACTTGGTTA	GTTCAATTTT	TGATCCCCTT	TCTACTTAAT
TTACATTAAT	GCTCTTTTTT	AGTATGTTCT	TTAATGCTGG	ATCACAGACA
GCTCATTTTC	TCAGTTTTTT	GGTATTTAAA	CCATTGCATT	GCAGTAGCAT
CATTTTAAAA	AATGCACCTT	TTTATTTATT	TATTTTTGGC	TAGGGAGTTT
ATCCCTTTTT	CGAATTATTT	TTAAGAAGAT	GCCAATATAA	TTTTTGTAAG
AAGGCAGTAA	CCTTTCATCA	TGATCATAGG	CAGTTGAAAA	ATTTTTACAC
CTTTTTTTTC	ACATTTTACA	TAAATAATAA	TGCTTTGCCA	GCAGTACGTG
GTAGCCACAA	TTGCACAATA	TATTTTCTTA	AAAAATACCA	GCAGTTACTC
ATGGAATATA	TTCTGCGTTT	ATAAAACTAG	TTTTTAAGAA	GAAATTTTTT
TTGGCCTATG	AAATTGTTAA	ACCTGGAACA	TGACATTGTT	AATCATATAA
TAATGATTCT	TAAATGCTGT	ATGGTTTATT	ATTTAAATGG	GTAAAGCCAT
TTACATAATA	TAGAAAGATA	TGCATATATC	TAGAAGGTAT	GTGGCATTTA
TTTGGATAAA	ATTCTCAATT	CAGAGAAATC	ATCTGATGTT	TCTATAGTCA
CTTTGCCAGC	TCAAAAGAAA	ACAATACCCT	ATGTAGTTGT	GGAAGTTTAT

GCTAATATTG	TGTAACTGAT	ATTAAACCTA	AATGTTCTGC	CTACCCTGTT
GGTATAAAGA	TATTTTGAGC	AGACTGTAAA	CAAGAAAAAA	AAAATCATGC
ATTCTTAGCA	AAATTGCCTA	GTATGTTAAT	TTGCTCAAAA	TACAATGTTT
GATTTTATGC	ACTTTGTCGC	TATTAACATC	CTTTTTTTCA	TGTAGATTTC
AATAATTGAG	TAATTTTAGA	AGCATTATTT	TAGGAATATA	TAGTTGTCAC
AGTAAATATC	TTGTTTTTTC	TATGTACATT	GTACAAATTT	TTCATTCCTT
TTGCTCTTTG	TGGTTGGATC	TAACACTAAC	TGTATTGTTT	TGTTACATCA
AATAAACATC	TTCTGTGGAC	CAGGAAAAAA	AAAAAAAAAA	AAA

Green: Primer complementary sequences

Red: AU-rich element

8.4 Appendix IV: Alignment of the sequencing result of the constructed vectors to the predicted sequences

3'UTR-HIF-1α pGL3-control (sense)

Plasmid (S): sequence of the 3'UTR-HIF-1α pGL3-control (sense) plasmid (insert) as analyzed by the sequencing reaction

3'UTR-HIF (S): predicted sequence of the HIF-1α 3'UTR (sense) insert

```
Plasmid (S)     ------------------------------CAC----------CTACCTAAAG-AGTCT
3'UTR-HIF (S)   GCTTTTTCTTAATTTCATTCCTTTTTTTGGACACTGGTGGCTCACTACCTAAAGCAGTCT
                                        ***          ********** *****

Plasmid (S)     ATTTATATTTTCTACATCTAATTTTAGAAGCCTGGCTACAATACTGCACAAACTTGGTTA
3'UTR-HIF (S)   ATTTATATTTTCTACATCTAATTTTAGAAGCCTGGCTACAATACTGCACAAACTTGGTTA
                ************************************************************

Plasmid (S)     GTTCAATTTT-GATCCCCTTTCTACTTAATTTACATTAATGCTCTTTTTTAGTATGTTCT
3'UTR-HIF (S)   GTTCAATTTTTGATCCCCTTTCTACTTAATTTACATTAATGCTCTTTTTTAGTATGTTCT
                ********** ***************************************************

Plasmid (S)     TTAATGCTGGATCACAGACAGCTCATTTTCTCAGTTTTTTGGTATTTAAACCATTGCATT
3'UTR-HIF (S)   TTAATGCTGGATCACAGACAGCTCATTTTCTCAGTTTTTTGGTATTTAAACCATTGCATT
                ************************************************************

Plasmid (S)     GCAGTAGCATCATTTTAAAAAATGCACCTTTTTATTTATTTATTTTTGGCTAGGGAGTTT
3'UTR-HIF (S)   GCAGTAGCATCATTTTAAAAAATGCACCTTTTTATTTATTTATTTTTGGCTAGGGAGTTT
                ************************************************************

Plasmid (S)     ATCCCTTTTTCGAATTATTTTTAAGAAGATGCCAATATAATTTTTGTAAGAAGGCAGTAA
3'UTR-HIF (S)   ATCCCTTTTTCGAATTATTTTTAAGAAGATGCCAATATAATTTTTGTAAGAAGGCAGTAA
                ************************************************************

Plasmid (S)     CCTTTCATCATGATCATAGGCAGTTGAAAAATTTTTACACCTTTTTTTTCACATTTTACA
3'UTR-HIF (S)   CCTTTCATCATGATCATAGGCAGTTGAAAAATTTTTACACCTTTTTTTTCACATTTTACA
                ************************************************************

Plasmid (S)     TAAATAATAATGCTTTGCCAGCAGTACGTGGTAGCCACAATTGCACAATATATTTTCTTA
3'UTR-HIF (S)   TAAATAATAATGCTTTGCCAGCAGTACGTGGTAGCCACAATTGCACAATATATTTTCTTA
                ************************************************************
```

```
Plasmid (S)     AAAAATACCAGCAGTTACTCATGGAATATATTCTGCGTTTATAAAACTAGTTTTTAAGAA
3’UTR-HIF (S)   AAAAATACCAGCAGTTACTCATGGAATATATTCTGCGTTTATAAAACTAGTTTTTAAGAA
                ************************************************************

Plasmid (S)     GAAATTTTTTTTGGCCTATGAAATTGTTAAACCTGGAACATGACATTGTTAATCATATAA
3’UTR-HIF (S)   GAAATTTTTTTTGGCCTATGAAATTGTTAAACCTGGAACATGACATTGTTAATCATATAA
                ************************************************************

Plasmid (S)     TAATGATTCTTAAATGCTGTATGGTTTATTATTTAAATGGGGTAAAGCCATTTACATAAT
3’UTR-HIF (S)   TAATGATTCTTAAATGCTGTATGGTTTATTATTTAAATGGG-TAAAGCCATTTACATAAT
                ************************************** ******************

Plasmid (S)     ATAGAAAGATATGCATATATCTAGAAGGTATGTGGCATTTATTTGGATAAAATTCTCAAT
3’UTR-HIF (S)   ATAGAAAGATATGCATATATCTAGAAGGTATGTGGCATTTATTTGGATAAAATTCTCAAT
                ************************************************************

Plasmid (S)     TCAGAGAAATCATCTGATGTTTCTATAGTCACTTTGCCAGCTCAA--GAAAACAATACCC
3’UTR-HIF (S)   TCAGAGAAATCATCTGATGTTTCTATAGTCACTTTGCCAGCTCAAAAGAAAACAATACCC
                ******************************************  *************

Plasmid (S)     TATGTAGT-GTGGAAGGTTATGCTA-TATTGTGTAACT----------------------
3’UTR-HIF (S)   TATGTAGTTGTGGAAGTTTATGCTAATATTGTGTAACTGATATTAAACCTAAATGTTCTG
                ******** ******* ******** ************

Plasmid (S)     ------------------------------------------------------------
3’UTR-HIF (S)   CCTACCCTGTTGGTATAAAGATATTTTGAGCAGACTGTAAACAAGAAAAAAAAAATCATG


Plasmid (S)     ------------------------------------------------------------
3’UTR-HIF (S)   CATTCTTAGCAAAATTGCCTAGTATGTTAATTTGCTCAAAATACAATGTTTGATTTTATG


Plasmid (S)     ------------------------------------------------------------
3’UTR-HIF (S)   CACTTTGTCGCTATTAACATCCTTTTTTTCATGTAGATTTCAATAATTGAGTAATTTTAG


Plasmid (S)     ------------------------------------------------------------
3’UTR-HIF (S)   AAGCATTATTTTAGGAATATATAGTTGTCACAGTAAATATCTTGTTTTTTCTATGTACAT


Plasmid (S)     ------------------------------------------------------------
3’UTR-HIF (S)   TGTACAAATTTTTCATTCCTTTTGCTCTTTGTGGTTGGATCTAACACTAACTGTATTGTT


Plasmid (S)     ----------------------------------------------------
3’UTR-HIF (S)   TTGTTACATCAAATAAACATCTTCTGTGGACCAGGAAAAAAAAAAAAAAAAA
```

3'UTR-HIF-1α pGL3-control (anti-sense)

Plasmid (AS): sequence of the 3'UTR-HIF-1α pGL3-control (anti-sense) plasmid (insert) as analyzed by the sequencing reaction

3'UTR-HIF (AS): predicted sequence of the HIF-1α 3'UTR (sense) insert

```
PLASMID (AS)    ------------------------------------------------------------
3'UTR-HIF (AS)  GCTTTTTCTTAATTTCATTCCTTTTTTTGGACACTGGTGGCTCACTACCTAAAGCAGTCT

PLASMID (AS)    ------------------------------------------------------------
3'UTR-HIF (AS)  ATTTATATTTTCTACATCTAATTTTAGAAGCCTGGCTACAATACTGCACAAACTTGGTTA

PLASMID (AS)    ------------------------------------------------------------
3'UTR-HIF (AS)  GTTCAATTTTTGATCCCCTTTCTACTTAATTTACATTAATGCTCTTTTTTAGTATGTTCT

PLASMID (AS)    ------------------------------------------------------------
3'UTR-HIF (AS)  TTAATGCTGGATCACAGACAGCTCATTTTCTCAGTTTTTTGGTATTTAAACCATTGCATT

PLASMID (AS)    ------------------------------------------------------------
3'UTR-HIF (AS)  GCAGTAGCATCATTTTAAAAAATGCACCTTTTTATTTATTTATTTTTGGCTAGGGAGTTT

PLASMID (AS)    ------------------------------------------------------------
3'UTR-HIF (AS)  ATCCCTTTTTCGAATTATTTTTAAGAAGATGCCAATATAATTTTTGTAAGAAGGCAGTAA

PLASMID (AS)    ------------------------------------------------------------
3'UTR-HIF (AS)  CCTTTCATCATGATCATAGGCAGTTGAAAAATTTTTACACCTTTTTTTTCACATTTTACA

PLASMID (AS)    ----------------------------------------------------ATTTTCGT-
3'UTR-HIF (AS)  TAAATAATAATGCTTTGCCAGCAGTACGTGGTAGCCACAATTGCACAATATATTTTCTTA
                                                                 ****** *

PLASMID (AS)    -------------------------------------TATAAA--TAGTTTTTA-GAA
3'UTR-HIF (AS)  AAAAATACCAGCAGTTACTCATGGAATATATTCTGCGTTTATAAAACTAGTTTTTAAGAA
                                                    ******  ********* ***

PLASMID (AS)    GAA--TTTTTTTGGC-TA-GAAATTTT---ACCTG--ACAG--CATTGTTAATCA-ATAA
3'UTR-HIF (AS)  GAAATTTTTTTTGGCCTATGAAATTGTTAAACCTGGAACATGACATTGTTAATCATATAA
                ***  ********** ** ****** *   *****  ***   ************ ****
```

```
PLASMID (AS)    TAATGATTCT-AAATG-TGTATG-TTTAT-ATTTAAA-GGGTAA-GCCATTT-CATAATA
3'UTR-HIF (AS)  TAATGATTCTTAAATGCTGTATGGTTTATTATTTAAATGGGTAAAGCCATTTACATAATA
                ********** ***** ****** ***** ******* ****** ******* *******


PLASMID (AS)    TAGAAAGATAT-CATATATCTAGAAGGTATGTGGCATTTATTTGGATAAAATTCTCA-TT
3'UTR-HIF (AS)  TAGAAAGATATGCATATATCTAGAAGGTATGTGGCATTTATTTGGATAAAATTCTCAATT
                *********** ********************************************** **

PLASMID (AS)    CAGAGAAATCATCTGATGTTTCTATAGTCACTTT-CCAGCTCAAAAGAAAACAATACCCT
3'UTR-HIF (AS)  CAGAGAAATCATCTGATGTTTCTATAGTCACTTTGCCAGCTCAAAAGAAAACAATACCCT
                ********************************** ************************

PLASMID (AS)    ATGTAGTTGTGGAAGTTTATGCTAATATCGTGTAACTGATATTAAACCTAAATGTTCTGC
3'UTR-HIF (AS)  ATGTAGTTGTGGAAGTTTATGCTAATATTGTGTAACTGATATTAAACCTAAATGTTCTGC
                **************************** ******************************

PLASMID (AS)    CTACCCTGTTGGTATAAAGATATTTTGAGCAG-CTGTAAACAAGAAAAAAAGAAATCATG
3'UTR-HIF (AS)  CTACCCTGTTGGTATAAAGATATTTTGAGCAGACTGTAAACAAGAAAAAAA-AAATCATG
                ******************************** ***************** ********

PLASMID (AS)    CATT-TTAGCAAAATTGC-TAGTATGTTAATTTGCTCGAAATACAATGTTTGATTTTATG
3'UTR-HIF (AS)  CATTCTTAGCAAAATTGCCTAGTATGTTAATTTGCTCAAAATACAATGTTTGATTTTATG
                **** ************* ***************** **********************

PLASMID (AS)    CACTTTGTCGCTATTAACATCCTTTTTTTCATGTAGATTTCAATAATTGAGTAATTTT-G
3'UTR-HIF (AS)  CACTTTGTCGCTATTAACATCCTTTTTTTCATGTAGATTTCAATAATTGAGTAATTTTAG
                ********************************************************** *

PLASMID (AS)    AAGCATTATTTTAGGAATATATAGGTGGTCACAGTAAAGATCTT-TTTTTTCGAGG----
3'UTR-HIF (AS)  AAGCATTATTTTAGGAATATATAG-TTGTCACAGTAAATATCTTGTTTTTTCTATGTACA
                ************************ * *********** ***** ******* * *

PLASMID (AS)    ------------------------------------------------------------
3'UTR-HIF (AS)  TTGTACAAATTTTTCATTCCTTTTGCTCTTTGTGGTTGGATCTAACACTAACTGTATTGT


PLASMID (AS)    ------------------------------------------------------
3'UTR-HIF (AS)  TTTGTTACATCAAATAAACATCTTCTGTGGACCAGGAAAAAAAAAAAAAAAAAA
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9 Publications

Papers

Zhou, J., Schmid, T., Brüne, B. (2004). HIF-1α and p53 as targets of NO in affecting cell proliferation, death and adaption. Curr. Mol. Med. (in press).

Schmid, T., Zhou, J., Köhl, R., Brüne, B. (2004). p300 relieves p53-evoked transcriptional repression of HIF-1. Biochem. J. **380**(1): pp. 289-95.

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Schmid, T., Callapina, M., Zhou, J., Brüne, B. (2003). Anoxia causes accumulation, transcriptional activation and cross-talk between p53 and HIF-1. Molecular and Cellular Proteomics **2**(9): p. 991 (no. 124.33).

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10 Curriculum Vitae

Personal information

Name	Tobias Schmid
Date of birth	27. 02. 1974
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Nationality	German
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Education and employment

2004	Anticipated completion of the Ph.D. thesis
2002 – 2004	Ph.D. studies in Prof. Brüne's lab, Department of Cell Biology, Technical University of Kaiserslautern Title of the thesis: Regulation of the hypoxia inducible factor-1 by prolonged and severe low oxygen tension
07. 2001 – 12. 2001	Research assistant in Prof. Brüne's lab, Department of Nephrology, Medical Clinic IV, University of Erlangen-Nürnberg
01. 2001 – 05. 2001	Research assistant in Prof. Dietrich's lab, Environmental Toxicology Group, University of Konstanz
1999 - 2000	Master`s degree of Biology (Diploma) in the Environmental Toxicology Group, University of Konstanz, and the Ecotoxicology Department, Novartis Crop Protection AG (Basel, Switzerland) Title of the thesis: Determination of the half-life of endocrine indicators in fish by means of a bioaccumulation system
1995 - 1996	Academic year at York University, Toronto, Canada
1993 - 2000	University Studies in Biology at the University of Konstanz Areas of specialization: Ecotoxicology, Molecular Toxicology
1990 - 1993	Keplergymnasium Freudenstadt (Abitur)
1984 - 1990	Progymnasium Baiersbronn
1980 - 1984	Grund- und Hauptschule Klosterreichenbach

11 Erklärung

Hiermit versichere ich, dass die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden. Ergebnisse beteiligter Mitarbeiter und anderer Autoren habe ich entsprechend gekennzeichnet.

Darüber hinaus erkläre ich, dass ich weder bereits früher ein Promotionsverfahren an einer anderen Hochschule beantragt habe, noch die Dissertationsschrift vollständig oder in Teilen einer anderen Fakultät mit dem Ziel, einen akademischen Grad zu erwerben, vorgelegt wurde.

Kaiserslautern, den 05. August, 2004