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**Hypoxic regulation of  
glyceraldehyde-3-phosphate dehydrogenase  
in HPV-positive non-tumorigenic and  
tumorigenic cells**

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Für meine Eltern

In Gedenken an

meinen Großvater Franz Ballik

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## Abstract

Metabolic adaptations of cancer to scarce oxygen conditions are believed to contribute to the tumor promoting effect of hypoxia. To assess this issue in the context of human papillomavirus induced cervical carcinogenesis, the hypoxic regulation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was investigated in non-tumorigenic HPV-positive HeLa x fibroblast hybrid cells (444), their tumorigenic segregants CGL 3 and the parental homologues IMR 90 and HeLa. 24 hours hypoxia lead to a transcriptional upregulation of GAPDH only in non-tumorigenic cells but not in their tumorigenic counterparts. As shown by ChIP analysis, the hypoxic increase of GAPDH was mediated by the transcription factors Net and HIF-2 $\alpha$ . Their binding to the GAPDH promoter was augmented upon hypoxia exclusively in non-tumorigenic cells. Moreover, GAPDH-reporter assays revealed a synergistic *trans*-activating effect of Net and HIF-2 $\alpha$  only in non-tumorigenic cells which was further enhanced by hypoxia. Intriguingly, GAPDH acts as a trigger between cell death and survival. To investigate whether the differential hypoxic regulation of GAPDH in non-tumorigenic and tumorigenic cells reflects their resistance to hypoxic conditions, cell viability was monitored by determining the cellular ATP content and by FACS analysis. Indeed, the hypoxic increase of GAPDH correlated with a better cell viability in non-tumorigenic 444 cells. Moreover, FACS assays revealed different responses to hypoxia in tumorigenic CGL 3 and HeLa cell. While HeLa substantially increased apoptosis upon hypoxia, CGL 3 underwent a different type of cell death, possibly necrosis. Apoptosis in HeLa was likely caused by an altered ROS homeostasis, as this cell line did not show the expected hypoxic increase of oxygen radicals. In contrast, CGL 3 probably died of irreversible energy depletion, as they depicted the lowest ATP levels of all tested cell lines upon hypoxia.

Another factor which might influence hypoxic survival are the viral oncoproteins E6 and E7. This study determined complete silencing of their expression upon hypoxia in both tumorigenic and non-tumorigenic cell lines. The main regulator of HPV transcription, the cellular transcription factor AP-1, was not responsible for this effect, as neither its dimeric composition nor the expression of its cognate subunits changed upon hypoxic treatment. Taken together, this study indicates a dysregulation of hypoxic GAPDH expression in cervical cancer cells which may influence survival of energy stress upon hypoxia. Whether the silencing of the HPV oncoproteins upon hypoxia contributes to this observation requires further investigation.

## Zusammenfassung

Hypoxie induziert metabolische Anpassungsprozesse in Krebszellen, die das Tumorwachstum fördern. Um diesen Zusammenhang am Model der HPV-induzierten zervikalen Karzinogenese zu erforschen, wurde die hypoxische Regulation des glykolytischen Enzyms Glyceraldehyd-3-Phosphat Dehydrogenase (GAPDH) in den nicht-tumorigenen HPV-positiven HeLa X Fibroblast Zellhybriden 444, in deren tumorigenen Abkömmlingen CGL 3 und in den parentalen Zelllinien IMR 90 und HeLa untersucht. Es stellte sich heraus, dass der hypoxische Anstieg der GAPDH Expression ausschließlich in nicht-tumorigenen Zellen zu beobachten ist. Er wird von den Transkriptionsfaktoren Net und HIF-2 $\alpha$  vermittelt, die unter Hypoxie nur in nicht-tumorigenen Zellen vermehrt an den GAPDH Promoter binden. Darüberhinaus zeigten GAPDH-Reporter Assays eine synergistische Aktivierung der GAPDH Transkription durch Net und HIF-2 $\alpha$ , die unter Hypoxie weiter verstärkt wird und ebenfalls auf nicht-tumorigene Zellen beschränkt ist. Um zu zeigen, dass die unterschiedliche GAPDH Regulation unter hypoxischen Bedingungen auf eine Diskrepanz der hypoxischen Resistenz in tumorigenen und nicht-tumorigenen Zellen hindeutet, wurde eine FACS Analyse und eine Untersuchung des zellulären ATP Gehalts durchgeführt. Die hypoxische Induktion der GAPDH Expression in IMR 90 und 444 korreliert tatsächlich mit einer besseren Lebensfähigkeit dieser Zellen unter diesen Bedingungen. Darüberhinaus enthüllte die FACS Analyse eine unterschiedliche hypoxische Reaktion der beiden tumorigenen Zelllinien. Während Hypoxie in HeLa vor allem Apoptose induziert, durchlaufen CGL 3 eine andere Form des Zelltods, bei dem es sich um Nekrose handeln könnte. Die Apoptose in HeLa wird wahrscheinlich durch eine vermehrte Empfindlichkeit auf Sauerstoffradikale hervorgerufen, da HeLa nicht die erwartete hypoxische Zunahme der Sauerstoffradikale zeigt. Im Gegensatz dazu sterben CGL 3 vermutlich an irreversibler Energiedefizienz, da sie unter Hypoxie die niedrigsten ATP Werte aller getesteten Zellen aufweisen. Weitere Faktoren, die das hypoxische Überleben der Zelle beeinflussen könnten, sind die viralen Onkoproteine E6 und E7. Diese Studie konnte ihre Hypoxie-vermittelte Suppression nachweisen und darüber hinaus zeigen, dass dies nicht durch den zellulären Transkriptionsfaktor AP-1 verursacht wird. Weder die Komposition des dimeren Faktors AP-1 noch die Expression seiner

Untereinheiten ändert sich unter Hypoxie. Zusammenfassend lässt sich sagen, dass Krebszellen eine Dysregulation der hypoxischen GAPDH Transkription aufweisen die das zelluläre Überleben unter hypoxischem Energiestress beeinflussen könnte. In wie weit sich die Stilllegung der viralen E6 und E7 Gene auf diese Beobachtung auswirkt, bedarf weiterer Forschung.

## 1. Introduction

Hypoxia denotes insufficient oxygen supply within a tissue. It is considered to be a major driving force for carcinogenesis (Papp-Szabò *et al.*, 2005) and in addition a prognostic factor in cancer patients indicating a decrease in disease-free patient survival (Höckel *et al.*, 1993). Metabolic adaptations of cancer cells are thought to play a major role in these processes (Vander Heiden *et al.*, 2009). To assess this issue in the context of human papillomavirus driven cervical carcinogenesis, the hypoxic regulation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was studied in HPV-positive tumorigenic and non-tumorigenic cell lines. Previously, the two transcription factors Net and HIF-2 $\alpha$  were suggested to bind to the GAPDH promoter (Hitschler, 2007). The impact of these factors was studied in more detail, since both of them were previously associated with tumor development (van Riggelen *et al.*, 2005; Imamura *et al.*, 2009) and play a fundamental role in mediating the hypoxic response (Gross *et al.*, 2008; O'Rourke *et al.*, 1999). In recent years, GAPDH was shown to be involved in many non-metabolic processes (Colell *et al.*, 2009). Indeed, GAPDH was suggested to act as a trigger between cell death and survival. Thus, a possible link between the differential GAPDH expression in non-tumorigenic and tumorigenic HPV-positive cells and their survival under hypoxic conditions was investigated. Another important factor determining the hypoxic tolerance of HPV-positive cells are the viral oncogenes E6 and E7, which exert pro-angiogenic (Toussaint-Smith *et al.*, 2004) and anti-apoptotic (Scheffner *et al.*, 1990) effects. To shed light on these issues, their hypoxic regulation was assessed in more detail.

### 1.1 Human papillomaviruses and cervical cancer

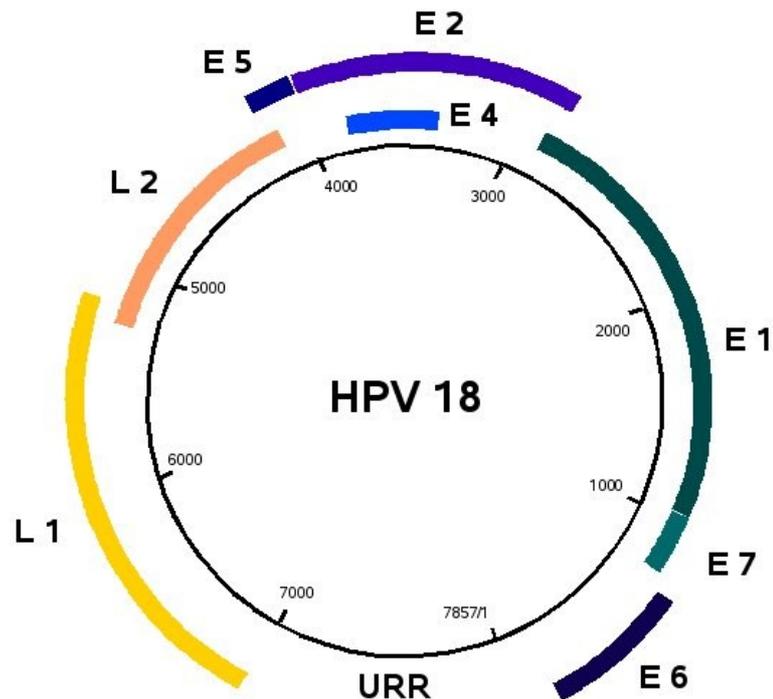
Human papillomaviruses (HPV) are non-enveloped, double-stranded DNA viruses belonging to the family of *Papillomaviridae*. To date, almost 200 different subtypes of HPVs are known and about 40 of them infect the anogenital tract causing benign lesions and sometimes malignancies (Bernard *et al.*, 2010). Tumorigenic progression is associated with so-called “high-risk” HPV types 16, 18 and 31, which are responsible for more than 95 % of cervical cancers (Muñoz *et al.*, 2003). The

transforming capacity of these types is mediated by the viral oncogenes E5 (Straight *et al.*, 1993), E6 and E7 (Münger *et al.*, 1989). Especially the latter ones bear oncogenic potential, since they promote the ubiquitin-proteasome dependent degradation or inactivation of the cellular tumor-suppressors p53 (Scheffner *et al.*, 1990) and pRb (Boyer *et al.*, 1996), respectively. Furthermore, they stimulate neo-vascularization (Chen *et al.*, 2007), which is important for early tumor angiogenesis. Transfection of primary human keratinocytes with high-risk E6 and E7 genes is sufficient for their immortalization *in vitro*. However, tumorigenic progression to cervical cancer requires further cancer promoting events like constitutive *c-fos* expression (Soto *et al.*, 1999).

### 1.1.1 HPV genome organization and viral life cycle

HPVs harbor a circular double-stranded DNA genome of about 8 kb which can be divided into three major regions:

- The 850 bp spanning upstream regulatory region (URR) (**Fig. 1.1**) harbors the origin of viral DNA replication as well as the epithelium-specific enhancer and the proximal promoter driving the transcription of the early genes (Butz and Hoppe-Seyler, 1993).
- The early region encodes six open reading frames (ORFs) (**Fig. 1.1**), among them E1 and E2 playing a role in viral DNA replication and the regulation of early transcription (Del Vecchio *et al.*, 1992; Bouvard *et al.*, 1994). E4 is involved in the breakdown of cellular integrity during viral release (Doorbar *et al.*, 1991). The viral oncogenes E5, E6 and E7 are associated with cell transformation and immortalization. An additional promoter, regulating the expression of the late genes, is located within the E7 ORF.
- The late region encodes the viral capsid proteins L1 and L2 necessary for the assembly of viral particles (Zhou *et al.*, 1991).



**Fig. 1.1: Genome organization of human papillomaviruses** (represented by HPV 18). The genome can be subdivided in three major regions: the upstream regulatory region (URR), the early region (E1 – E7) and the late region (L1 and L2).

HPVs infect dividing basal epithelial cells. Heparan sulfate proteoglycans (Shafti-Keramat, 2003) and certain integrins (Evander *et al.*, 1997) at the host cell surface mediate viral docking and their subsequent endocytic inclusion (Day *et al.*, 2003). After viral disassembly, the L2 protein triggers the nuclear translocation of viral DNA (Day *et al.*, 2004). The viral genome persists as a stable episome in basal epithelial cells which replicates together with cellular DNA during S-phase (Gilbert and Cohen, 1987). In this part of the viral life cycle, only the early genes are expressed. Progression of keratinocytes into suprabasal layers induces transcription of the late viral genes enabling the assembly and release of viral particles (Bodily and Meyers, 2005). The continued expression of E6 and E7 prevents terminal differentiation of the host cell and keeps the keratinocytes in cell cycle, resulting in the formation of benign lesions (Sherman *et al.*, 1997). During long-term infection, episomal HPV DNA tends

to integrate into the host genome, leading to the disruption of several ORFs (Schwarz *et al.*, 1985). Among them are E2, L1 and L2 which disables formation of viral particles.

### **1.1.2 Transcriptional regulation of the early HPV genes**

Regulation of viral transcription is especially important for HPVs, as it decisively contributes to their cell-type specificity. As mentioned above (chapter 1.1.1), the viral URR controls the transcription of the early genes. It can be subdivided into two domains: the tissue specific enhancer and the early promoter.

The early promoter, which is referred to as p105 in HPV18 and p97 in HPV16 mediates the recruitment of RNA polymerase II and the associated transcriptional machinery (Thierry *et al.*, 1987; Garcia-Carranca *et al.*, 1988). The main *cis*-regulatory elements are a tandem repeat of E2 binding-sites overlapping with an Sp1 binding site near the viral TATA box. It is believed, that during the episomal phase of the viral life-cycle, binding of viral E2 to the early promoter prevents interaction with Sp1, thus suppressing transcription of the early oncogenes E6/E7 (Tan *et al.*, 1994). Integration of the viral DNA into the host genome causes loss of E2 and thus subsequent upregulation of E6/E7. Other cellular factors which bind to the early promoter are activator protein 1 (AP-1), Ying Yang 1 (YY-1) and glucocorticoid receptors. Interestingly, the AP-1 binding site overlaps with several YY-1 binding sites, which enables the transcriptional inhibitor YY-1 to quench AP-1 mediated *trans*-activation of early gene transcription (O'Connor *et al.*, 1996).

The HPV enhancer mediates the epithelial specificity of HPV transcription. Although the exact mechanism is not yet clear, the composition and interplay of cellular transcription factors at the URR substantially contributes to this effect. The most prominent ones are Oct-1 (octamer binding protein 1), NF-1 (nuclear factor 1), PR (progesterone receptor), TEF-1 (Transcription enhancer factor 1), AP-1 and nucleolin. The roles of Oct-1 and NF-1 are highly dependent on the HPV type (Butz and Hoppe-Seyler, 1993; O'Connor and Bernard, 1995): Both factors seem to be essential for HPV16 but dispensable for HPV18. Transcriptional stimulation by the progesterone receptor is dependent on the progesterone type (Chen *et al.*, 1996). The keratinocyte specificity of viral transcription is mediated by TEF-1, AP-1 and to some extent by

nucleolin. The transcription factor TEF-1 is believed to mediate cell specificity of HPV transcription by the recruitment of tissue-specific co-activators (Ishiji *et al.*, 1992). Similarly, different compositions of the dimeric transcription factor AP-1 regulate the HPV transcription according to the cellular background. Mutation of the AP-1 binding site inhibits early transcription, making AP-1 a central *trans*-activator of viral gene expression. The important role of AP-1 will be outlined in chapter 1.1.3. A factor mediating the specificity of HPV transcription for dividing cells is nucleolin, whose expression correlates with cell proliferation rates (Grinstein *et al.*, 2002).

In conclusion, the HPV enhancer is activated by the presence of a cell type specific set of transcription factors. Bouallaga and colleagues propose a model, where a keratinocyte specific composition of the AP-1 complex is prerequisite for the formation of an enhanceosome over the HPV URR. The enhanceosome would accordingly consist of other transcription factors mediating efficient and host specific HPV transcription (Bouallaga *et al.*, 2000).

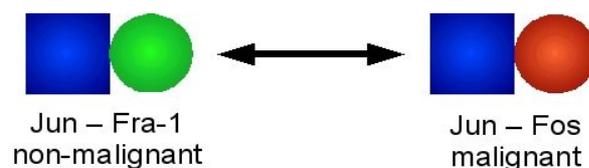
### **1.1.3 The central role of AP-1**

AP-1 is a dimeric transcription factor composed of Jun, Fos and ATF family members. Jun proteins either form homodimers or heterodimers with Fos and ATF family members. The composition of the AP-1 complex is regulated by various extracellular stimuli. Its *trans*-activating properties depend on the availability and post-translational modification of AP-1 subunits and the interaction with co-factors. The AP-1 complex composition determines binding to two different DNA response elements, the TRE (TPA responsive element) and the CRE (calcium/cAMP-responsive element). Thereby AP-1 enables the specific regulation of a certain set of genes in a very selective manner (Shaulian, 2010; Milde-Langosch, 2005).

AP-1 is essential for HPV expression since mutation of either AP-1 binding site completely abrogates HPV transcription (Butz and Hoppe-Seyler, 1993). Similarly, the antioxidants curcumin and pyrrolidine dithiocarbamate (PDTC) inhibit HPV expression by abolishing binding of AP-1 to the HPV URR (Rösl *et al.*, 1997; Prusty and Das, 2005). In addition, the composition of the AP-1 complex highly influences its effect on HPV transcription. In this regard, the Fos family members Fra-1 and c-Fos

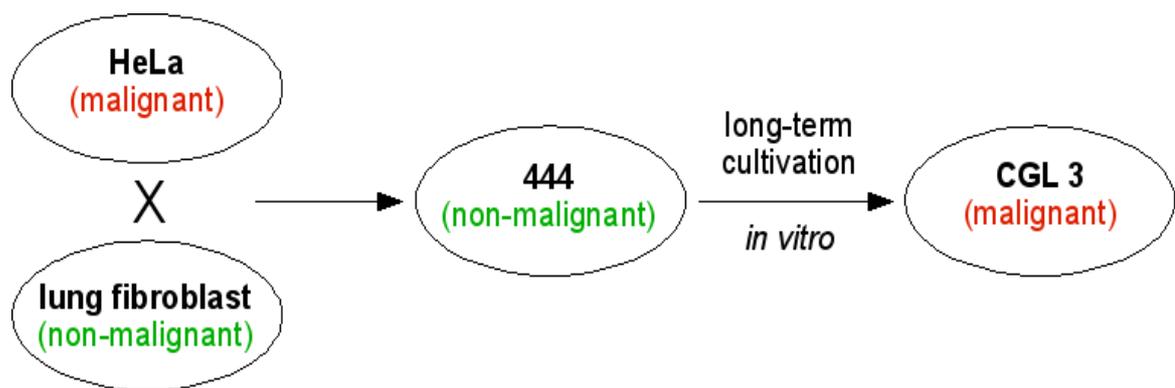
play an important role. In non-malignant cells, the prevalent AP-1 composition is Jun-Fra-1, which is not able to efficiently *trans*-activate early HPV transcription (Soto *et al.*, 1999). Cervical carcinogenesis is accompanied by an increased expression of the oncogene c-Fos and almost complete loss of Fra-1, which leads to a shift in the AP-1 composition (De Wilde *et al.*, 2008). In tumorigenic HPV-positive cells, the prevalent AP-1 composition is c-Jun – c-Fos (**Fig. 1.2**) leading to a high expression of E6/E7 genes.

The constitutive expression of *c-fos* in cervical cancer is mediated by changes in its transcriptional regulation. The ternary complex factor Net is bound to the *c-fos* promoter of non-malignant cells leading to a suppression of *c-fos* transcription. In contrast, Net is almost absent in malignant cells resulting in a high transcription of *c-fos*. Knock-down of Net in non-malignant cells promotes reexpression of *c-fos* (van Riggelen *et al.*, 2005).



**Fig. 1.2:** The ratio of Fos and Fra-1 changes during malignancy

An attractive model to study the impact of AP-1 composition on HPV transcription and cervical carcinogenesis are somatic cell hybrids. Fusion of the HPV18-positive cervical cancer cell line HeLa with non-tumorigenic lung fibroblasts resulted in a non-malignant hybrid (444). Upon long-term cultivation, spontaneous tumorigenic segregants (CGL 3) arose (Stanbridge, 1984) (**Fig. 1.3**). Non-tumorigenic 444 cells express high levels of Fra-1 which changes in favor to c-Fos in CGL 3. The importance of the correlation is emphasized by the fact that ectopic overexpression of c-Fos in 444 induces tumorigenicity (Soto *et al.*, 1999). However, over-expression of Fra-1 in CGL 3 neither reversed the tumorigenic phenotype nor changed the composition of the AP-1 complex (De Castro Arce *et al.*, 2004).



**Fig. 1.3:** Fusion of HeLa and lung fibroblasts results in non-malignant hybrids (444). Upon long-term cultivation, rare tumorigenic segregants (CGL 3) arose.

### 1.1.4 The Ets transcription factor family

The prototype of the Ets (E26 transformation specific) family, the oncogene *v-ets*, was first described in 1983 causing both erythroblastic and myeloblastic leukemias in chicken (Leprince *et al.*, 1983). Since then, many cellular homologues were isolated (Oikawa and Yamada, 2003) – the family of *ets* genes. Today, this family comprises approximately 30 members. The characteristic feature of Ets factors is their evolutionary conserved Ets domain, a stretch of about 85 amino acids which facilitates the binding to the EBS (Ets binding site) in the target promoters (Graves and Petersen, 1998). However it is still mainly unclear, what determines the specific recruitment of an Ets factor to a certain gene. Although multiple Ets factors can target a specific EBS (Hollenhorst *et al.*, 2007), there is evidence that interaction with other transcription factors mediates gene specific recruitment of a certain Ets factor (Aprelikova *et al.*, 2006; Hollenhorst *et al.*, 2009). Ets family proteins are transcription factors which integrate signal transduction cascades into the nucleus. Thus, post-translational modifications play an important role and influence subcellular localization, *trans*-activating properties, DNA binding, recruitment of interaction partners and protein stability (Sharrocks *et al.*, 2001). Ets factors play a crucial role in apoptosis, growth, development, differentiation and oncogenic transformation (Oikawa and Yamada, 2003).

One of the best studied subfamilies of Ets factors are the ternary complex factors (TCFs). Their characteristic feature is the formation of ternary complexes with a

serum response factor (SRF) dimer over a serum response element (SRE), a phenomenon which was first described at the *c-fos* promoter (Shaw *et al.*, 1989). By now, three TCFs have been identified: Elk-1 (Ets-like transcription factor 1) (Rao *et al.*, 1989), Sap-1 (SRF accessory protein 1)/Elk-4 (Dalton and Treisman, 1992) and Net (new Ets transcription factor)/Erp (ets related protein)/Sap-2/Elk-3 (Giovane *et al.*, 1994). The biological roles of the TCF are still unclear. Elk-1 knock-out mice display only minor neuronal defects and are viable (Cesari *et al.*, 2004). Sap-1 knock-out mice do not show a specific phenotype (Sadakata *et al.*, 2009). However, this could be explained by a high redundancy of Sap-1 and Elk-1 as recently indicated for thymocyte development (Costello *et al.*, 2010). Net knock-out results in severe vascular and lymphatic abnormalities and mice die of respiratory failure shortly after birth (Ayadi *et al.*, 2001).

### **1.1.5 Physiological role of Net and the impact on tumorigenesis**

As mentioned above, Net differs from the other ternary complex factors because its function seems to be indispensable for normal development. In the following section, the current knowledge about the role of Net in transcriptional regulation will be outlined.

Net acts as a negative regulator of the oncogene *c-fos* in a SRF-dependent manner (van Riggelen *et al.*, 2005). Overexpression of Net seems to retard cell proliferation in a *c-fos* positive pancreatic cancer model (Li *et al.*, 2008), making Net a key player in *c-fos*- driven tumorigenicity. Similarly, loss of Net and the concomitant *c-fos* upregulation is an important event during cervical carcinogenesis (van Riggelen *et al.*, 2005; de Wilde *et al.*, 2008)

Accumulating evidence suggests a role of Net during angiogenesis. Expression of VEGF, a potent activator of neo-vascularization, is negatively controlled by Net via an indirect mechanism (Zheng *et al.*, 2003). However, Net can exert pro-angiogenic properties suppressing the transcription of plasminogen activator inhibitor (PAI-1) in mice (Buchwalter *et al.*, 2005) and Egr-1 in humans (Ayadi *et al.*, 2001). PAI-1, a negative regulator of fibrinolysis, retards cell migration and wound closure. Egr-1 acts as a negative regulator of angiogenesis. Indeed, sustained Egr-1 expression

abrogates angiogenesis and thereby prevents tumor growth in murine fibrosarcomas (Lucerna *et al.*, 2006). Overall, the role of Net in angiogenesis is still unclear, as it inhibits the expression of both pro- and anti-angiogenic mediators.

Furthermore, Net participates in the inflammatory response. Net was shown to repress the transcription of two inflammatory mediators, nitric oxide synthase 2 (NOS2) (Chen *et al.*, 2003) and heme oxygenase 1 (HO-1) (Chung *et al.*, 2006). In addition to its anti-inflammatory properties, HO-1 stimulates angiogenesis. Overexpression of HO-1 promotes pancreatic cancer progression and metastasis (Sunamura *et al.*, 2003).

Emerging evidence suggests an important function of Net in the hypoxic response. Gross and colleagues report in a microarray study, that 75 % of Net target genes are in common with one of the main regulators of the hypoxic response, HIF-1 $\alpha$ . This suggests widely overlapping functions. In addition, Net and HIF-1 $\alpha$  are both regulated by a group of prolyl-hydroxylase-domain containing proteins (PHD), which act as sensors of cellular oxygen content (Jaakkola *et al.*, 2001; Gross *et al.*, 2007). Thus, Net and HIF-1 $\alpha$  not only share their target genes, but they are regulated by common signaling pathways, too.

### **1.1.6 Hypoxia and cancer**

Hypoxia, defined as inadequate oxygen supply ( $\leq 5\%$  O<sub>2</sub>) of a tissue, is found in a variety of pathophysiological conditions like stroke (Sulter *et al.*, 2000), inflammation (Murdoch *et al.*, 2005) and carcinogenesis (Bertout *et al.*, 2008). Red blood cells are responsible for distributing the oxygen throughout the body. Diffusion of oxygen into the neighboring tissue enables an adequate oxygen supply within 150  $\mu\text{m}$  distance of the blood vessel (Helmlinger *et al.*, 1997). Thomlinson and colleagues reported already in the 1950s that viable tumor cells were only found in the direct neighborhood of blood vessels. Tumor areas distant from arteriolar supply were necrotic. In fact, there is an oxygen gradient from well oxygenated cells next to the vessels to anoxic areas where oxygen is almost absent. Today it is generally accepted that cancer cells are exposed to repeated cycles of hypoxia due to the chaotic and

rapidly changing tumor vascularization (Brown, 1979). However, the duration of these cycles largely varies. Thus, scientists discriminate between “acute hypoxia” lasting from minutes to hours and “chronic hypoxia” lasting from hours to days.

In general, hypoxia is associated with decreased survival of patients in a variety of cancer types like cervical (Höckel *et al.*, 1993), head and neck (Nordsmark *et al.*, 2005) and soft-tissues sarcoma (Nordsmark *et al.*, 2001). The specific contributions of the different hypoxia characteristics to the aetiopathology of cancer are however still under debate. Acutely hypoxic cells were shown to quickly reenter the cell cycle upon reoxygenation in a p53-dependent manner (Pires *et al.*, 2010). Reoxygenation of these cells is accompanied by a burst of reactive oxygen species (ROS), which induces massive DNA-damage and subsequently p53 accumulation and apoptosis. However, most cancers display an impaired p53 functionality, which enables a quick resumption of the cell cycle in the presence of DNA damage (Aguilera and Gomez-Gonzales, 2008). Chronic hypoxia was as well reported to drive a more tumorigenic phenotype. When comparing a chronic and an acute hypoxic prostate cancer model with its parental, tumorigenic cell line, the chronic hypoxia model displayed the highest clonogenic capacity and the strongest VEGF secretion (Alqawi *et al.*, 2007). In addition, only prolonged hypoxic treatment of a neuroblastoma model resulted in reduced susceptibility to chemotherapeutic drugs (Hussein *et al.*, 2006). In line with these observations, Ameri and colleagues demonstrated in a breast cancer model, that circulating tumor cells, causative for metastasis, displayed an increased colony formation under chronic hypoxia, indicating a preceding adaptation to prolonged oxygen deprivation (Ameri *et al.*, 2010).

Overall, the cellular consequences of hypoxia regarding tumorigenicity and cellular survival might be highly dependent on the duration and extent of oxygen deprivation as well as the genetic background of the host. To illustrate the hypoxic response, its key players will be highlighted in the following section.

### 1.1.7 Keyplayers of the hypoxic response

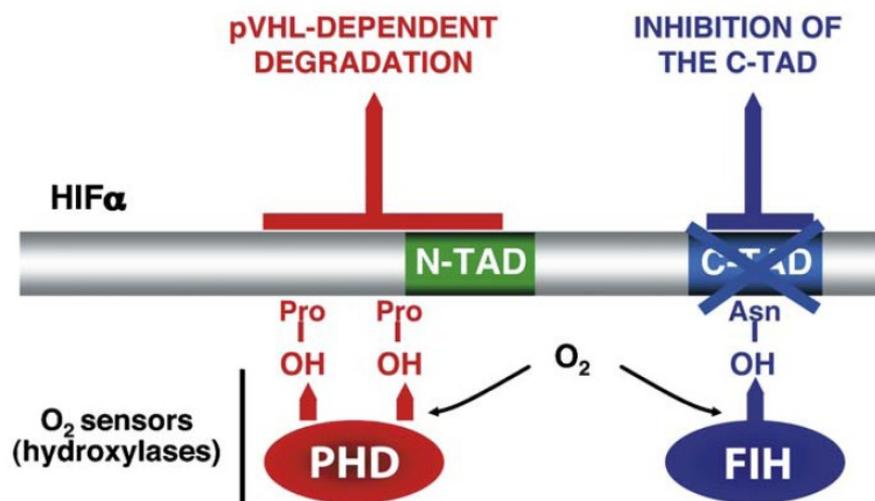
#### Hypoxia inducible factors (HIF)

The best studied mediators of the hypoxic response are the hypoxia inducible factors. These dimeric transcription factors belong to the basic Helix-Loop-Helix (bHLH) protein family and consist of an oxygen-sensitive alpha subunit and a constitutively expressed beta-subunit (HIF-1 $\beta$ , also known as Aryl Hydrocarbon Receptor Nuclear Translocator) (Wang *et al.*, 1995). To date, three different alpha-subunits have been described: The best studied and ubiquitously expressed is HIF-1 $\alpha$ . Basic studies describing its hypoxic *trans*-activating capacity were conducted on the EPO promoter (Wang and Semenza, 1993b). Since then, two other members were discovered which are encoded in separate genes. HIF-2 $\alpha$  shares 48 % homology with HIF-1 $\alpha$  (Hu *et al.*, 2007) but it is expressed only in certain tissues and cancer types (Wiesener *et al.*, 2003). Both, HIF-1 $\alpha$  and HIF-2 $\alpha$  harbor two *trans*-activation domains (TAD) which enable them to strongly induce transcription of hypoxia-inducible genes. In contrast, the third member HIF-3 $\alpha$  holds only the N-terminal TAD, thus exhibiting a low *trans*-activation potential. When competing with the other alpha-subunits, it was shown to repress transcription of the respective target genes (Hara *et al.*, 2001).

As mentioned above, all HIF $\alpha$  subunits are sensitive to oxygen-dependent degradation which is mediated by their shared oxygen-dependent degradation domain (ODD). When oxygen levels rise, a family of prolyl-hydroxylase-domain containing proteins (PHD) mediates the hydroxylation of distinct prolin residues (**Fig. 1.5**). This enables the interaction of HIF $\alpha$  subunits with the E3 ubiquitin-ligase VHL (von-Hippel-Landau factor) and subsequent proteasomal degradation (Jaakkola *et al.* 2001; Appelhoff *et al.*, 2004). Under hypoxic conditions, PHDs lack the essential oxygen for hydroxylation. In addition, they themselves are subject of enhanced proteasomal degradation mediated by the E3 ubiquitin-ligases Siah 1 and Siah 2 ("Seven in absentia homologues" 1 and 2) (Nakayama *et al.*, 2004).

In the case of HIF-1 $\alpha$ , the activity of the c-terminal TAD can be fine-tuned in an oxygen dependent manner. Factor inhibiting HIF-1 (FIH) hydroxylates a distinct asparagine residue in HIF-1 $\alpha$  leading to the inhibition of the c-terminal TAD (Lando *et al.*, 2002) (**Fig 1.5**). HIF-2 $\alpha$  is as well hydroxylated by FIH, but there is only a minor effect on its *trans*-activating potential (Yan *et al.*, 2007).

Presence of the viral oncogenes E6 and E7 of both high- and low-risk HPVs was shown to enhance stabilization of HIF-1 $\alpha$  upon hypoxia, resulting in the increased activation of HIF-1 target genes (Nakamura *et al.*, 2009; Tang *et al.*, 2007).



(Dayan *et al.*, 2009)

**Fig. 1.5: Oxygen dependent regulation of Hypoxia-inducible factors.** Under normoxic conditions, PHDs hydroxylate two proline (Pro) residues of the HIF $\alpha$  subunits. This facilitates ubiquitination by the VHL and subsequent proteasomal degradation. In contrast, FIH mediates oxygen dependent fine-tuning, especially of HIF-1 $\alpha$ . By hydroxylating an asparagine residue, FIH blocks the activity of the c-terminal TAD (C-TAD) domain and enables a distinct *trans*-activation of genes more susceptible to the N-terminal TAD (N-TAD).

All HIF dimers were shown to bind to hypoxia-responsible elements (HRE) located at the promoter of hypoxia sensitive genes. The target genes of HIF-1 and HIF-2 are mainly redundant. However, growing evidence suggests, that both factors indeed vary in the specificity to distinct groups of target genes. HIF-1 was shown to preferentially *trans*-activate glycolytic genes (Hu *et al.*, 2003), while HIF-2 was shown to induce for example transforming growth factor  $\alpha$  (TGF $\alpha$ ) (Raval *et al.*, 2005), octamer binding transcription factor 4 (Oct-4) (Covello *et al.*, 2006) and vascular

endothelial growth factor receptor 2 (VEGFR2) (Kappel et al., 1999). The target gene specificity is conferred by two main factors: the duration and extent of hypoxia as well as the cellular background and the availability of co-factors. Concerning the influence of oxygen availability, HIF-1 $\alpha$  preferentially mediates the acute response to hypoxia, while HIF-2 $\alpha$  takes over its function in case of prolonged hypoxia (Holmquist-Mengelbier *et al.*, 2006). In the context of co-factor mediated target gene specificity, the transcription factor family of Ets factors plays an important role: Ets-1 (Elvert *et al.*, 2003) and Elk-1 (Aprelikova *et al.*, 2006) recruit HIF-2 $\alpha$  to target genes. In contrast, GATA-2 was shown to be required for HIF-1 recruitment (Yamashita *et al.*, 2001). This mechanism facilitates target gene specificity in an elegant way despite the redundant DNA binding motif of HIF-1 and HIF-2.

### **Reactive oxygen species (ROS)**

Accumulating evidence suggests that mitochondria are one of the central oxygen sensors of the cell. It seems that reactive oxygen species produced by mitochondria upon oxygen deprivation are essential for the hypoxic response. Indeed, hypoxic induction of HIFs is abolished by antioxidant treatment. In a similar way, mitochondria depleted cells fail to induce HIFs upon hypoxia. (Chandel *et al.*, 1998)

The main source for mitochondrial ROS is complex III, where the enzyme ubiquinone (co-enzyme Q) is oxidized in a two-step process. In the intermediate step, radical ubisemiquinone is formed, which is capable of donating its free electron to molecular oxygen (Turrens *et al.*, 1985). The resulting mitochondrial radical, the superoxide-anion ( $O_2^{\cdot-}$ ), is subsequently distributed from the mitochondria to the cytoplasm (Muller *et al.*, 2004). Hypoxia is generally accompanied by an increase of ROS. The underlying mechanism is however still under debate. Mitochondrial peroxidases which are able to neutralize the radicals within the mitochondria, may display a reduced activity under hypoxia (Murphy 2009).

ROS exert a multitude of cellular effects, many of them linked to carcinogenesis. Most apparently, they induce multiple forms of DNA damage which increases genomic instability in cancer cells. In addition, ROS regulate the activity of several

kinases, among them extracellular signal regulated kinase (ERK1/2) and phosphoinositide 3-kinase (PI3K) (Triantafyllou *et al.*, 2006) whose dysregulation is considered to promote tumorigenesis.

## 1.2 Metabolism and cancer

Carcinogenesis is accompanied by a shift in energy metabolism. In the presence of sufficient amounts of oxygen, most differentiated cells optimize their metabolism to yield maximal ATP and minimal lactate, a ratio which changes only in case of scarce oxygenation. Already in the 1920s, Otto Warburg observed that most cancer cells catabolize glucose to lactate even in the presence of sufficient amounts of oxygen which is termed “aerobic glycolysis”. Concerning the resulting ATP, aerobic glycolysis seems to be much less efficient than mitochondrial oxidative phosphorylation: while the latter one results in 36 M ATP, a conversion of glucose to lactate renders only 2 M ATP (Lehninger *et al.*, 1993). Recently, scientists began to unravel the advantage of aerobic glycolysis for cancer cells. Several glycolytic intermediates are precursors for the synthesis of lipids, amino acids and nucleotides. All of these macromolecules are essential for highly proliferating cancer cells, since they need to double their biomass for each cell division.

An important example is the pentose phosphate pathway. It reroutes glycolytic intermediates to fuel the production of NADPH and ribose-5-phosphate which are precursors for fatty acid and nucleotide synthesis, respectively. Furthermore, NADPH is one of the most important co-factors of ROS-neutralizing enzymes (Pollack *et al.*, 2007). ROS induced DNA damage is especially harmful during proliferation and may be causative for the accumulation of mutations.

To allow a rerouting of glycolytic intermediates into the pentose phosphate pathway, cancer cells take control over their glycolytic flux by regulating the last step of glycolysis: the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase. In contrast to normal cells, which use the highly active pyruvate kinase isoenzyme 1 (PK-1M), cancer cells preferentially express the substrate-inducible variant PK-2M which displays a low activity and can be inhibited by tyrosine kinase signalling

(Christofk *et al.*, 2008). The preferential expression of PK-2M enables a reduction of glycolytic throughput. This leads to the accumulation of glycolytic intermediates and induces their redirection into the pentose phosphate pathway.

How do cancer cells reprogram their metabolism? Vander Heiden and colleagues propose a model, where oncogenic pathways promote a proliferative metabolism including cell-autonomous nutrient uptake (Vander Heiden *et al.*, 2009). In contrast, tumor suppressive signaling is thought to shut-down anabolic processes. A well studied example for this theory is the tumor suppressor p53. Its target gene TIGAR controls the rerouting of glycolytic intermediates into the pentose phosphate pathway (Bensaad *et al.*, 2006). Another example for oncogenes which reprogram the cellular metabolism is c-myc, which is involved in glutamine metabolism. Glutamine withdrawal of c-myc transformed cells in fact induces apoptosis (Yuneva *et al.*, 2007).

### **1.3 The manifold properties of GAPDH**

For a long time, GAPDH was simply seen as a central enzyme of the glycolytic pathway, catalyzing the oxidation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate (Racker et Krimsky, 1952). GAPDH is often referred to as a housekeeping gene due to its ubiquitous and high expression levels. Today, GAPDH is known to play a role in a variety of cellular functions. Indeed, most of the recently discovered properties of GAPDH are unrelated to its glycolytic function. The enzyme is involved in cytoskeletal dynamics (Huitorel and Pantaloni, 1985), it regulates RNA stability, thus controlling protein expression (Zhou *et al.*, 2008) and it acts as a cell-cycle dependent transcription factor (Zheng *et al.*, 2003).

More intriguingly, GAPDH serves as a trigger between cell death and survival. Nuclear accumulation of GAPDH directly precedes induction of apoptosis which can be efficiently antagonized by over-expression of the anti-apoptotic Bcl2 (Dastoor and Dreyer 2001; Maruyama *et al.*, 2001). Indeed, nuclear translocation of GAPDH is tightly regulated, as the enzyme itself does not harbor a nuclear localization signal. Upon apoptotic stimuli, GAPDH interacts with Siah1 which facilitates subsequent nuclear transport (Hara *et al.*, 2005). Here, GAPDH acts as a transcription factor recruiting the co-activator CBP/p300. This enhances CBP/p300 *trans*-activation capacity and leads to the activation of pro-apoptotic mediators like p53 (Sen *et al.*,

2008). Siah-1 in turn is stabilized by GAPDH which increases Siah1 nuclear levels and accelerates proteasomal degradation of its nuclear targets. In addition, GAPDH exerts pro-apoptotic functions in the cytoplasm. The active site of GAPDH dehydrogenase activity is highly susceptible to oxidation (Hwang *et al.*, 2009). Thus it is not surprising, that GAPDH acts as a trigger in oxidant induced cell death: Severe oxidation can induce the formation of inter-molecular disulfide-bonds, leading to amyloid-like GAPDH aggregation and subsequent cell death. On the other hand, inactivation of GAPDH blocks the glycolytic flux which leads to a rerouting of glycolytic intermediates into the pentose phosphate pathway and fuels the anti-oxidant defense (Ralser *et al.*, 2007).

Indeed, GAPDH has several pro-survival functions. This notion is supported by the fact that cancers often display an over-expression of GAPDH, which was associated with poor patient outcome (Révillion *et al.*, 2000; Lavallard *et al.*, 2009). Tumor cells often depict defects in the apoptotic machinery, rendering them resistant to apoptotic triggers. Under these circumstances, GAPDH can prevent caspase independent cell death (CICD) (Colell *et al.*, 2007) which is characterized by mitochondrial membrane permeabilization and subsequent cell death in the absence of caspase activity. GAPDH was shown to prevent CICD in two ways: it enhances the expression of autophagic genes to clear damaged mitochondria and enables the maintenance of cellular energy homeostasis via its glycolytic properties. In addition, GAPDH can inactivate the mTOR pathway, an important trigger for autophagy (Lee *et al.*, 2009). Another pro-survival function is assigned to the genome protecting properties of GAPDH. Its binding to the chromosomal telomeres is required for their maintenance and might be associated with cellular immortalization (Sundaraj *et al.*, 2004). Additionally, GAPDH stabilizes theapurinic endonuclease 1 (APE1). This enzyme repairs drug- or oxidant-induced DNA damages thus enabling the maintenance of genomic integrity under harsh environmental conditions (Azam *et al.*, 2008).

Taken together, the function of GAPDH is highly dependent on the cellular background and its post-translational modifications. Colell and colleagues propose a GAPDH mediated metabolic checkpoint, which triggers pro-apoptotic and pro-

survival machineries. Overcome this checkpoint would be beneficial in the process of tumorigenesis, when cells are getting more resistant to oxidative stress and scarce nutrient conditions.

## 2. Objectives

During cervical carcinogenesis, hypoxia drives the development of a more aggressive cancer phenotype which is accompanied by metabolic alterations (Höckel *et al.*, 1993; Vander Heiden *et al.*, 2009). Indeed, the expression of the glycolytic enzyme GAPDH is often deregulated in cancers but the underlying mechanisms remain unknown (Révillion *et al.*, 2000). Interestingly, the two transcription factors Net and HIF-2 $\alpha$  associated with carcinogenesis and cellular hypoxic response were suggested to bind to the GAPDH promoter (Hitschler, 2007). This study aims to elucidate, whether they are involved in the frequently observed deregulation of GAPDH during tumorigenesis. Considering the crucial role of this enzyme as a trigger between cell death and survival (Colell *et al.*, 2009), GAPDH impacts cellular resistance to metabolic stress caused by hypoxia. Thus, as a second objective, the hypoxic tolerance of tumorigenic and non-tumorigenic HPV-positive cells will be investigated. Another important factor affecting the hypoxic response are the viral oncoproteins E6 and E7 which exert anti-apoptotic and pro-angiogenic effects (Scheffner *et al.*, 1990; Toussaint-Smith *et al.*, 2004). To shed light on this issues, the hypoxic regulation of these oncogenes will be assessed in more detail.

### 3. Material and Methods

#### 3.1 Abbreviations

Ad 5	Adenovirus 5
AGE	Agarose gel electrophoresis
AMPK	AMP activated protein kinase
AP-1	Activator protein 1
APS	Ammonium persulfate
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATF	Activating transcription factor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine 5-triphosphate
ATR	Ataxia telangiectasia and Rad3-related
bHLH	Basic helix-loop-helix
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
bp	Basepair
BSA	Bovine serum albumin
C-fos	human homolog of the Finkel-Biskis-Jinkins osteosarcoma virus oncogene
CAMKK2	Calcium-calmodulin-dependent kinase 2
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
CICD	Caspase independent cell death
CRE	Calcium/cAMP-responsive element
DAPI	4',6-Diamidin-2-phenylindol
DEPC	Diethyl-pyrocarbonate
DHE	Dihydroethidium
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	2 -Deoxynucleosine-5 -triphosphate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EBS	Ets binding site
ECL-Reagent	Enhanced chemiluminescence reagent
EDTA	Ethylenediaminetetraacetic acid
Egr-1	Early growth response protein-1
EGTA	Ethylene glycol tetraacetic acid
Elk-1	Ets-like transcription factor 1
EMSA	Electrophoresis mobility shift assay
ERK	Extracellular signal-regulated kinase
Erp	Ets related protein
Ets	E26 transformation-specific
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum

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Fra-1	Fos related antigene 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
HBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
HIPK2	Homeodomain-interacting protein kinase 2
HO-1	Heme oxygenase 1
HPV	Human papillomavirus
HRE	Hypoxia response element
HRP	Horse radish peroxidase
IgG	Immunoglobuline G
IP	Immunoprecipitation
Kbp	Kilobasepair
KDa	Kilodalton
LAR II	Luciferase assay reagent II
LB	Luria Bertoni
MAPK	Mitogen activated protein kinase
MOPS	Morpholine propanesulfonic acid
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
Net	New Ets transcription factor
NF-1	Nuclear factor 1
NOS2	Nitric oxide synthase 2
NP-40	Nonidet P-40
NTP	Nucleoside triphosphate
Oct-1	Octamer-binding transcription factor 1
ODD	Oxygen-dependent degradation domain
ORF	Open reading frame
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PDTC	Pyrrolidine dithiocarbamate
PHD	Prolyl hydroxylase
PI3K	Phosphoinositide 3-kinase
PIAS1	Protein inhibitor of activated STAT1
PML	Promyelocytic leukaemia tumor suppressor
PR	Progesterone receptor
PVDF	Polyvinylidene difluoride
RIPA	Radioactive immunoprecipitation assay
RLU	Relative luciferase units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
Sap-1	SRF accessory protein 1
SDS	Sodium dodecyl sulfate

SDS-PAGE	Denaturant polyacrylamide gel electrophoresis
Siah1	Seven-in-absentia homologue
SOC	Super optimal broth with catabolite repression
Sp-1	Specificity protein 1
SRE	Serum response element
SRF	Serum response factor
SR101	Sulforhodamine 101
TAD	<i>Trans</i> -activation domain
TAE	Tris-acetate-EDTA
TBP	TATA binding protein
TBST	Tris-buffered saline with Tween
TCF	Ternary complex factor
TE	Tris-EDTA
TEF-1	Transcription enhancer factor 1
TEMED	N, N, N, N -Tetramethylethylenediamine
TK	Thymidine kinase
TPA	12-O-tetradecanoylphorbol-13-acetate
TPE	TPA responsive element
Tris	Tris(hydroxymethyl)-aminomethane
TSC	Tuberous sclerosis protein
URR	Upstream regulatory region
VEGF	Vascular endothelial growth factor
VHL	Von-Hippel-Landau factor
v/v	Volume percentage
w/v	Weight percentage
YY-1	Yin Yang 1

## 3.2 Material

### 3.2.1 Antibodies

Antibody	Epitope	Reference	Use
Actin (Clone 4) mouse monoclonal IgG cat # 691001	Chicken gizzard actin	MP Biomedical Inc., Eschwege	Western 1:10000
Anti-Flag M2 mouse monoclonal IgG cat # F3165	Flag peptide sequence	Sigma-Aldrich, Munich	Western 1:5000
Anti-HA High Affinity (Clone 3F10) rat monoclonal IgG cat # 1867423	HA peptide sequence	Roche Diagnostics, Mannheim	Western 1:1000

### 3. Material and Methods

Antibody	Epitope	Reference	Use
Anti-mouse IgG HRP, cat # W4021	Mouse IgG heavy and light chain	Promega, Mannheim	Western 1:10000
Anti-rabbit IgG HRP, cat # W401B	Rabbit IgG heavy and light chain	Promega, Mannheim	Western 1:10000
Anti-rat IgG HRP	Rat IgG heavy and light chain	Dianova, Hamburg	Western 1:10000
c-Fos rabbit polyclonal IgG cat # 06-341 lot # 23255	amino acids 3-16 of human c-Fos	Upstate Cell Signaling, Hamburg	Western 1:10000
Fra-1 rabbit polyclonal IgG cat # sc-605X lot # H022	N-terminus of murine Fra-1	Santa Cruz Biotech, Heidelberg	Western 1:5000
HIF-1 $\alpha$ (H-206) cat # sc-10790 X lot # L0905	amino acids 575-780 mapping near the C- terminus of human HIF-1 $\alpha$	Santa Cruz Biotech, Heidelberg	Western 1:1500
HIF-2 $\alpha$ rabbit polyclonal IgG cat # NB100-122	C-terminus of murine/human HIF- 2 $\alpha$	Novus Biologicals, Cambridge (UK)	Western 1:2000 ChIP 1,5 $\mu$ g
Net antisera 2005 and 2007	Human Net	Dr. Bohdan Wasylyk, IGBMC Illkirch, France	ChIP 1:50 Western 1:2000
Normal rabbit IgG, cat # sc-2027	Control IgG	Santa Cruz Biotech., Heidelberg	ChIP, 2 $\mu$ g
RNA polymerase II rabbit polyclonal IgG cat # ab5131-50	RNA polymerase II, CTD repeat YSPTSPS (phospho S5)	Abcam, Cambridge (UK)	ChIP, 2 $\mu$ g

### 3.2.2 PCR primers

#### Primers for promoter analysis

Gene	Sequence	Annealing temperature	PCR cycles
adh5	5'-CTGGAACGCACAACCTTAGCAGCA-3'	60 °C	35
	5'-ATAGCAGCCTAGTCCCATGCC-3'		
gapdh	5'-CCCAACTTTCCCGCCTCTC-3'	62°C	35
	5'-CAGCCGCCTGGTTCAACTG-3'		
pai-1	5'-CAACCTCAGCCAGACAAGGT-3'	55°C	35
	5'-ACCTCCATCAAACGTGGAA-3'		

#### Primers for cDNA analysis

Gene	Sequence	Annealing temperature	PCR cycles
gapdh	5'-ATATTGTTGCCATCAATGACC-3'	65°C	20
	5'-GATGGCATGGACTGTGGTCATG-3'		
HPV18 E6/E7	5'-CGGAACTGAACACTTCACTGC-3'	65°C	18
	5'-TGCGTCGTTGGAGTCTTTC-3'		
pai-1	5'-CTCCGAGAATCCCACACAG-3'	60°C (Buchwalter <i>et al.</i> , 2005)	35
	5'-ACTTTGAATCCCATAGCATC-3'		
β-Tub	5'-TCTGTTCGCTCAGGTCCTTT-3'	59°C	35
	5'-TTCATGATGCGATCAGGGTA-3'		
VEGF	5'-TCCTCACACCATTGAAACCA-3'	53°C	30
	5'-CACCGATCAGGGAGAGAGAG-3'		

### 3.2.3 Oligos for EMSA

Oligo	Sequence	Reference
AP-1 consensus	5'-CGCTTGATGACTCAGCCGGAA-3'	Collagenase gene (Lee <i>et al.</i> , 1987)

## 3.2.4 Plasmids

Plasmid	Insert	Reference
pcDNA3.1	-	Invitrogen, Karlsruhe
pcDNA3-HIF-1 $\alpha$ <sup>P564A</sup>	Human HIF-1 $\alpha$ cDNA, substitution at P564 for increased stability	Ph.D. Olga Aprelikova, NIH, Bethesda (USA)
pcDNA-HIF-2 $\alpha$ <sup>P531A</sup>	Human HIF-1 $\alpha$ cDNA, substitution at P531 for increased stability	Ph.D. Olga Aprelikova, NIH, Bethesda (USA)
pcDNA3-Flag-HIPK2	Human HIPK2 cDNA with N-terminal Flag-tag	Dr. Thomas Hofmann, DKFZ, Heidelberg
pcDNA-HA-Siah1	Human Siah1 cDNA with N-terminal HA-tag	Dr. Thomas Hofmann, DKFZ, Heidelberg
pcDNA-HA-Siah1 <sup>C44S</sup>	Human Siah1, E3-ligase deficient mutant due to substitution at C44; encodes N-terminal HA-tag	Dr. Thomas Hofmann, DKFZ, Heidelberg
pEGFP	Optimized variant of wild-type GFP cDNA	Clontech, Saint-Germain-en-Laye (France)
pGL3(-1112)GAPDH	1112 bp of the human GAPDH promoter driving firefly luciferase	Ph.D. Shan Lu, University of Cincinnati, Cincinnati (USA)
pRL-TK	herpes simplex virus thymidine kinase (HSV-TK) promoter driving renilla luciferase	Promega GmbH, Mannheim
pSG5	-	Agilent Technologies, Waldbronn
pTL2-Net	Human Net cDNA	Dr. Bohadan Wasyluk, IGBMC, Strasbourg (France)
pCRII-TOPO-Tub	271 bp cDNA fragment of human $\beta$ -Tubulin	-

### 3.2.5 Buffers and solutions

#### Agarose gel electrophoresis

DEPC water	0.1 %(v/v) DEPC
MOPS (20X) pH 7,0	400 mM MOPS 100 mM Sodium acetate 20 mM EDTA pH 7.0 (DEPC water) Store light protected
RNA loading buffer (2X)	50 % Formamide 2.2 M Formaldehyde 1 % (w/v) Ficoll (type 400) 0.02 % (w/v) Bromophenol blue 1X MOPS buffer pH 7.0 (DEPC water) Store at -20 °C
TAE (50X) pH 7.8	2 M Tris Base 250 mM NaAc 50 mM EDTA pH 8.0

#### Bacteria culture

Ampicillin	100 mg/ml in water Store at -20 °C
Kanamycin	50 mg/ml in water Store at -20 °C
LB-ampicillin plates	LB medium 2 % (w/v) Bacto-Agar 100 mg/l ampicillin
LB-kanamycin plates	LB medium 2 % (w/v) Bacto-Agar 50 mg/l kanamycin
LB medium pH 7,2	10 % (w/v) NaCl 10 % (w/v) Bacto-Trypton 5 % (w/v) yeast extract

SOC medium

- 2 % (w/v) Bacto-Trypton
- 0.5 % (w/v) yeast extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM MgCl<sub>2</sub>
- 10 mM MgSO<sub>4</sub>
- 20 mM Glucose

#### Calcium phosphate precipitation

CaCl<sub>2</sub>

- 2 M CaCl<sub>2</sub>
- Sterile filter before use

HBS (2X)

- 280 mM NaCl
- 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>
- 50 mM HEPES
- Adjust to pH 7.05
- Sterile filter before use

#### Cell culture

DMEM complete

- 500 ml DMEM
- 10 % (v/v) FCS
- 100 U/ml Penicillin
- 100 µg/ml Streptomycin

Freezing DMEM

- 60 % (v/v) DMEM
- 30 % (v/v) FCS
- 10 % (v/v) DMSO

#### Chromatin immunoprecipitation

Cell lysis buffer

- 85 mM KCl
- 5 mM HEPES pH 8.0
- 0.5 % (v/v) NP-40
- Before use add protease and phosphatase inhibitors

ChIP buffer	167 mM NaCl 16.7 mM Tris/HCl pH 8.0 1.2 mM EDTA pH 8.0 1.1 % (v/v) Triton X 0.01 % (v/v) SDS Before use add protease and phosphatase inhibitors
Chloroform/Isoamylalcohol (24:1)	24 parts chloroform 1 part isoamylalcohol Store light protected at 4 °C
Digestion and decrosslinking cocktail	320 mM NaCl 80 mM Tris/HCl pH 8 20 mM EDTA pH 8 80 µg/ml Proteinase K Diluted in elution buffer Need to be prepared freshly
Elution buffer	95 mM NaHCO <sub>3</sub> 1 % v/v SDS Need to be prepared freshly
High salt buffer	500 mM NaCl 50 mM Tris/HCl pH 8.0 5 mM EDTA pH 8.0 0.5 % (v/v) NP-40
Lithium chloride buffer	250 mM LiCl 10 mM Tris/HCl pH 8.0 1 mM EDTA pH 8.0 0.5 % (v/v) NP-40 0.5 % (w/v) Sodium Desoxycholate
Low salt buffer	0.1 % (v/v) SDS 1 % (v/v) Triton X 20 mM Tris/HCl pH 8.0 150 mM NaCl
TE (1X)	10 mM Tris Base 1 mM EDTA pH 8.0

#### **EMSA**

TBE (10X)	0.9 M Tris Base 0.9 M Boric Acid 0.02 M EDTA pH 8.0
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TNE (1X) 100 mM Sodium chloride  
Dissolved in 1X TE pH 8  
Adjust to pH 7.4

**Immunofluorescence**

Mowiol 10 % 10 % Mowiol 4-88  
25 % Glycerol  
100 mM Tris/HCl pH 8.5

Paraformaldehyde 4 % 4 % Paraformaldehyde  
Dissolved in PBS  
Adjust to pH 7.2

**Nuclear protein preparation**

Buffer A 10 mM HEPES pH 7.9  
10 mM KCl  
0.1 mM EDTA pH 8.0  
0.1 mM EGTA pH 7.9  
Store at -20 °C  
Before use add protease and  
phosphatase inhibitors

Buffer C 25 % (v/v) glycerol 99.5 %  
20 mM HEPES pH 7.5  
400 mM Sodium chloride  
1 mM EDTA pH 7.9  
Store at -20 °C  
Before use add protease and  
phosphatase inhibitors

**Protease and phosphatase inhibitors**

Complete protease inhibitor 50X stock solution  
Store at -20 °C

DTT 0.1 M stock solution  
Store at -20 °C

MG-132 20 mM stock solution  
Dissolve in DMSO  
Store at -80 °C

Sodium fluoride 500 mM NaF  
Store at -20 °C

Sodium ortho-vanadate pH 10 10 mM NaVO<sub>4</sub>  
Store at -20 °C

**Western blot**

APS 10 % (w/v) APS

Blocking Buffer (Western Blot) TBST  
5 % (w/v) Milk powder  
Store at 4 °C

Laemmli buffer (10X) 0.25 M Tris Base  
1.9 M Glycine  
1 % (w/v) SDS

SDS loading buffer (5X) 10 % (v/v) SDS  
5 mg Bromophenol blue  
12.5 % (v/v) 2-Mercaptoethanol  
5 mM EDTA pH 8.0  
50 % (v/v) Glycerol  
300 mM Tris/HCl pH 6.8  
Store at -20 °C

TBS (1X) 10 mM Tris /HCl pH 7.5  
100 mM NaCl

TBST 1X TBS pH 7.6  
0.1 % (v/v) Tween 20

Towbin (1X) 25 mM Tris Base  
192 mM Glycine  
0.1 % (w/v) SDS  
15 % (v/v) Methanol

**Whole cell protein extraction**

RIPA buffer 10 mM Tris/HCl pH 8  
150 mM NaCl  
1 mM EDTA pH 8  
1 % NP-40  
0.1 % SDS  
Before use add protease and  
phosphatase inhibitors

### 3.2.6 Cell lines

Cell line	Characteristics	Reference
444	HeLa x fibroblast hybrids <i>Viral status</i> : HPV 18 <i>In vivo phenotype</i> : non-tumorigenic after s.c. injection into nu <sup>-/-</sup> mice	(Stanbridge, 1984)
CGL 3	HeLa x fibroblast hybrids (segregant of 444) <i>Viral status</i> : HPV 18 <i>In vivo phenotype</i> : tumorigenic after s.c. injection into nu <sup>-/-</sup> mice	(Stanbridge, 1984)
HEK 293	<i>Species</i> : human, female, embryonic <i>Tissue</i> : kidney <i>Viral status</i> : partial sequence from Ad 5 genome <i>In vivo phenotype</i> : tumorigenic after s.c. injection into nu <sup>-/-</sup> mice	(Graham <i>et al.</i> , 1977)
HeLa	<i>Species</i> : human, female, adult <i>Tissue</i> : cervix, adenocarcinoma <i>Viral status</i> : HPV 18 <i>In vivo phenotype</i> : tumorigenic after s.c. injection into nu <sup>-/-</sup> mice	(Boshart <i>et al.</i> , 1984; Schwarz <i>et al.</i> , 1985)
IMR 90	<i>Species</i> : human, female, embryo <i>Tissue</i> : lung, fibroblast <i>Viral status</i> : negative <i>In vivo phenotype</i> : non-tumorigenic after s.c. injection into nu <sup>-/-</sup> mice	(Nichols <i>et al.</i> , 1977)

### 3.2.7 Chemicals and reagents

Acrylamide/bis-Acrylamide (29:1), 30 %	Sigma-Aldrich, Munich
Agarose	Merck, Darmstadt
Ammonium persulfate	Sigma-Aldrich, Munich
Ampicillin	Roche Diagnostics, Mannheim
Bacto-Agar	Invitrogen, Karlsruhe
Bacto-Tryptone	BD Diagnostics, Heidelberg
Bovine Serum Albumin	Sigma-Aldrich, Munich
Bradford-Reagent (Bio-Rad Protein Assay)	Bio-Rad Laboratories, Munich
Bromophenol Blue	Sigma-Aldrich, Munich
Chloric acid	Merck, Darmstadt
Chloroform	Merck, Darmstadt
Complete Protease inhibitor, EDTA-free	Roche, Mannheim

Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, Munich
Dihydroethidium 5 mM in DMSO	Invitrogen, Karlsruhe
dNTP set	Invitrogen, Karlsruhe
Dithiothreitol (DTT)	Sigma-Aldrich, Munich
DNA 6X loading buffer	Fermentas, St. Leon-Rot
Dulbecco's modified Eagle's Medium (DMEM)	Invitrogen, Karlsruhe
ECL-Reagent	PerkinElmer, Rodgau-Jügesheim
EDTA	Roche Diagnostics, Mannheim
EGTA	Sigma-Aldrich, Munich
Ethanol absolute	Merck, Darmstadt
Ethidiumbromide	Fluka BioChemika, Buchs (CH)
Fetal calf serum	Invitrogen, Karlsruhe
Ficoll 400	Serva, Heidelberg
Formaldehyde	Merck, Darmstadt
Glycerol	Merck, Darmstadt
Glycine	Sigma-Aldrich, Munich
HEPES	Gerbu, Gaiberg
Hoechst 33258	Sigma-Aldrich, Munich
Kanamycin	Roche Diagnostics, Mannheim
Lithium chloride	Fluka BioChemika, Buchs (CH)
Magnesium chloride	Merck, Darmstadt
2-Mercaptoethanol	Sigma-Aldrich, Munich
Methanol	Merck, Darmstadt
MG-132	Biomol, Hamburg
Milk powder	Roth, Karlsruhe
MOPS	Gerbu, Gaiberg
Mowiol 4-88	Roth, Karlsruhe
Nonidet-P40	Roche Diagnostics, Mannheim
PBS	Invitrogen, Karlsruhe
Penicillin 10000U/ml	Invitrogen, Karlsruhe
Poly(dI-dC).poly(dI-dC)	Amersham-Pharmacia, Freiburg
Potassium chloride	Merck, Darmstadt
Protein A Agarose	Roche Diagnostics, Mannheim
Random primers p(dN) <sub>6</sub>	Boehringer, Mannheim
RNasin Plus RNase Inhibitor, 40 U/μl	Promega, Mannheim
Roti-Phenol	Roth, Karlsruhe
Salmon Sperm	Sigma-Aldrich, Munich
Sodium acetate	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium desoxycholate	Merck, Darmstadt

Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Munich
Sodium fluoride	Merck, Darmstadt
Sodium hydrogen carbonate	Merck, Darmstadt
Sodium ortho-vanadate	Sigma-Aldrich, Munich
Streptomycin 10000 mg/ml	Invitrogen, Karlsruhe
TEMED	Invitrogen, Karlsruhe
Trypsin/EDTA 0.25 %	Invitrogen, Karlsruhe
Triton X-100	Sigma-Aldrich, Munich
Trizma-Base	Sigma-Aldrich, Munich
Trypan blue 0.5 % (w/v) in PBS	Biochrom, Berlin
Tween 20	Sigma-Aldrich, Munich

### 3.2.8 Enzymes

DreamTaq Green DNA Master Mix (2X)	Fermentas, St. Leon-Rot
GoTaq Green PCR Master Mix	Promega, Mannheim
$\lambda$ -Phosphatase 400000 U/ml	NEB, Frankfurt a.M.
Proteinase K	Roche Diagnostics, Mannheim
RQ1 RNase-free DNase	Promega, Mannheim
SuperScript II (RNase H (-) 200 U/ $\mu$ l)	Invitrogen, Karlsruhe
T4 Polynucleotide kinase (10000U/ml)	NEB, Frankfurt a.M.

### 3.2.9 Kits

CellTiter-Glo Luminescent Cell Viability Assay	Promega, Mannheim
Dual Luciferase Reporter Assay System	Promega, Mannheim
Effectene Transfection Reagent	Qiagen Hilden
LightCycler FastStart DNA Master <sup>PLUS</sup> SYBR Green I	Roche, Mannheim
MiniPrep Kit	Qiagen, Hilden
One Shot Top 10	Invitrogen, Karlsruhe
RNeasy Mini Kit	Qiagen, Hilden
TOPO TA Cloning kit	Invitrogen, Karlsruhe

### 3.2.10 Size Marker

Gene Ruler DNA-Ladder Mix	Fermentas, St. Leon-Rot
PageRuler Plus Prestained Protein Ladder	Fermentas, St. Leon-Rot

### 3.2.11 Other Consumables

Cell culture flasks	(Corning) Sigma-Aldrich, Munich
Cell culture plates	Greiner Bio-One, Wemmel
Cell scraper	(Corning) Sigma-Aldrich, Munich
Coverslips	Thermo-Scientific, Braunschweig
Eppendorf Tubes	Eppendorf, Hamburg
Films Hyperfilm ECL	Amersham Bioscience, Freiburg
Graduated pipettes	Hirschmann, Eberstadt
Microscope slide	Langenbrinck, Emmendingen
One Shot TOP10 Chemically Competent <i>E. coli</i>	Invitrogen, Karlsruhe
Photometer plastic cuvette	Cuvetteser, Nürtingen
Polypropilene conical tubes	(Falcon) BD, Heidelberg
PVDF-membranes (Immobilon P)	Millipore, Eschborn
Sterile Needles, Microlance 3	BD, Heidelberg
Transfer Membrane Hybond LFP PVDF	GE Healthcare, Munich
Whatman 3 mm paper filter	Schleicher and Schüll,
Dassel	

### 3.2.12 Laboratory Equipment

Analytic scale AE 160	Mettler, Gießen
Analytic scale basic	Sartorius, Göttingen
Autoradiography cassettes	Kodak, Stuttgart
Bacteria shaker G25	Infors, Bottmingen (Schweiz)
BioPhotometer	Eppendorf, Hamburg
Bioruptor	Diagenode, Liège (B)
Bio-Trap electrophoresis chamber	Renner, Dannstadt
Blotting chamber, semi-dry	Hoefer Pharmacia Biotech,
San	Francisco (USA)
Centrifuge Biofuge, Varifuge RF	Heraeus, Hanau
Centrifuge 5415 R	Eppendorf, Hamburg
Developer machine Curix 60	AGFA, Cologne
DNA Engine DYAD and Tetrad 2 Cyclor	Bio-Rad, Munich
Fluoview FV1000	Olympus, Hamburg
Gel documentation system EASY 429 K	Herolab, Wiesloch
Gel dryer model 583	Bio-Rad, Munich
Incubator C16	Labotect, Göttingen
Microscope CKX 41	Olympus, Hamburg
Microscope Leitz DM RBE	Leica, Bensheim
Minifuge	Heraeus, Hanau
Mini-PROTEAN II (minigels western)	BioRad, Munich

NanoDrop ND-1000	Nanodrop Technologies, Wilmington (USA)
Neubauer count chamber	Bender and Hobein, Bruchsal
pH-meter Calimatic 765	Knick, Egelsbach
Pipette boy acu	Hirschmann, Eberstadt
Pipet-Lite	Mettler-Toledo, Gießen
Plate reader Mithras LB 940	Berthold Technologies, Bad Wildbad
Power supply PHERO-stab 500	Biotech-Fischer, Reiskirchen
Power supply Power Pac HC	Bio-Rad, Munich
Polymax 2040	Heidolph, Schwabach
Scale 1216 MP	Sartorius, Göttingen
Sonifier 250	Branson/Heinemann, Gmünd
Schwäbisch	Baker Company, Sandford
Sterile hood (BioGard Hood)	Heraeus, Hanau
Steri Cult 200 incubator	Eppendorf, Hamburg
Thermomixer compact	Vetter, Wiesloch
Trans-illuminator 254-366 nm	Invitrogen, Darmstadt
Vertical gel electrophoresis apparatus V15-17	Heidolf, Rust
Vortex	Julabo, Seelbach
Water bath	

#### 3.2.13 Analysis software

ImageJ 1.41o	National Institute of Health (USA)
Mikrowin 2000	Mikrotek Laborsysteme, Overath

### 3.3 Methods

#### 3.3.1 Cell culture

##### Cell culture

All cells were cultured in complete DMEM (see buffer lists). When reaching 80 % to 90 % confluence, cells were split. They were washed once with PBS and detached by incubating them 5-10 min in 0.25 % Trypsin/EDTA at room temperature. To stop the trypsinization, complete DMEM was added at a ratio of 5:1. Finally, cells were diluted between 1/10 and 1/40, depending on cell line and future experiments.

To minimize stress for the cells, all used reagents were adjusted to room temperature.

##### Cell counting

After trypsinization was stopped as described above, 15  $\mu$ l of the cell suspension were mixed 1:1 with Trypan blue and loaded into the Neubauer Chamber for cell counting. The final cell concentration (cells per milliliter) was calculated as described below:

$$\text{cells}(ml) = \frac{\text{\textcircled{O}} \text{ counted cells}}{\text{\textcircled{O}} \text{ counted squares}} \cdot \text{dilution factor} \cdot 10^4$$

Depending on the experimental approach, the following amount of cells were seeded:

**Tab. 3.1:** Amount of cells per cell culture dish

	<b>96-well plate</b>	<b>24-well plate</b>	<b>6-well plate</b>	<b>20 cm<sup>2</sup> dish</b>	<b>60 cm<sup>2</sup> dish</b>
<b>444</b>	$1.5 \cdot 10^4$	$8.0 \cdot 10^4$	$3.0 \cdot 10^5$	$4.0 \cdot 10^5$	$2.5 \cdot 10^6$
<b>CGL 3</b>	$2.5 \cdot 10^4$	$1.6 \cdot 10^5$	$8.0 \cdot 10^5$	$9.0 \cdot 10^5$	$5.0 \cdot 10^6$
<b>HEK 293</b>	-	-	-	$4.0 \cdot 10^5$	-
<b>HeLa</b>	$2.0 \cdot 10^4$	$1.4 \cdot 10^5$	$6.0 \cdot 10^5$	$7.0 \cdot 10^5$	$3.5 \cdot 10^6$
<b>IMR 90</b>	$2.0 \cdot 10^4$	$1.4 \cdot 10^5$	$6.0 \cdot 10^5$	$7.0 \cdot 10^5$	$3.5 \cdot 10^6$

### **Transient transfection using the calcium phosphate precipitation**

The calcium phosphate transfection is based on the reaction of sodium hydrogen phosphate and calcium chloride to crystalline calcium phosphate (Graham and van der Eb, 1973). This precipitate binds the plasmid-DNA (pDNA) at its surface and the complex enters the cell via endocytosis.

The following protocol was used to transfect HEK 293 grown in 20 cm<sup>2</sup> dishes. 8 µg pDNA were diluted in 0.2 M CaCl<sub>2</sub> solution. HBS 2X was added in a 1:1 ratio and the reaction was vortexed vigorously before incubating 30 min at room temperature. Finally, the calcium phosphate crystals were broken up by intensive pipetting and the mix was transferred drop-wise to the cells. HEK 293 cells were harvested 2 days after transfection.

### **Transient transfection using the Effectene Transfection Reagent**

The Effectene Transfection Reagent (Qiagen) is based on a non-liposomal two-step approach. First, the DNA is tightly condensed by the salt-containing Enhancer and later on coated with cationic lipids by the addition of the Effectene Transfection Reagent, allowing an efficient DNA transfer into eukaryotic cells.

The Effectene Transfection Reagent was used according to the manufacturer's instructions. Cells grown in 20 cm<sup>2</sup> were transfected with 1 µg pDNA diluted in 150 µl EC buffer. To condense the DNA, 8 µl Enhancer were included and the reaction was incubated at room temperature for 5 min. Then, 25 µl Effectene Transfection Reagent were added, the mix was vortexed for 10 s and incubated for further 10 min at room temperature. Finally, the reaction was diluted in 1 ml DMEM and added drop-wise to the cells. Dependent on the scheduled experiment, the transfection mix was removed 6-8 h after transfection.

### **Hypoxia**

Generally, cells are grown at 21 % O<sub>2</sub> and 5 % CO<sub>2</sub> which is termed normoxia and used as control condition within this study. To monitor the cellular hypoxic reaction, cells were exposed to 1.5-2 % O<sub>2</sub> in the incubator C16 (Labotect, Göttingen) one day

after seeding. Depending on the experimental approach, the hypoxia treatment lasted from 6-24 hours. During that time, the incubator was always closed to prevent reoxygenation of the cells.

### **Cryoconservation and reactivation of eukaryotic cells**

Subconfluent cells were trypsinized and the reaction was stopped as described in the “cell culture” section above. The cell suspension was pelleted at 1000 rpm for 2 min and the resulting supernatant was discarded. Next, cells were resuspended in freezing DMEM (precooled to 4 °C ), aliquoted into cryotubes and slowly cooled down to -80 °C. For long-term storage, the frozen cells were transferred to liquid nitrogen.

To thaw cells, the cryosuspension was defrozen at room temperature and cells were washed once in DMEM with additives. Initially, the reactivated cells were cultured in a 25 cm<sup>2</sup> flask to allow cell-cell contact.

### **3.3.2 Preparation and analysis of proteins**

#### **Nuclear protein preparation**

The method of Schreiber *et al.* was used to prepare nuclear protein extracts (Schreiber *et al.*, 1989). Phosphatases and proteases inhibitors were added to the buffers A and C as described in Tab. 3.2. Prior to nuclear protein preparation, the cells grown in 60 cm<sup>2</sup> plates were subjected to hypoxia (1.5-2 % O<sub>2</sub>) or control conditions for 24 h.

**Tab. 3.2:** Amount of cells per cell culture dish

<b>Agent</b>	<b>Function</b>	<b>Final concentration</b>
<b>DTT</b>	Reducing agent	1 mM
<b>Complete protease inhibitor</b>	Protease inhibitor	1X
<b>MG-132</b>	Proteasome inhibitor	1 μM
<b>NaF</b>	Phosphatase inhibitor	1 mM
<b>Sodium ortho-vanadate</b>	Phosphatase inhibitor	0.2 mM

For harvesting, cells were washed twice with ice-cold PBS, scraped in 1.2 ml hypotonic buffer A and transferred to a 1.5 ml Eppendorf tube. Following 15 min swelling on ice, 75  $\mu$ l of the non-ionic detergent NP-40 were added, the samples were vortexed 10 s to complete the plasma membrane lysis and centrifuged 1 min at 4 °C and 15500 g. The supernatant (cytoplasmic extract) was stored for subsequent RNA extraction and the pellet was resuspended in 100  $\mu$ l ice-cold buffer C to extract the nuclear proteins. Then, the samples were incubated another 15 min on ice, being mixed every 2 min. After 5 min centrifugation, the supernatant containing the nuclear protein extract was recovered and stored at -80 °C. To determine the protein concentration of the extract, a Bradford protein assay (Bradford, 1976) was performed.

#### **Whole cell protein preparation (RIPA) (Klotz *et al.*, 1999)**

For RIPA extract, cells were grown in 20 cm<sup>2</sup> cell culture dishes and subjected to 24 h hypoxic (1.5 % O<sub>2</sub>) or normoxic conditions prior to harvesting. The protein preparation was performed in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors as described in Tab. 3.2.

To prepare whole cell protein extract, cells were washed twice with PBS, scraped in 250  $\mu$ l RIPA buffer and transferred to 1.5 ml Eppendorf tubes. To lyse cellular membranes, cell suspensions were sonified twice for 10 s, with Sonifier 250 set to: timer "hold", duty cycle "50 %" and output control "5". Subsequently, suspensions were incubated 20 min on ice to allow complete protein extraction. After brief vortexing and 5 min centrifugation at 13,000 rpm, supernatants containing whole cell proteins were transferred into fresh tubes and stored at -80 °C. Protein concentrations were determined by Bradford protein assay.

### **Bradford protein assay** (Bradford, 1976)

The following reaction was prepared for every sample, including the calibration line:

- 800  $\mu$ l Bio-Rad Protein Assay reagent
- 200  $\mu$ l *aqua dest.*
- 2  $\mu$ l sample

For the standard curve, 1-12  $\mu$ g BSA were measured. All samples were quantified at 595 nm (the absorbance maximum of the protein bound “Bio-Rad Protein Assay reagent”), using the BioPhotometer. 800  $\mu$ l “Bio-Rad Protein Assay reagent” and 200  $\mu$ l *aqua dest.* were set to zero point.

### **$\lambda$ -Phosphatase treatment**

To identify post-translational protein phosphorylation, cellular protein extracts were treated with  $\lambda$ -phosphatase (NEB). For this purpose, RIPA extracts were prepared in the absence of phosphatase inhibitors. 50  $\mu$ g of protein extract were incubated with 2000 U  $\lambda$ -phosphatase, 1X reaction buffer and 1 mM  $\text{MnCl}_2$  at 30 °C for 30 min. Subsequently, 1X SDS-loading buffer was added, the reaction mix was cooked for 10 min at 99 °C and subjected to SDS-PAGE.

### **SDS-PAGE** (Laemmli, 1970; Hames and Rickwood, 1990)

Prior to the separation of proteins by SDS-PAGE, they were denaturated by cooking 10 min at 99 °C in 1X  $\beta$ -mercaptoethanol containing SDS-loading-buffer.

With SDS-polyacrylamide-gel electrophoresis (SDS-PAGE), proteins were separated according to their size. The polyacrylamide gel is composed of a low-percentage stacking gel and a high-percentage running gel. The stacking gel concentrates the proteins whereas they are separated in the running gel. The negatively charged detergent SDS in loading buffer and gel attaches to the proteins, neutralizes their positive charges and denaturates them. The scaffold of the gels, the polyacrylamide, builds a net-like structure separating proteins according to their size. Resolution

depends on the concentration of polyacrylamide. For an optimal separation, 10 % running gels for proteins of 40-70 kDa and 7 % running gels for larger proteins were used:

**Tab. 3.3:** Composition of polyacrylamide gels used for SDS-PAGE

	<b>Stacking gel</b>	<b>7 % running gel</b>	<b>10 % running gel</b>
<b>Tris/HCl, pH 6,8</b>	0.126 M	-	-
<b>Tris/HCl, pH 8,8</b>	-	0.376 M	0.376 M
<b>SDS</b>	0.1 % (w/v)	0.1 % (w/v)	0.1 % (w/v)
<b>Acrylamide/bis-Acrylamide (29:1)</b>	3 % (w/v)	7 % (w/v)	10 % (w/v)
<b>APS</b>	0.05 % (w/v)	0.05 % (w/v)	0.05 % (w/v)
<b>TEMED</b>	0.12 % (w/v)	0.06 % (w/v)	0.06 % (w/v)

The SDS-PAGE was run at 15 mA until the samples reached the separating gel. Then amperage was increased to 30 mA. The separation was stopped, when the blue coloured running front left the gel.

#### **Western blot “semi-dry”** (Gallagher *et al.*, 1997)

Whatman Paper was soaked in Towbin buffer including 10 % methanol. To activate the PVDF-membrane (Immobilon P, Millipore or Hybond LFP, GE Healthcare), it was hydrated in 100 % methanol for 1 min, in *aqua dest.* for 5 min and finally, the membrane was incubated further 15 min in Towbin buffer including 10 % methanol. After setting up the blotting system, the transfer proceeded 70 min at 4 °C and 1.2 mA per cm<sup>2</sup>.

Then the membrane was blocked in blocking solution (5 % milk powder in TBST) for at least one hour. The first antibody was diluted according to the manufacturers' recommendations in blocking solution and incubated with the membrane over night at 4 °C. Having washed the membrane thrice in TBST for 15-20 min each, the second antibody (diluted as well in blocking solution) was added and incubated for 1 h at room temperature. Finally, the washing was repeated and the ECL-reaction

was performed. In order to use the same membrane for reincubation with additional antibodies, it was “stripped” in 200 mM NaOH for 5 min and washed with water and TBST.

### **3.3.3 Preparation and analysis of nucleic acids**

#### **DNA electrophoresis (agarose gel electrophoresis)**

DNA fragments like PCR products were visualized by agarose gel electrophoresis. Depending on the fragment size, 1-2 % agarose gels were prepared. The gels were run in 1X TAE buffer at constant voltage.

#### **DNase digest**

Prior to RT-PCR, residual traces of DNA were removed from RNA preparations by RQ1 DNase digest. For this purpose, 10 µg RNA were added to the reaction mix (20 U RNAsin, 2 U RQ1 DNase, 10 mM Tris/HCl pH 7.5, 10 mM MgCl and 50 mM KCl) and incubated for 20 min at 37 °C. By the addition of 200 mM sodium acetate pH 4, the DNase digestion was stopped.

Subsequently, the RNA was purified by the RNeasy Mini Kit. Briefly, the sample volume was adjusted to 100 µl with DEPC-H<sub>2</sub>O, mixed with 350 µl RLT buffer, 250 µl 100 % ethanol and 3.5 µl 99 % β-Mercaptoethanol and loaded onto the column. Washing was performed according to the manufacturer's recommendations.

#### **Quantification of nucleic acids**

To quantify nucleic acids, 2 µl of each sample were subjected to spectrophotometrical analysis by the NanoDrop ND-1000 according to the manufacturer's recommendations.

#### **RNA electrophoresis**

In order to inactivate putative RNases, all used equipment like gel chambers and combs were incubated 15 min in 50 mM NaOH. After extensive washing, a 1 % agarose gel in 1X MOPS was prepared. 10 µl 1X RNA loading buffer were added to 1 µg RNA and incubated 10 min at 65 °C. After chilling on ice for 3 min, samples were loaded to the gel.

#### **RNA extraction using the RNeasy Mini Kit (Qiagen)**

In order to obtain RNA and protein from the same samples, cytoplasmic extracts remaining from nuclear protein preparation was mixed with 0.44 M RLT buffer, 22 % Ethanol and 63.5 mM β-Mercaptoethanol. The sample was transferred sequentially to a RNeasy mini column, spinned 15 s at 8000 g, and washed according to the manufacturers recommendations. After drying the column, RNA was eluted in 30 µl DEPC-water by centrifugation. To monitor RNA integrity, a 1 % AGE-MOPS gel was run. RNA was stored at -80 °C.

#### **Reverse transcription polymerase chain reaction (RT-PCR)**

To obtain cDNA, SuperScript II reverse transcriptase and random primers were used according to the manufacturer's instructions. Briefly, 1 µg of DNase-purified RNA and 15 mM random primers were incubated 10 min at 70 °C and immediately transferred onto ice. For primer annealing, 1X reverse transcription (RT) buffer, 10 mM DTT, 500 mM dNTPs were added and incubated at 25 °C for 10 min. After including 100 U SuperScript II, synthesis of the first cDNA strand was carried out 50 min at 42 °C followed by 15 min at 70 °C. cDNA was stored at -20 °C.

#### **Semi-quantitative PCR**

PCR was performed in a final volume of 25 µl. For ChIP analysis, GoTaq Green Master Mix (Promega) was used. The final concentrations were: 1X Promega Go Taq Green Master Mix, 4 % DMSO and 400 nM of each primer. Two different amounts of template DNA (1 µl and 3 µl) were used, in order to monitor the quantitative range of

the PCR.

For amplification of cDNA, DreamTaq Green Master Mix (Fermentas) was applied: The relative amounts were 1X DreamTaq Green Master Mix, 400 nM of each primer and 1  $\mu$ l cDNA.

For number of cycles and annealing temperatures of the specific primers see chapter 3.2.2. The following standard PCR program was used:

**Tab. 3.4:** Semi-quantitative PCR program

PCR program		Temperature	Continuance
<b>Denaturation</b>		95 °C	5 min
<b>Cycles (gene specific)</b>	<b>Denaturation</b>	95 °C	30 s
	<b>Annealing</b>	Primer specific	30 s
	<b>Elongation</b>	72 °C	30 s
<b>Final elongation</b>		72 °C	10 min
<b>Cooling</b>		4 °C	forever

PCR products were analyzed in 2 % agarose gels.

### Quantitative PCR (qPCR)

The relative amount of GAPDH cDNA was determined by quantitative RT-PCR using LightCycler FastStart DNA Master<sup>PLUS</sup> SYBR Green I according to the manufacturer's instructions. The cyanine dye SYBR Green I forms complexes with dsDNA which absorb blue light at 494 nm and emit green light at 521 nm. The quantification of the PCR product is enabled by the linear correlation of fluorescence intensity and amount of DNA. As a reference, 5 distinct concentrations of pCLII-TOPO-Tub (7.5 ng, 750 pg, 75 pg, 7.5 pg, 0.75 pg) were included in every qPCR run, enabling the generation of a calibration line with a minimal error of <0.07. GAPDH cDNA was relatively quantified by first subtracting the background (sample with water instead of cDNA) and then by normalizing against Tubulin to correct for differences concerning quality and amount of cDNA. Finally, values were displayed as percentages compared to normoxia set to 100 %. PCRs were performed in 10  $\mu$ l total volume with 1X LightCycler FastStart DNA Master<sup>PLUS</sup> SYBR Green I, 2  $\mu$ M Primer and 1  $\mu$ l cDNA. qPCRs were run following this program:

Tab. 3.5: qPCR program

qPCR program		Temperature	Hold time	Acquisition
<b>Denaturation</b>		95 °C	10 min	-
<b>50 cycles</b>	<b>Denaturation</b>	95 °C	10 s	-
	<b>Amplification</b>	59 °C	5 s	-
	<b>Elongation</b>	72 °C	20 s	single
<b>Melting Curve</b>		95 °C	0 s	-
		50 °C-95 °C	15 s	-
		95 °C	0 s	continuous
<b>Cooling</b>		40 °C	30 s	-

### Cloning of PCR products

To create a quantitative reference for qPCR analysis, a 271 bp fragment of the human  $\beta$ -Tubulin cDNA was cloned into the pCLII-TOPO vector. Initially, the fragment was amplified from 2  $\mu$ g 444 cDNA using the DreamTaq Green Master Mix (Fermentas) according to the manufacturer's recommendations and the  $\beta$ -Tub primers described in 3.2.2. 4  $\mu$ l of the PCR reaction were mixed with 1  $\mu$ l TOPO TA vector and 1  $\mu$ l salt solution derived from the TOPO TA cloning kit (Invitrogen). To allow the ligation of TOPO TA vector and PCR product, the reaction was incubated for 30 min at room temperature. Subsequently, chemical competent TOP10 *E.coli* were transformed.

### Propagation of pDNA by competent TOP10 *E.coli*

To propagate the vectors needed for transfection of eukaryotic cells, chemically competent TOP10 *E.coli* (Invitrogen) were transformed according to the manufacturer's recommendations. Briefly, TOP10 bacteria were slowly thawed on ice. After adding the vector solution, bacteria were additionally incubated on ice for 30 min, heat shocked at 42 °C for 30 s and immediately returned to ice. 250  $\mu$ l of SOC medium were added, bacteria were shaken for 1 h at 37 °C and subsequently propagated on antibiotics containing LB dishes over night. Single colonies were

picked and liquid cultures were inoculated. After 24 h incubation at 37 °C, plasmid DNA was isolated using the MiniPrep kit or the MaxiPrep kit (Qiagen) according to the manufacturers' instructions.

#### **Sequencing of plasmids**

All plasmids used in this study were sequenced prior to their first usage by GATC Biotech AG (Konstanz) to ensure absence of point mutations and correct open reading frames.

#### **3.3.4 Protein-DNA interaction**

##### **Chromatin Immunoprecipitation (ChIP)** (Weinmann *et al.*, 2001)

*Formaldehyde crosslinking and cell harvesting:* Cells grown in 60 cm<sup>2</sup> dishes were subjected to 2 % O<sub>2</sub> or control conditions for 24 h. For harvesting, the growth medium was exchanged to DMEM without additives, Formaldehyde was added to 1 % final concentration and the cells were cross-linked at room temperature for 10 min. The reaction was stopped with 0.125 M glycine. After 5 min incubation, the cells were washed twice with PBS, trypsinized for 20 min at 37 °C, scraped and transferred to an Eppendorf tube. Cell pellets were washed once with PBS.

*Estimation of chromatin amount:* The cell pellets were resuspended in 1 ml Cell Lysis Buffer, incubated on ice for 20 min and vortexed 1 min to break the cellular membranes. To estimate the amount of chromatin, nuclei were counted in the Neubauer chamber:

$$10^7 \text{ nuclei} \approx 150 \mu\text{g chromatin}$$

*Chromatin shearing by sonification:* The nuclei were pelleted by centrifugation (1 min at 15500 g, 4 °C) and resuspended in 500 µl ChIP buffer. The suspension was

passed 10 times through a Microlance needle to break up the nuclei and incubated at least 15 min on ice. After 10 min sonification (constant output, 10 % duty) in an ice bath, nuclear debris was removed by centrifuging 10 min at 4 °C and 15500 g.

*Analysis of chromatin shearing:* 2 µg of sheared chromatin were decrosslinked over night in 0.3 M sodium chloride, shaking at 65 °C. After 1:1 phenol-chloroform extraction, DNA was purified by ethanol precipitation (100 % ethanol, 3 M sodium acetate and 1 µg glycogen). Samples were resuspended in 20 µl water and analyzed in an 1.5 % agarose gel.

*Preclearing and ChIP:* For preclearing, 25 µg sonified chromatin were mixed with 40 µg salmon sperm and 40 µl of blocked protein A agarose beads (50 % slurry) and rocked for 2 h at 4 °C. Agarose beads were removed by centrifugation (2 min at 15500 g, 4 °C). Subsequently 2 µg of unspecific control antibody or RNA polymerase II antibody were included. After over night rotation at 4 °C, the antigen-antibody complex was pulled down by 40 µl protein A agarose beads during 3 h at room temperature.

Complexes were washed using 500 µl high salt buffer. After 5 min incubation, the complexes were pelleted by centrifugation (2 min at 4 °C, 15500 g). The washing procedure was repeated twice with low salt buffer, twice with lithium chloride buffer and twice with TE buffer.

To elute the formaldehyde cross-linked protein-DNA complexes from beads and antibodies, the mixture was rocked 40 min in 50 µl elution buffer at room temperature.

For the second IP, the eluate was transferred to a new Eppendorf tube and completed to 500 µl using ChIP buffer. 2 µg of unspecific control antibody were added to the samples precipitated with control antibody. To samples precipitated with RNA pol II antibody, Net antiserum 2007 (concentration 1:50) or 1.5 µg of HIF-2α antibody were included. Precipitation was carried out over night at 4 °C and antigen-antibody complexes were pulled down and washed as described above. For final elution, 250 µl elution buffer were added and the protein-DNA complexes were eluted 20 min at room temperature.

*Decrosslinking and purification of DNA:* To remove remaining proteins and to release the formaldehyde cross-linking, a digestion and decrosslinking cocktail was included in a 1:1 ratio to the DNA-protein containing supernatant. From here on, the input chromatin sample was included as a positive control for PCR and processed in the same way as the IP chromatin. During the following two hours, the proteins were digested by Proteinase K at 37 °C. Then, temperature was increased to 65 °C which inactivated the Proteinase K and DNA complexes were decrosslinked over night. Finally, the DNA was purified by phenol : chloroform extraction and ethanol precipitated using 3 M sodium acetate and 1 µg glycogen as a carrier. DNA pellets were resuspended in 60 µl water. Purified ChIP DNA was stored at -20 °C.

#### **Electrophoresis Mobility Shift Assay (EMSA)**

The interaction of DNA and DNA-binding proteins like transcription factors can be determined by electrophoresis mobility shift assay (EMSA). This technique is based on the different migration properties of protein-DNA complexes and DNA alone in a non-denaturing polyacrylamide gel: Transcription factor binding to a double-stranded oligonucleotide leads to a retarded migration due to an increased size of the protein-DNA complex. In order to identify specific proteins involved in the complex formation, antibodies can be applied prior to PAGE which leads to a further retardation of the protein-DNA-antibody complex.

Oligonucleotide strands (sense and anti-sense) were annealed in TNE in a PCR machine using the following PCR program: 85 °C for 10 min followed by a sequential temperature decrease of 0.5 °C every 30 s. 200 ng of annealed oligonucleotides were end-labeled with 3,000 Ci/mmol [ $\gamma$ -<sup>32</sup>P] dATP by T4 polynucleotide kinase at 37 °C for 30 min. To remove non-labeled oligonucleotides, the mix was subjected to PAGE: 15 % polyacrylamide/1X TBE-gel electrophoresis at 200 V for 90 min. Finally, the radioactively labeled oligonucleotides were excised from the gel and extracted in TNE.

The EMSA was performed in a 20 µl reaction volume containing 1X EMSA binding buffer supplemented with phosphatase and protease inhibitors, 2 µg poly(dI/dC).poly(dI/dC), 2 µg nuclear protein extract and 10,000 cpm of 5' end-

labeled probe. The reaction was incubated for 30 min at room temperature. To confirm the specificity of the complex formation, the unlabeled oligonucleotide probe was included in molar excess to compete with the labeled probe for binding to the protein complexes. For supershift analysis, 2 µg of the specific antibody were added and the reaction mix was further incubated at 4 °C for 1 h. Finally, the different complexes were separated in a 5.5 % non-denaturing polyacrylamide/1X TBE-gel which was subsequently dried on a Whatmann 3MM filter paper and exposed to X-ray films at -80 °C for hours to days depending on the intensity of the signal.

#### **3.3.5 Reporter assays**

##### **ATP assay (CellTiter-Glo Luminescent Cell Viability Assay, Promega)**

The ATP assay can be used to determine cellular survival as there is a direct relationship between ATP content and cell viability. To quantify the ATP, the applied CellTiter-Glo cell viability assay takes advantage of a firefly luciferase based system. The firefly luciferase requires the ATP to catalyze the oxidative carboxylation of the substrate luciferin. The product of this reaction emits light at a wavelength of 537 nm which can be quantified.

The CellTiter-Glo Luminescent Cell Viability Assay was used according to the manufacturer's recommendations with slight modifications. Cells were grown in opaque 96-well plates, in triplicate for each experiment. The CellTiter-Glo assay was carried out at 37 °C. After 24 h hypoxia (1.5 % O<sub>2</sub>) or normoxia, 80 µl CellTiter-Glo reagent were added to each well and the plate was rocked for 2 min to allow complete permeabilization of the cellular membrane. To stabilize the luciferin signal, the reaction was incubated for further 10 min. Finally, the light emission was quantified by the Mithras LB 940 plate reader using the Mikrowin2000 software.

Graphics depict the percental change of the ATP level after 6 h hypoxia compared to normoxia.

**GAPDH reporter assay (Dual Luciferase Reporter Assay System, Promega)**

To monitor the transcriptional regulation of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, the Dual Luciferase Reporter Assay System (Promega) was applied. Here, the activity of a GAPDH promoter driven firefly-luciferase and a HSV-Thymidinkinase driven renilla-luciferase were quantified one after the other to exclude variations caused by different transfection efficiencies.

444 and CGL 3 grown in 20 cm<sup>2</sup> dishes were transfected with the reporter pGL3-GAPDH(-1112), the normalization vector pRL-TK, a combination of vectors expressing HIF-1 $\alpha$ , HIF-2 $\alpha$ , Net, Siah1 or Siah1<sup>C44S</sup> and pEGFP. 6 h after transfection, cells were split 1:3 in 6-well plates. To monitor overall transfection efficiency by EGFP expression, 1:15 of the transfected cells were grown on cover slides and later on analyzed by immunofluorescence. When indicated, cells were subjected to 2 % O<sub>2</sub> or control conditions for 24 h the next day.

To prepare the cellular extract for the reporter gene analysis, cells were washed twice with ice-cold PBS and scraped in 1X Passive Lysis Buffer. After brief vortexing, cell debris was removed by centrifugation. The extract was stored at -80 °C.

For read-out of the Luciferase signal, 10  $\mu$ l of each extract were loaded onto a 96-well plate. LARII and Stop&Glow buffers were prepared according to the manufacturer's instructions. The signal was quantified in the Mithras LB 940 plate reader, using the following program: injection of 50  $\mu$ l LARII, 2 s incubation, 10 s reading, injection of 50  $\mu$ l Stop&Glow, 2 s incubation and 10 s reading. *firefly*-luciferase readings were normalized to *Renilla*-luciferase readings. Graphics show the fold-induction of the GAPDH reporter compared to the induction of the GAPDH reporter observed in a control transfection (without transcription factors) set to 100 %.

### 3.3.6 Immunofluorescence

#### **ROS detection by dihydroethidium (DHE) (Bucana *et al.*, 1986; Carter *et al.*, 1994)**

Intracellular ROS can be visualized by oxidation of DHE. When excited, the resulting product emits light at a broad spectrum from 560-610 nm. For ROS detection, 2.5  $\mu$ M DHE, also known as hydroethidine, were added to the cells 30 min prior to fixation. Until harvesting, cells were subjected again to hypoxic or normoxic conditions, depending on the previous treatment.

#### **Fixation and Hoechst staining**

Cells grown on cover slides were rinsed with PBS and fixed in 4 % Paraformaldehyde for 20 min at room temperature. After washing 5 min in PBS, nuclei were stained for 20 min at room temperature with Hoechst 33258 diluted 1:10000 in PBS. Finally, samples were washed three times in PBS and one time in water, rinsed in 100 % Ethanol, mounted on glass slides in 10 % mowiol and dried over night in the dark.

#### **Microscopic analysis**

Previously stained and fixed cells were analyzed for GFP expression to ensure equal transfection efficiency, Hoechst 33258 staining of the nuclei or DHE oxidation by reactive oxygen species (ROS).

GFP was first isolated from the jelly fish *Aequorea victoria*. Since then, it has been genetically modified to obtain a better thermostability and improve the handling. When excited by blue light, it has a major emission peak at 509 nm. To distinguish between the total number of cells and the number of transfected cells, the nuclei were stained with Hoechst 33258. This dye binds to DNA and emits cyan light when stimulated at 350 nm (ultraviolet spectrum). For GFP and Hoechst 33258 analysis, the microscope Leitz DM RBE (Leica) was used.

To monitor intracellular ROS levels under normoxic and hypoxic conditions, cells

were stained with DHE. When oxidized, the product can be excited at 490 nm wavelength and emits a broad spectrum between 560-610 nm. Here, emission was recorded at 605 nm. For ROS detection, the Microscope CKX 41 was used.

#### **3.3.7 Cell cycle analysis**

##### **Fluorescence activated cell sorting (FACS)**

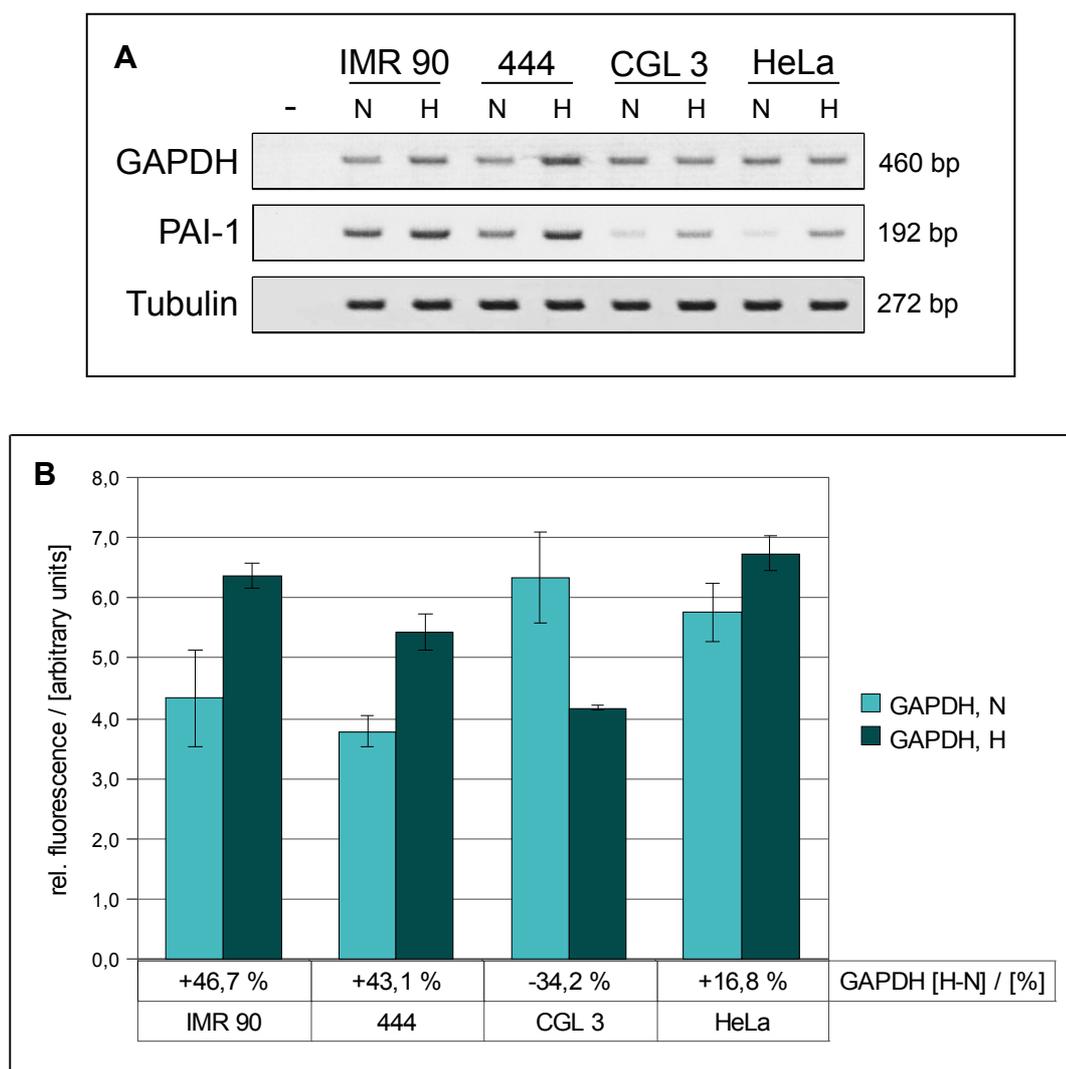
Cells grown in 6-well dishes were trypsinized, washed twice with PBS and fixed in 70 % ethanol at least over night. For FACS analysis, the fixation solution was removed by centrifugation and the cell pellet was resuspended in the DNA staining solution containing DAPI to stain the DNA and sulforhodamine 101 (SR101) to stain the protein following a protocol published by Stoehr and Goertler in 1979. Processing, cell cycle analysis and quantification of apoptotic cells was performed according to Dean and Jett, 1974.

## **4. Results**

### **4.1 Net and HIF-2 $\alpha$ synergistically regulate GAPDH transcription**

#### **4.1.1 Hypoxic GAPDH regulation changes in cervical cancer cells**

Hypoxia is known to regulate GAPDH transcription in a cell type specific manner (Graven *et al.*, 2003). GAPDH expression increases upon hypoxia in multiple cell lines which is mediated by two hypoxia response elements within its promoter (Graven *et al.*, 1999; Lu *et al.*, 2002).



**Fig. 4.1: Hypoxia exerts a cell type dependent effect on GAPDH expression.** IMR 90, 444, CGL 3 and HeLa were subjected to hypoxia (1.5 % O<sub>2</sub>) (“H”) or normoxia (“N”) for 24 h. RNA was extracted, purified from DNA and reverse transcribed. The resulting cDNA was analyzed by semi-quantitative PCR for PAI-1, GAPDH and Tubulin (**A**). In addition, GAPDH expression was monitored by qPCR (**B**). The GAPDH expression data was normalized to the control Tubulin. Error bars represent the standard deviation between three independent experiments. The table in the lower part of (B) depicts the percentaged changes of GAPDH expression upon hypoxia.

To monitor the hypoxic response of GAPDH in the fibroblasts IMR 90, the HeLa x fibroblast hybrids 444, their tumorigenic segregants CGL 3 and the cervical cancer cell line HeLa, cells were subjected to 1.5 % O<sub>2</sub> for 24 h. Subsequently, the expression of GAPDH was monitored by semi-quantitative and quantitative PCR.

According to **Fig. 4.1 A** and **B**, GAPDH mRNA levels are differentially regulated under hypoxia in non-tumorigenic and tumorigenic cell lines. Whereas the non-tumorigenic IMR 90 and 444 upregulate their GAPDH expression upon oxygen

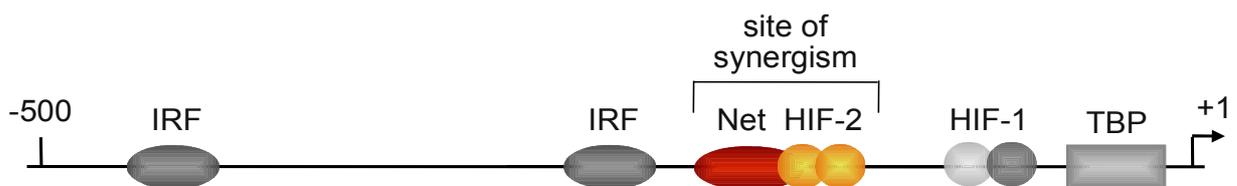
deprivation, the tumorigenic cell lines HeLa and CGL 3 display either no significant change of GAPDH mRNA levels or even a decrease (**Fig. 4.1 B**), respectively. This tendency is conserved in three independent experiments.

To exclude genome wide transcriptional variations or differences in concentration and quality of the cDNA, the expression of  $\beta$ -tubulin and PAI-1 were analyzed (**Fig. 4.1 A**). As a loading control and a gene, which is not influenced by hypoxia,  $\beta$ -tubulin is equally expressed in all tested samples. In contrast, PAI-1 is known to be a hypoxia responsive gene (Liao *et al.*, 2007). As shown in **Fig. 4.1 A**, its expression is upregulated under hypoxia in all tested cell lines – which emphasizes the cell type specific effect of GAPDH regulation depicted in **Fig. 4.1 B**.

In conclusion, GAPDH expression is regulated in a cell type dependent manner. The present data suggests a connection of tumorigenicity and an altered hypoxic GAPDH regulation.

#### 4.1.2 Net and HIF-2 $\alpha$ bind to the GAPDH promoter

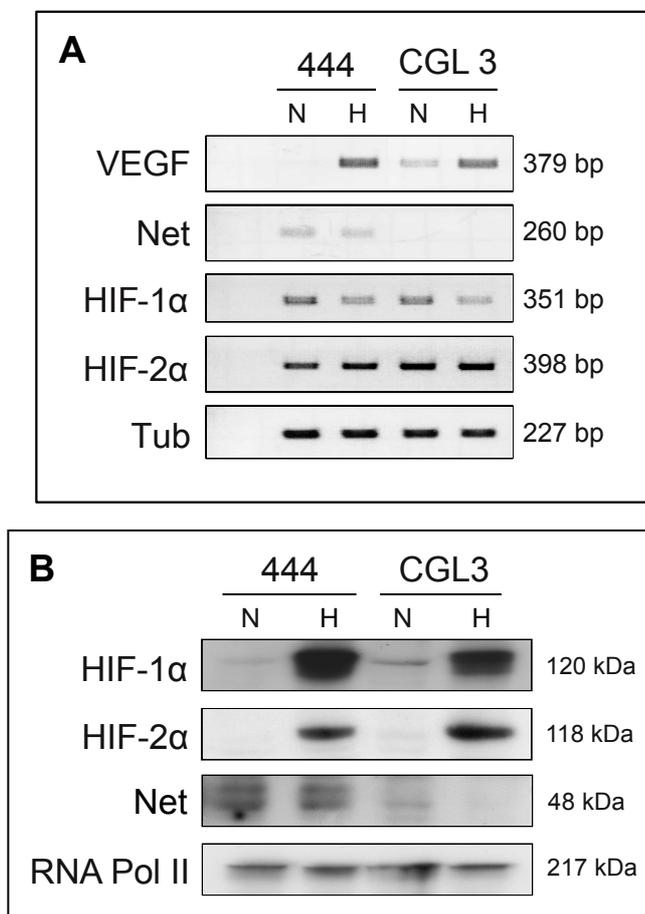
Hypoxic changes of GAPDH mRNA levels could be regulated on transcriptional level. Previous *in silico* analysis of the GAPDH promoter suggests a Net binding site in close proximity to a previously reported HRE which could be bound by HIF-2 $\alpha$  (**Fig.**



**Fig. 4.2: GAPDH promoter model.** Displayed are previously identified *cis*-regulatory elements for insulin response factors (IRF), HIF-1, HIF-2 and TATA binding protein (TBP) located within 500 bp upstream of the GAPDH transcriptional start site (Nasrin *et al.*, 1990). The indicated Net binding site was predicted by *in silico* analysis.

Both transcription factors play an important role in the hypoxic response (Gross *et al.*, 2008; Wiesener *et al.*, 1998). Moreover, the location of their response elements at the GAPDH promoter (**Fig. 4.2**) would enable a synergistic *trans*-activating effect, which was previously described for Net related proteins and HIF-2 $\alpha$  (Elvert *et al.*,

2003; Aprelikova *et al.*, 2006). However, the expression of Net and HIF-2 $\alpha$  is cell type dependent. Therefore, the mRNA and protein levels of both genes were analyzed in



**Fig. 4.3: Net and HIF expression under normoxic and hypoxic conditions** 444 and CGL 3 were cultured 24 h under normoxia or 2 % O<sub>2</sub>. Subsequently, nuclear protein extract and RNA were prepared. **(A)** RNA was purified from DNA contamination and reverse transcribed. The expression of VEGF, Net, HIF-1 $\alpha$ , HIF-2 $\alpha$  and  $\beta$ -Tubulin was analyzed by semi-quantitative PCR. **(B)** 25  $\mu$ g nuclear protein extract were subjected to Western Blot. The protein levels of Net, HIF-1 $\alpha$ , HIF-2 $\alpha$  and RNA polymerase II were determined.

According to **Fig. 4.3 A** and **B**, 444 and CGL 3 differ essentially in their Net mRNA and protein levels: 444 expresses higher Net levels compared to CGL 3, which is shown on both mRNA and protein level and indicates different transcriptional activity of the Net gene in both cell lines. The low Net protein levels in CGL 3 are further reduced upon hypoxia, while they remain unchanged in 444.

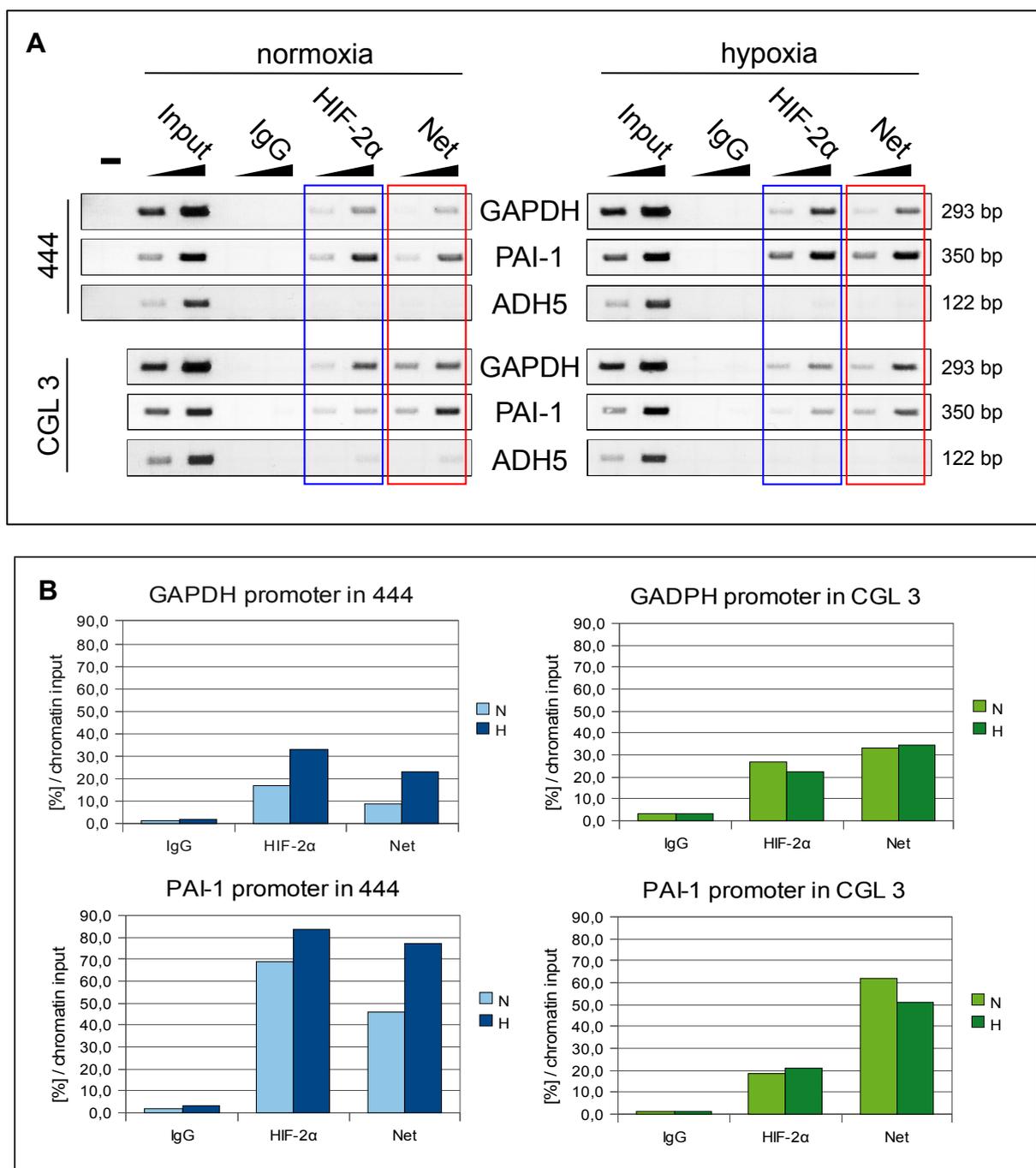
Concerning HIF-1 $\alpha$  and HIF-2 $\alpha$ , the protein levels do not correlate with the amount of RNA detected. While the RNA levels of HIF-1 $\alpha$  are slightly reduced upon hypoxia in both cell lines, HIF-2 $\alpha$  remains unchanged in CGL 3 or it is slightly upregulated in 444 (**Fig 4.3 A**). In contrast, hypoxia has a tremendous effect on HIF protein levels: while HIF-1 $\alpha$  and HIF-2 $\alpha$  are almost undetectable under normoxia, they strongly accumulate upon hypoxia (**Fig 4.3 B**). The hypoxic stabilization of HIF proteins is considered to be a major event in the cellular hypoxic response and was described previously (Wang and Semenza 1993; Wiesener *et al.*, 1998). Comparing the cell lines 444 and CGL 3, they slightly differ in their expression levels for both HIF factors. While 444 depict more HIF-1 $\alpha$  than CGL 3, this ratio changes in the case of HIF-2 $\alpha$  (**Fig 4.3 B**).

Vascular endothelial growth factor (VEGF) expression was analyzed as a hypoxic control gene. VEGF is known to be transcriptionally upregulated under hypoxia (Shweiki *et al.*, 1992), which is depicted as well in **Fig. 4.3 A**. CGL 3 constitutively express VEGF under normoxia, which is a well-studied feature of tumorigenic cells (Kodama *et al.*, 1999).

In conclusion, both Net and HIF-2 $\alpha$  are expressed in 444 and CGL 3. 444 shows high Net RNA and protein levels, while Net expression in CGL 3 is close to the detection limit. HIFs proteins massively accumulate under hypoxia, which is an expected, post-translational event. There are only minor variations of HIF RNA levels. HIF-2 $\alpha$  expression is slightly higher in CGL 3, while there is more HIF-1 $\alpha$  in 444.

#### **4.1.3 Net and HIF-2 $\alpha$ bind to the GAPDH promoter**

As outlined above, Net and HIF-2 $\alpha$  may be involved in GAPDH transcriptional regulation. The location of their *in silico* predicted binding sites at the GAPDH promoter may enable a common *trans*-activating effect which was previously reported for Net related proteins and HIF-2 $\alpha$ . Considering their important role in the hypoxic response, an impact of Net and HIF-2 $\alpha$  on the hypoxic regulation of GAPDH is likely. Initially, the promoter binding of both factors was analyzed under normoxic and hypoxic conditions by chromatin immunoprecipitation.



**Fig. 4.4: ChIP showing differential binding of Net and HIF-2 $\alpha$  at the GAPDH promoter.** The displayed ChIP involves two immunoprecipitation steps: A first one using an antibody directed against active RNA Polymerase II to select for actively transcribed genes. And the second one to specifically determine the binding of Net and HIF-2 $\alpha$ . Following elution and purification, the resulting DNA was analyzed by semi-quantitative PCR. Depicted are the results for the GAPDH, PAI-1 and ADH 5 promoter (**A**). Net and HIF-2 $\alpha$  indicate the specific antibodies, IgG stands for the unspecific IP control, Input represents non-IP chromatin. Prior to ChIP, 444 and CGL 3 were grown under normoxia ("N") or 2 % O<sub>2</sub> ("H") for 24 h. (**B**) The semi-quantitative ChIP results for GAPDH and PAI-1 shown in (A) were quantified by the ImageJ software. IgG, HIF-2 $\alpha$  and Net IP values were normalized to the non-IP chromatin referred to as "Input" in (A).

These results indicate, that both Net and HIF-2 $\alpha$  bind to the promoter of GAPDH in 444 and CGL 3 (**Fig. 4.4 A and B**). However, their binding seems to be differentially regulated under hypoxia: While the binding of Net and HIF-2 $\alpha$  at the GAPDH promoter is rather low in 444 under normoxic conditions, it increases upon hypoxic treatment (**Fig. 4.4 B**). In contrast, the promoter binding of both factors in CGL 3 does not change upon oxygen deprivation. The presence of HIF-2 $\alpha$  at the GAPDH promoter under normoxic conditions does not correlate with the overall protein levels (**Fig. 4.3**): Although its low expression levels under normoxia, it binds to the GAPDH promoter in both 444 and CGL 3 in significant amounts. Similar, the nuclear accumulation of HIF protein upon hypoxia is not reflected by its GAPDH promoter binding.

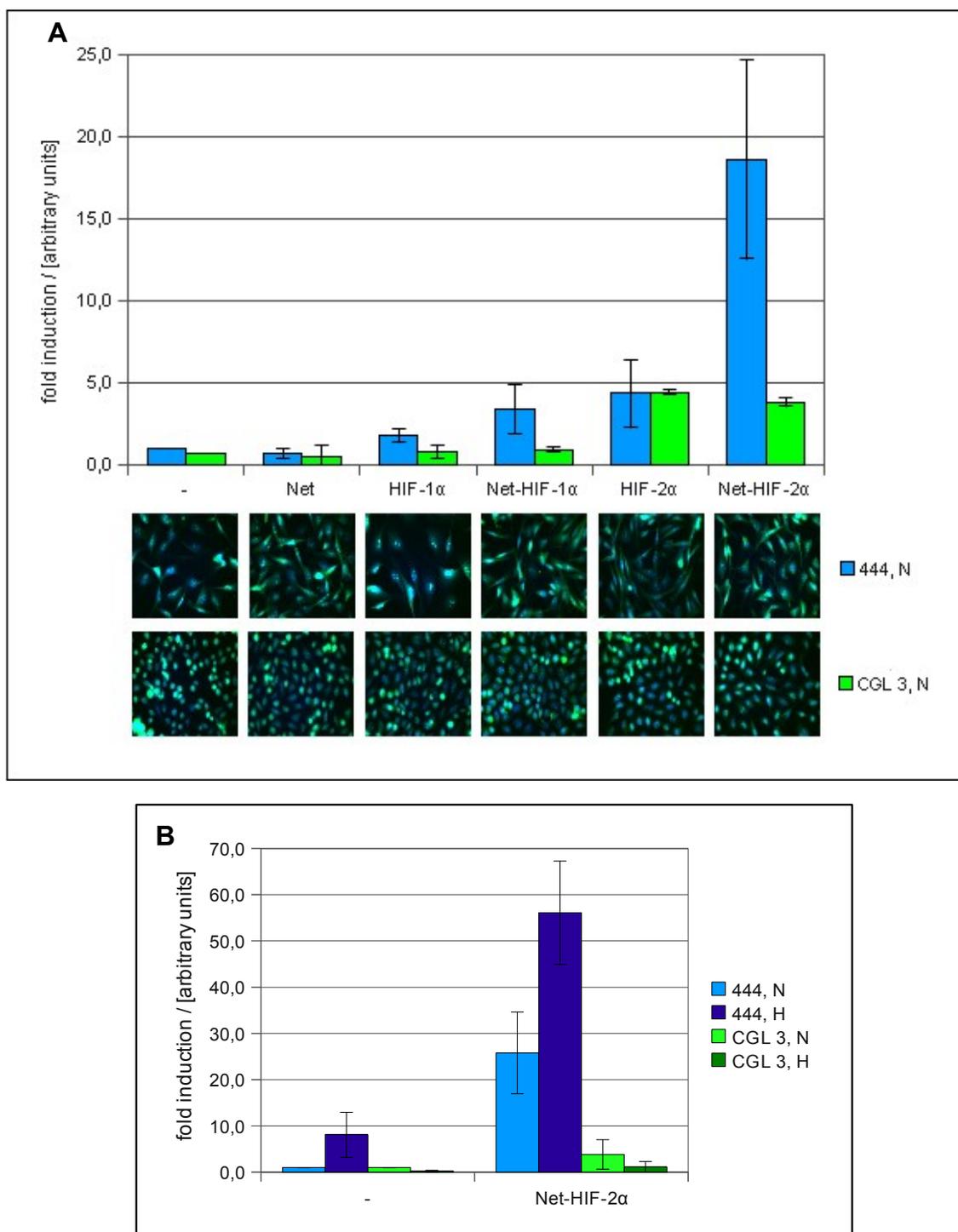
As a positive control, the promoter of plasminogen activator inhibitor 1 (PAI-1) was analyzed. This gene is known to be regulated by Net (Buchwalter *et al.*, 2005) and HIF-2 $\alpha$  (Liao *et al.*, 2007). Here, the promoter binding of Net and HIF-2 $\alpha$  shows a similar pattern compared to GAPDH: The binding of both transcription factors to the PAI-1 promoter in 444 is increased under hypoxia, with the relative increase of Net being higher than the relative increase of HIF-2 $\alpha$  (**Fig. 4.4 B**). In CGL 3, the presence of Net and HIF-2 $\alpha$  at the PAI-1 promoter shows the opposite: Hypoxic HIF-2 $\alpha$  levels remain unchanged while the binding of Net is diminished.

The promoter of alcohol dehydrogenase 5 (ADH5) was included as a negative control for ChIP. As a housekeeping gene constitutively transcribed by RNA polymerase II (Hur and Edenberg, 1992), but neither regulated by Net nor by HIF-2 $\alpha$  it enabled the specificity control for the second IP step.

Summing up, it could be shown that Net and HIF-2 $\alpha$  bind to the promoters of GAPDH and PAI-1 under normoxic and hypoxic conditions. However, Net and HIF-2 $\alpha$  binding seems to be differentially regulated in 444 and CGL 3 under hypoxic conditions.

#### 4.1.4 Net and HIF-2 $\alpha$ synergistically act on a GAPDH reporter

To determine the function of Net and HIF-2 $\alpha$  in GAPDH transcriptional regulation, luciferase assays were performed. As depicted in **Fig. 4.5**, the effect of different co-transfected transcription factors on a GAPDH-luciferase-reporter harboring 1112 bp of the endogenous GAPDH promoter was monitored.



**Fig. 4.5: Net and HIF-2 $\alpha$  synergistically activate the GAPDH reporter.** To monitor the effect of Net, HIF-1 $\alpha$ , and HIF-2 $\alpha$  on a GAPDH-promoter driven firefly-luciferase reporter, the mentioned transcription factors either alone or in the indicated combinations were co-transfected in 444 and CGL 3 cells (**A**) and subjected to 24 h hypoxia (**B**). The displayed values represent relative changes in the GAPDH-reporter activity normalized to normoxic control samples and a co-transfected TK-renilla-luciferase reporter, which is not regulated by Net or HIFs. “N” and “H” indicate normoxia and 2 % O<sub>2</sub>, respectively. Error bars represent variations within three independent experiments. A GFP expression plasmid was included (**A**) to rule out potential variations in the transfection efficiency. Presented are merged pictures of nuclear Hoechst 33258 staining and GFP.

Only the co-transfection of Net and HIF-2 $\alpha$  induced a significant synergistic upregulation of the GAPDH-luciferase-reporter in 444 cells (**Fig. 4.5 A**). This effect is further boosted under hypoxic conditions (**Fig. 4.5 B**). Neither Net, HIF-1 $\alpha$  or HIF-2 $\alpha$  alone nor a combination of Net and HIF-1 $\alpha$  was able to induce the reporter to this extent. Interestingly, the synergism could be only demonstrated in non-tumorigenic 444 cells. In tumorigenic CGL 3, the co-transfection of HIF-2 $\alpha$  alone or in combination with Net induced a less than 5-fold induction of the GAPDH-reporter in comparison to a 18-fold induction in 444. Hypoxia does not significantly induce the GAPDH reporter in CGL 3 cells, too (**Fig 4.5 B**). This data indicates a different regulation of GAPDH transcription upon hypoxia in both cell lines.

An unintended reason for the different regulation of the GAPDH reporter in 444 and CGL 3 could be differences in the transfection efficiency. To exclude this possibility, a GFP expressing plasmid was co-transfected and the expression of GFP was later-on monitored by fluorescence at 509 nm (**Fig. 4.5 A**). The results indicate, that the inability of Net and HIF-2 $\alpha$  to activate the GAPDH-reporter in CGL 3 is not due to different transfection efficiencies.

Overall, Net and HIF-2 $\alpha$  exert a synergistic effect on GAPDH transcription which seems to play a role in the hypoxic regulation of the gene. Their *trans*-activating properties are restricted to non-tumorigenic 444 cells.

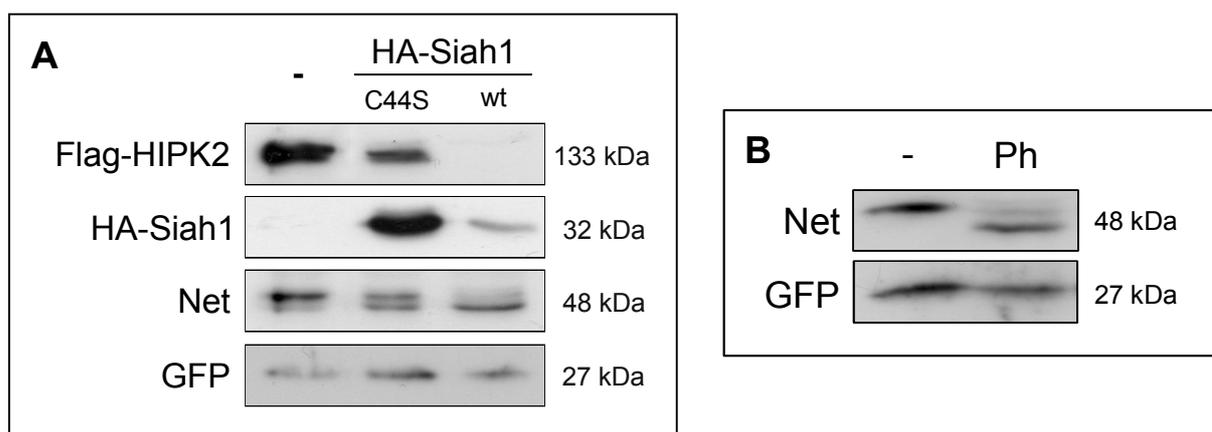
#### **4.1.5 Siah 1 is not involved in the Net-HIF-2 $\alpha$ mediated GAPDH transcriptional regulation**

As outlined above, the Net-HIF-2 $\alpha$  mediated transcriptional regulation of GAPDH is exclusively observed in non-tumorigenic cells. Two explanations are possible: first, the post-translational modifications of Net and HIF-2 $\alpha$  could be different in the investigated cell lines which would in turn influence the putative interaction of both factors or the recruitment of other *trans*-activating factors. Alternatively, the different response could be determined by the availability of a third, so far unknown factor or its post-translational modification status.

To investigate the second possibility, the impact of the “seven-in-absentia homologue” Siah 1 on the Net and HIF-2 $\alpha$  mediated synergism was studied. Several

properties of Siah 1 support this model: Siah 1 and Siah 2 are both involved in the mediation of the hypoxic response (Nakayama *et al.*, 2004). These E3-ubiquitin ligases indirectly mediate the stabilization of HIFs via the ubiquitination and subsequent degradation of their major upstream regulatory factors, the prolyl-hydroxylases (PHDs). In addition, they have been suggested to play a so far unknown role in the hypoxic regulation of Net (Gross *et al.*, 2008). Altogether, Siah 1 may be involved in the Net-HIF-2 $\alpha$  mediated synergism of GAPDH transcription.

Initially, a possible role for Siah 1 in the post-translational modification of Net was investigated. Therefore, co-transfections of Net, Siah 1 or a ligase-deficient mutant, Siah 1<sup>C44S</sup> and homeo-domain-interacting protein kinase 2 (HIPK2) were performed. HIPK2 is a well-known target of Siah 1-mediated proteasomal degradation and was included as a positive control.



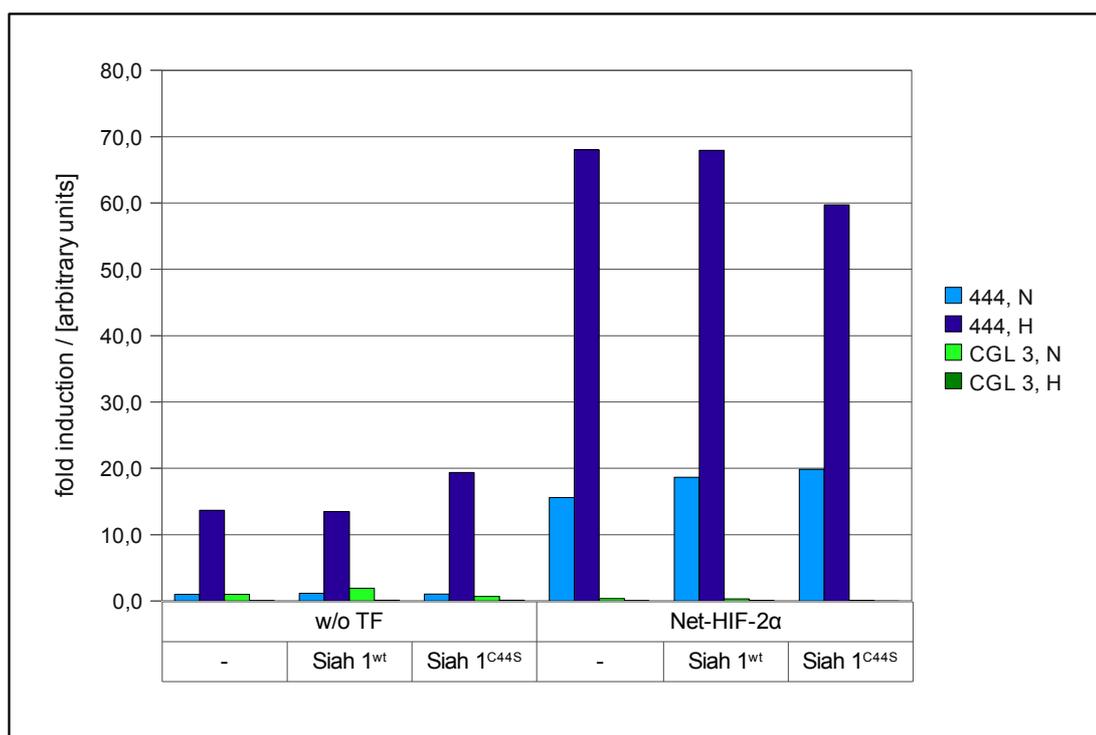
**Fig. 4.6: Siah 1 regulates post-translational modifications of Net.** Co-transfections of Net, Flag-HIPK2, HA-Siah 1<sup>wt</sup> (“wt”) and its E3-ligase deficient mutant HA-Siah 1<sup>C44S</sup> (“C44S”) were performed in HEK 293. GFP was included to control the transfection efficiency. **(A)** 48 h after transfection, whole cell extracts were prepared and analyzed by Western Blot. “-” indicates control transfections without Siah 1. Flag-HIPK2 and HA-Siah 1 were detected with their appropriate protein-tag specific antibodies. **(B)** To determine, whether the doublet-band observed in the case of Net represents phosphorylated and unphosphorylated species, the control sample was subjected to  $\lambda$ -phosphatase treatment. Subsequently, equal amounts of dephosphorylated (“Ph”) and untreated (“-”) sample were subjected to Western Blot analysis.

The Net antiserum applied in **Figure 4.3 B** and **Figure 4.6** detects two differential post-translationally modified subspecies of Net. In the absence of over-expressed Siah 1 preferentially the upper migrating form of Net is detectable (**Fig. 4.6 A and B**). This changes fundamentally upon co-expression of Siah 1<sup>wt</sup>: Mainly the lower migrating form of Net is visible. Co-transfection of the E3 ligase-deficient mutant

Siah 1<sup>C44S</sup> results in an intermediate band pattern. To identify the underlying post-translational modification,  $\lambda$ -phosphatase treatment was performed. Dephosphorylation lead to an accumulation of the lower migrating band (**Fig. 4.6 B**). Thus, the band-doublet represents phosphorylated and non-phosphorylated forms of Net, respectively.

HIPK2 was included in the transfections as a positive control. Its ubiquitination and subsequent proteasomal degradation is mediated by Siah 1. Thus, the majority of HIPK2 is detectable in the absence of over-expressed Siah 1 (**Fig. 4.6 A**).

Although equal amounts of Siah 1<sup>wt</sup> and its mutant were transfected, the mutant seems to be more abundant than Siah 1<sup>wt</sup> (**Fig. 4.6 A**). However, this is expected as Siah 1 is capable to auto-ubiquitinate itself and thus mediate its own degradation (Lorick *et al.*, 1999). Taken together, Siah 1 is involved in the post-translational regulation of Net.



**Fig 4.7: Siah 1 does not have an impact on the Net-HIF-2 $\alpha$  mediated transcriptional regulation of GAPDH.** To monitor the effect of Siah 1 on a GAPDH-promoter driven firefly-luciferase reporter, either Siah 1<sup>wt</sup> or Siah 1<sup>C44S</sup> alone or in combination with HIF-2 $\alpha$  and Net were co-transfected in 444 and CGL 3 and subjected to 24 h hypoxia (2 % O<sub>2</sub>), when indicated. The displayed values represent relative changes in the GAPDH-reporter activity normalized to a normoxic control sample and a co-transfected TK-renilla-luciferase reporter, which is not regulated by Net and HIF-2 $\alpha$ . “N” and “H” indicate normoxia and 2 % O<sub>2</sub>, respectively.

To address the role of Siah 1 in the Net and HIF-2 $\alpha$  mediated transcriptional regulation of GAPDH, luciferase assays were performed. According to **Fig. 4.7**, Siah 1 is not involved in the transcriptional regulation of GAPDH. Co-transfection of Siah 1 or its E3-ligase deficient mutant Siah 1<sup>C44S</sup> did not significantly change the GAPDH reporter activity neither under normoxic nor under hypoxic conditions. Furthermore, the different response of the GAPDH-reporter in 444 and CGL 3 is not affected by Siah 1. As already shown in **Figure 4.5 B**, only hypoxia and the co-transfection of Net and HIF-2 $\alpha$  lead to a substantial induction of the GAPDH reporter in non-tumorigenic 444.

Summing up, Siah 1 does not seem to be involved in the transcriptional regulation of GAPDH in non of the tested conditions. It may however play a role in the post-translational regulation of Net phosphorylation.

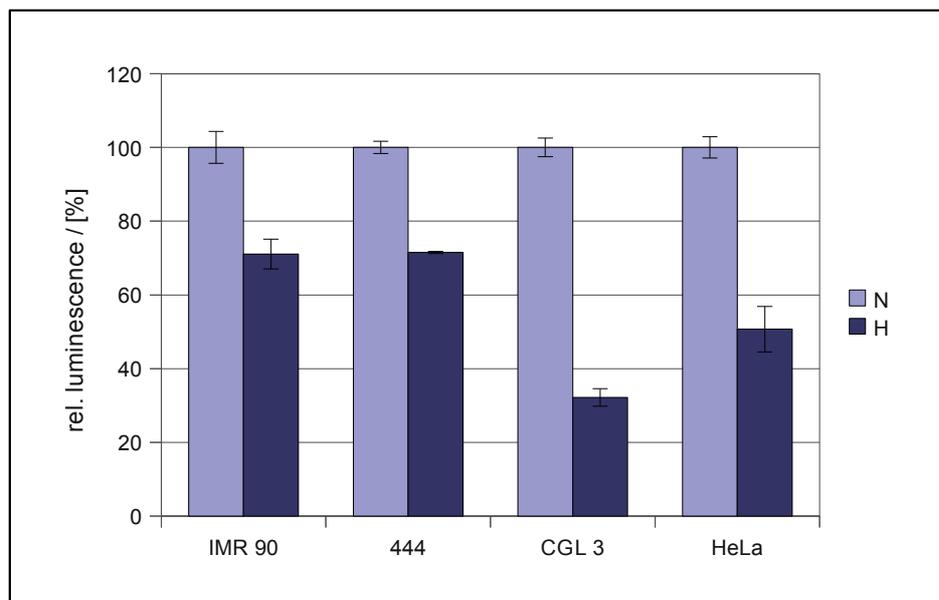
## **4.2 Tumorigenic and non-tumorigenic cells differ in their response to chronic hypoxia**

### **4.2.1 Impaired viability of tumorigenic cells upon hypoxia**

Hypoxia, a severe reduction of the oxygen tension within the tissue, induces a variety of cellular adaption processes concerning for example metabolism, proliferation and angiogenesis. While it is generally accepted, that cancer cells develop mechanisms to effectively cope with short term and intermittent hypoxia, the situation is more complex in the case of chronic hypoxia, lasting from several hours to days (Bristow and Hill, 2008).

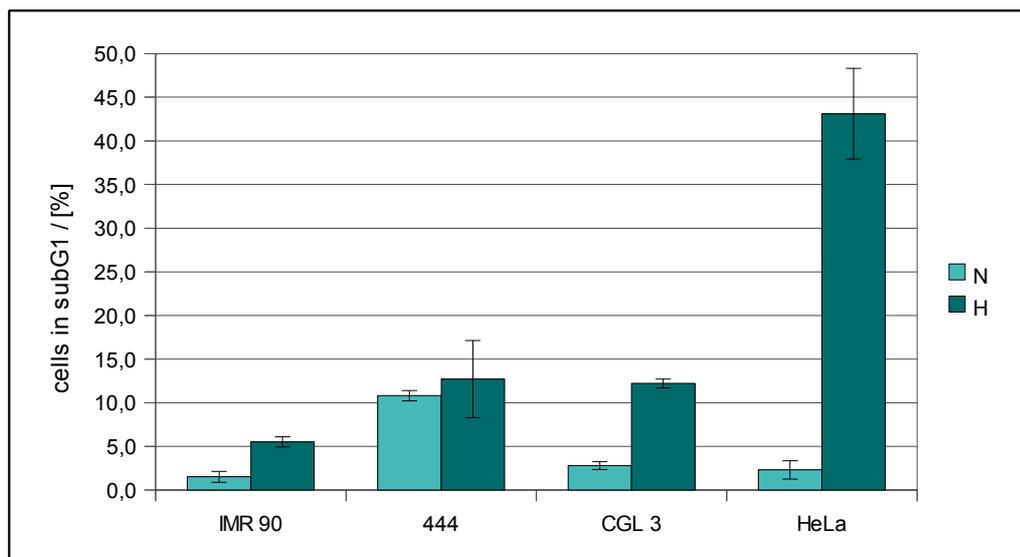
Previously presented results already indicate a different hypoxic response of tumorigenic and non-tumorigenic cells. To decipher this observation in more detail, different assays were performed to monitor the hypoxic tolerance of the used cell lines.

The universal cellular energy carrier ATP can be considered as a parameter reflecting cell viability. Considering that viable cells show only minor variations in their ATP levels, relative ATP levels could be used to determine the amount of viable cells in a culture (Izyumov *et al.*, 2004). To determine the viability of the cell lines IMR 90, 444, CGL 3 and HeLa upon hypoxia, relative ATP levels were analyzed after 8 h at 1.5 %



**Fig. 4.8: Cellular ATP levels of CGL 3 and HeLa are largely impaired after 8 h hypoxia.** IMR 90, 444, CGL 3 and HeLa were subjected to 8 h at 1.5 % O<sub>2</sub> (“H”) or control conditions (“N”). Subsequently, ATP was quantified by a Luciferase-reporter based method using the CellTiter Glo kit (Promega). Luciferase units were normalized to the number of cells seeded. Depicted is the relative decrease as compared to the normoxic Luciferase activity.

**Figure 4.8** indicates decreased ATP levels and thus a decrease of viable cells for all tested cell lines. However, the decrease is more prominent in the tumorigenic cell lines CGL 3 (67.3 %) and HeLa (49.3 %) than in the non-tumorigenic IMR 90 (29.9 %) and 444 (29.5 %). To distinguish, whether these results simply reflect hypoxic growth arrest or induction of apoptosis, a FACS analysis of normoxic and hypoxic cells was performed.



**Fig. 4.9: HeLa cells show a dramatic increase of apoptosis upon hypoxia.** IMR 90, 444, CGL 3 and HeLa were subjected to 24 h hypoxia at 1.5 % O<sub>2</sub> (“H”) or normoxia (“N”). Subsequently, cells were fixed for FACS analysis. Depicted are the percentage of cells in subG1. Error bars represent standard deviation of three independent experiments.

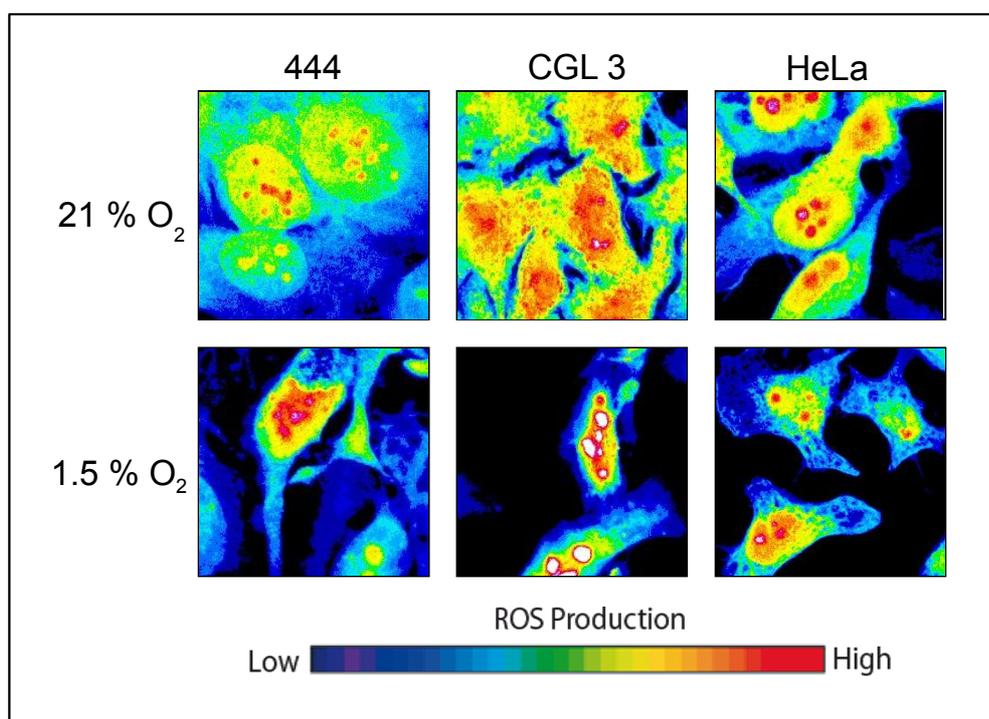
The FACS analysis (**Fig. 4.9**) provides a more detailed picture of cell viability. Upon hypoxia, the non-tumorigenic cell lines IMR 90 and 444 show only a small increase of apoptosis: 4.4 % increase in IMR 90 and 1.9 % in 444. Notably, 444 already exhibits elevated levels of apoptosis under normoxia. The situation of the tumorigenic cells CGL 3 and HeLa is more complex: CGL 3 displays an 9.4 % increase of apoptotic cells upon hypoxia. But the most striking difference was observed in HeLa: after 24 h at 1.5 % O<sub>2</sub>, they show a 40.8 % increase of apoptosis as compared to normoxia. Altogether, ATP assay and FACS analysis point to a compromised cell viability in the tumorigenic CGL 3 and HeLa, with HeLa undergoing apoptosis to a large extent. The non-tumorigenic IMR 90 and 444 display only minimal increase of apoptosis and their ATP levels were less compromised under hypoxia.

#### 4.2.2 Elevated ROS levels in tumorigenic cells

A severe side effect of hypoxia is the occurrence of reactive oxygen species (ROS) at mitochondrial complex III (Guzy *et al.*, 2005). During short term and intermittent hypoxia (lasting from minutes to hours) cancer cells develop efficient mechanisms to deal with the elevated ROS levels and the limited oxygen supply. Moreover, repeated

cycles of hypoxia and reoxygenation drive genomic instability resulting in an even more aggressive phenotype (Cairns *et al.*, 2001). But is this also true for chronic hypoxia?

FACS analysis (**Fig. 4.9**) and ATP assay (**Fig. 4.8**) point to a reduced survival of tumorigenic cells under hypoxia. To figure out, whether ROS are involved in this process, 444, CGL 3 and HeLa were monitored for reactive oxygen species under normoxic and hypoxic conditions.



**Fig. 4.10: Tumorigenic cells exhibit elevated ROS levels.** 444, CGL 3 and HeLa cells grown on cover slides were subjected to 24 h hypoxia (1.5 % O<sub>2</sub>) or normoxia. Prior to fixation for immunofluorescence, 2.5  $\mu$ M dihydroethidium (DHE) was added. ROS oxidized the DHE to a fluorescent dye, which was detected at 605 nm. The resulting images were converted to 16-colour code by the analysis software ImageJ. The scale in the lower part of the figure represents the semi-quantitative code for ROS levels.

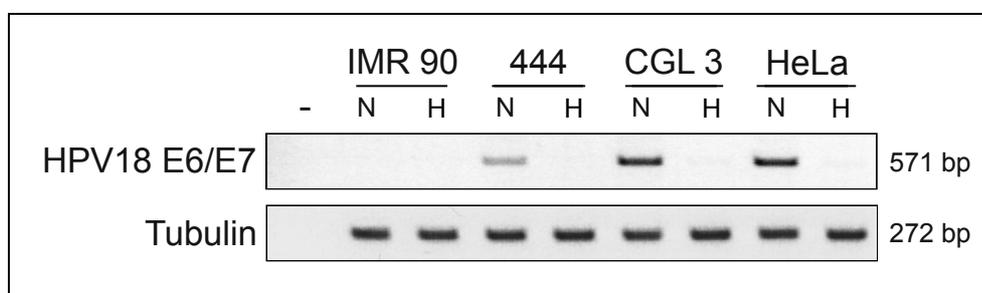
Compared to non-tumorigenic cells, ROS levels are already elevated in the tumorigenic cell lines under normoxic conditions (**Fig. 4.10**): The majority of CGL 3 and HeLa cells appears in yellow to red staining, indicating elevated ROS production. On the other hand, 444 cells show mainly a green to yellow staining reflecting a medium ROS production under normoxia (**Fig. 4.10, upper part**). This changes upon hypoxic treatment (**Fig. 4.10, lower part**): Here, 444 are intensively red coloured indicating a strong increase of ROS. This is accompanied by a morphological change

towards a more elongated-spiky form which reflects the cellular stress. ROS levels in CGL 3 increase to an extent, which exceeds the quantitative range of the assay. Morphologically, CGL 3 do not change as dramatic as 444. In HeLa cells, the ROS levels seem to be unchanged. The hypoxic shape of HeLa cells resembles the one of CGL 3. A closer look, however, reveals first signs of membrane blebbing in hypoxic HeLa cells which indicates apoptosis and confirms the FACS data (**Fig. 4.9**).

### 4.3 HPV and hypoxia

#### 4.3.1 Viral gene expression under hypoxia

In the case of cervical cancer, intratumoral hypoxia serves as a prognostic factor associated with a poor prognosis for patients. Despite the accumulating knowledge on therapeutic implications in the treatment of hypoxic tumors, little is known about the regulation of HPV gene expression under these conditions. To get first insights into the expression of the early genes E6 and E7, semi-quantitative PCR was performed.



**Fig. 4.11: HPV E6/E7 expression is silenced upon hypoxia.** Cells were subjected to 24 h normoxia ("N") or 1.5 % O<sub>2</sub> ("H"), cytosolic RNA was prepared, purified from DNA contamination and reverse transcribed. The expression of a HPV E6/E7 transcript and  $\beta$ -Tubulin was analyzed by semi-quantitative PCR.

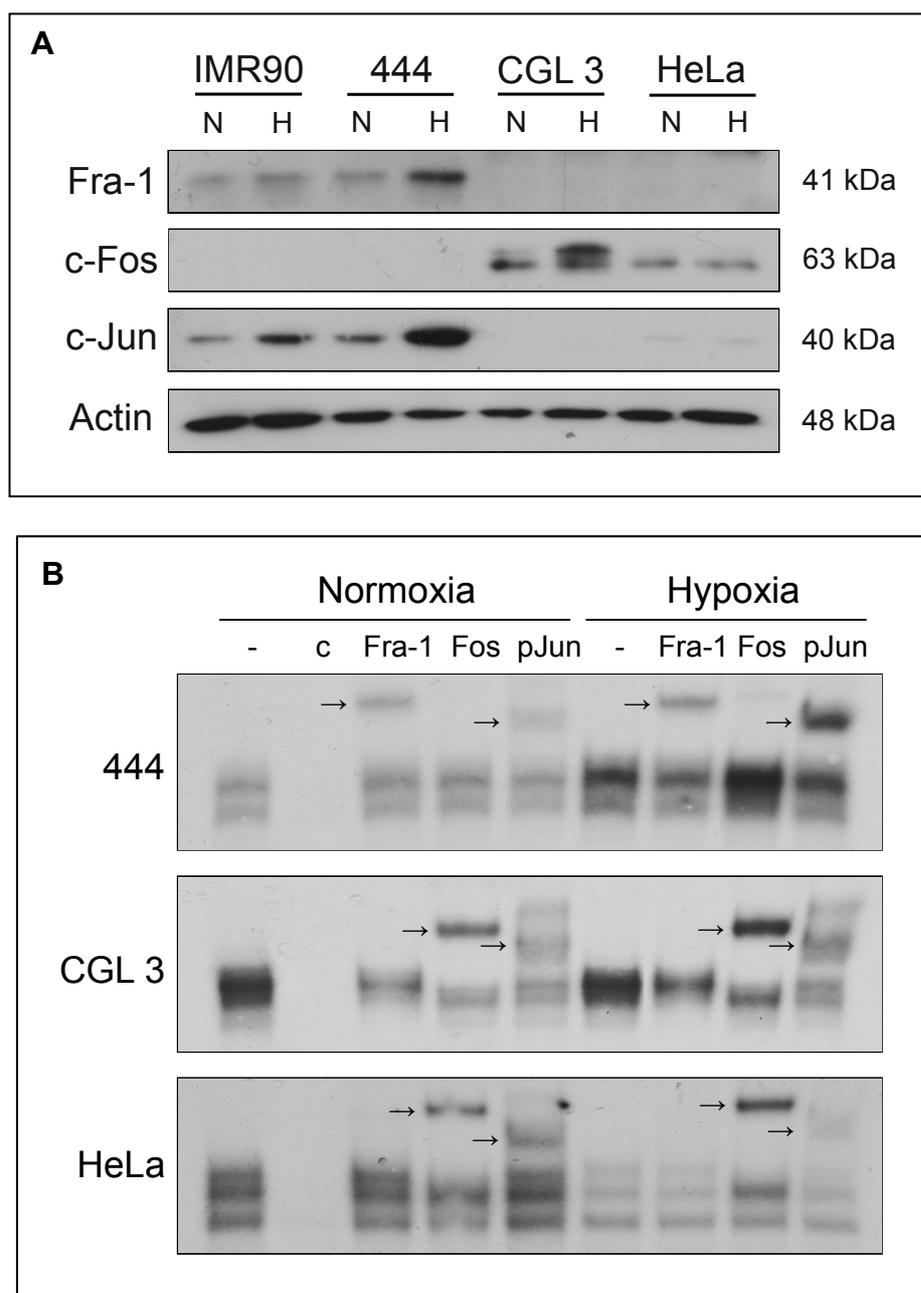
Strikingly, the expression of HPV E6/E7 is silenced upon 24 h hypoxia. Moreover, all HPV-positive cells show this effect. Comparing the normoxic E6/E7 expression of 444, CGL 3 and HeLa, the tumorigenic cells display increased levels of the E6/E7 transcript. As a normal fibroblast, IMR 90 does not show HPV expression, indicating PCR specificity.

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### 4.3.2 The impact of AP-1 in the hypoxic regulation of HPV expression

The cellular transcription factor AP-1 plays a fundamental role in the transcriptional regulation of HPV (Butz and Hoppe-Seyler, 1993). In particular, its dimeric composition is essential: While there is a high HPV E6/E7 expression expected in the presence of Jun-Fos dimers, the transcription of the early genes is reduced, when preferentially Fra-1 is incorporated into the AP-1 complex (Rösl *et al.*, 1997; Soto *et al.*, 1999). To unravel, whether AP-1 is involved in the hypoxic down-regulation of HPV E6/E7, electromobility shift assays (EMSA) were performed. Additionally, the cellular expression of the cognate AP-1 subunits was monitored by Western Blot.

As shown in **Fig. 4.12 A**, the analyzed cell lines IMR 90, 444, CGL 3 and HeLa do not fundamentally change their expression pattern of Fra-1, Fos and Jun. Rather, there is in some cases an increased expression of the AP-1 subunits which are already detectable under normoxia. This is in particular the case for Fra-1 in 444, Fos in CGL 3 and Jun in IMR 90 and 444. The upregulation of Fos in CGL 3 is accompanied by a change in the post-translational modifications, which might effect the *trans*-activating capacity of this transcription factor. Taken together, if Fra-1, Fos and Jun are not detectable under normoxia in the tested cell lines, hypoxia does not induce their expression.



**Fig. 4.12: Hypoxia does not change the AP-1 composition.** Cells were exposed to 1.5 % O<sub>2</sub> ("H") or normoxia ("N") for 24 h and nuclear protein extract was prepared. 25 µg and 2 µg protein were analyzed by Western Blot (A) and EMSA (B), respectively. Fra-1, Fos, Jun, phospho-Jun ("pJun") and Actin indicate antibodies used for immunodetection and supershift. To determine the AP-1 composition by EMSA, the <sup>32</sup>P-labeled AP-1 consensus oligonucleotide was initially incubated with the nuclear extract. To monitor the specific binding of AP-1 to the oligonucleotide, the binding was competed with an identical but unlabeled probe ("c"). Subsequently, the antibody was included to target the indicated AP-1 subunit. Finally, the reaction mix was separated by PAGE, dried and exposed to a film. Supershifts are indicated by arrows.

However, the protein levels of the AP-1 subunits do not necessarily reflect the composition of the dimeric complex. Thus, an EMSA-supershift was performed (**Fig. 4.12 B**). It confirms the tendency of the Western Blot: There is not a change of the AP-1 composition. Rather, the overall AP-1 content in the cells changes preserving the original composition. In 444, the AP-1 composition is mainly pJun-Fra-1. The incorporation of both factors is increased upon hypoxia, reflecting the absolute increase of AP-1 complex formation (**Fig. 4.12 B, lane 1 and 6**). In CGL 3, the amount of AP-1 complexes is similar under normoxia and hypoxia (**lane 1 and 6**). However, there seems to be an increased incorporation of Fos upon hypoxia (**lane 4 and 8**), while there is only a minor change concerning pJun (**lane 5 and 9**). In HeLa, the EMSA even indicates a reduction of AP-1 complexes upon hypoxia (**lane 1 and 6**), which is reflected by lower pJun incorporation (**lane 5 and 9**). However, the EMSA depicts an increased Fos incorporation into the AP-1 dimer upon hypoxia in HeLa (**lane 4 and 8**).

Taken together, neither the expression of the tested AP-1 subunits nor the composition of the AP-1 complex is basically changed upon hypoxia. Variations occur exclusively in the expression level with a concomitant enhanced AP-1 binding. However, the composition of the complex remains identical compared to normoxia.

## 5. Discussion

GAPDH, for a long time exclusively known as a glycolytic, ubiquitously expressed gene, gained attention in the last decade when scientists unraveled its multiple functions in cell death and survival (Hara *et al.*, 2005; Colell *et al.*, 2007). Therefore it is not surprising that GAPDH expression seems to be deregulated in a variety of cancer types, including cervical cancer (Kim *et al.*, 1998). To get insights into the underlying mechanisms, this study aimed to investigate the transcriptional regulation of GAPDH by the transcription factors HIF-2 $\alpha$  and Net, as previous studies suggested their binding at the GAPDH promoter (Graven *et al.*, 2003; A. Hitschler, 2007). Moreover, Net and HIF-2 $\alpha$  were linked to cancer development and mediate the hypoxic response (Wiesener *et al.*, 1998; Gross *et al.*, 2008). Considering the role of GAPDH as a trigger of cell death, its deregulation in cancer cells might be reflected by a worse survival of chronic hypoxia. In the context of HPV-positive cells, the viral oncogenes E6 and E7 may determine the hypoxic resistance. To get first insights, their expression and their transcriptional regulation was studied under chronic oxygen deprivation.

### 5.1 Net and HIF-2 $\alpha$ synergistically activate GAPDH transcription

#### 5.1.1 GAPDH is differentially expressed in tumorigenic and non-tumorigenic cell lines

As mentioned above, a different expression of GAPDH has been reported for multiple types of cancer, including breast cancer (Révillion *et al.*, 2000), prostate cancer (Epner *et al.*, 1993) and cervical cancer (Kim *et al.*, 1998). Although the basal expression of GAPDH is frequently elevated in tumors, an impact of this deregulation on hypoxic GAPDH expression remains elusive so far. In the context of HPV-associated cervical cancer, tumor hypoxia serves as a prognostic marker indicating poor patient outcome (Höckel *et al.*, 1993). Considering that GAPDH is involved in many metabolic processes and acts as a trigger between cell death and survival, a deregulated GAPDH expression under hypoxic conditions seems likely to occur in cervical cancer cells.

In order to unravel the relevance of an altered hypoxic GAPDH regulation, a model of HPV-positive tumorigenic (CGL 3) and non-tumorigenic (444) HeLa x fibroblast hybrids (Stanbridge, 1984) and their parental homologues HeLa and IMR 90 were chosen. The hybrid model enables the comparison of molecular events in a genetically identical background, as tumorigenic CGL 3 are spontaneous segregants of non-tumorigenic 444 after long-term cultivation. The parental cell lines IMR 90 and HeLa were included to allow conclusions about the origin of an observed phenotype. Furthermore, this model reflects different stages of carcinogenesis: IMR 90 is a primary fibroblast cell line, reflecting HPV-negative non-transformed tissue (Nichols *et al.*, 1977). 444 represents an intermediate state: as a HPV-positive hybrid cell line, it is immortal. Although these cells are non-tumorigenic, 444 display single oncogenic features like anchorage independent growth in soft agar (Stanbridge and Wilkinson, 1980). In contrast, their tumorigenic counterparts CGL 3 and HeLa are able to form tumors in nude mice.

Initially, GAPDH expression was monitored on RNA level in both semi-quantitative (**Fig 4.1 A**) and quantitative PCR (**Fig 4.1 B**). Consistent with a deregulation of basal GAPDH expression during carcinogenesis, GAPDH expression under normoxia is elevated in CGL 3 and HeLa compared to IMR 90 and 444 (**Fig 4.1 B**).

Hypoxic GAPDH regulation differs between non-tumorigenic and tumorigenic cell lines, too (**Fig 4.1 A**). It seems, that 24 h of 1.5 % O<sub>2</sub> induce a profound upregulation of GAPDH in the non-tumorigenic IMR 90 and 444 while the effect in their tumorigenic counterparts CGL 3 and HeLa is less pronounced. However, the semi-quantitative approach is error-prone. To verify these results, three independent sample sets were analyzed by qPCR (**Fig 4.1 B**). Overall, the quantitative data confirms the differences between tumorigenic and non-tumorigenic cell lines. The similar behavior of IMR 90 and 444 leads to the conclusion, that the hypoxic regulation of GAPDH is unaltered in both cell lines and mirrors the physiological response to hypoxia (Graven *et al.*, 1998). More intriguingly, the tumorigenic cell lines HeLa and CGL 3 differ in their hypoxic GAPDH expression: CGL 3 show reduced GAPDH expression upon hypoxia, whereas GAPDH expression in HeLa remains unchanged. This reflects the differences observed between HeLa and CGL 3 at normoxic GAPDH levels and points to cell line specific regulatory changes.

In conclusion, GAPDH transcription is deregulated in the tumorigenic cell lines HeLa and CGL 3. While basal GAPDH transcription seems to be generally elevated in the tumorigenic cells, CGL 3 and HeLa both lack the hypoxic GAPDH upregulation as observed in the non-tumorigenic cell lines IMR 90 and 444.

### **5.1.2 Net and HIF-2 $\alpha$ expression in HeLa x fibroblast hybrids is subject to multiple layers of regulation**

To investigate the proposed mechanism of GAPDH transcriptional regulation in more detail, expression and promoter binding of the transcription factors Net and HIF-2 $\alpha$  was monitored in the hybrid system 444 and CGL 3. As expected, both cell lines showed a substantial accumulation of HIF-1 $\alpha$  and HIF-2 $\alpha$  protein after hypoxia (**Fig. 4.3 B**). This effect is mediated by post-translational stabilization upon oxygen deprivation, as the changes on protein level are not reflected by mRNA levels (Wang and Semenza 1993a; Wiesener *et al.*, 1998). However, upon hypoxia, minor changes are observed in the mRNA levels of both HIFs (**Fig. 4.3 A**). In the case of HIF-1 $\alpha$ , mRNA levels slightly drop after 24 h chronic hypoxia in both 444 and CGL 3. In contrast, HIF-2 $\alpha$  levels seem to be slightly elevated in 444 and remain unchanged in CGL 3. There is evidence, that both transcription factors are transcriptionally upregulated upon acute, short-term hypoxia lasting from minutes to hours. While chronic hypoxia (hours to days) does not affect the expression of HIF-2 $\alpha$ , the mRNA stability of HIF-1 $\alpha$  might be reduced by miRNA mediated mechanisms (Trash-Bingham *et al.*, 1999). Interestingly, an auto-negative feedback loop was proposed for this mechanism, involving the induction of an antisense transcript by both HIF-1 $\alpha$  and HIF-2 $\alpha$  and resulting in the exclusive destabilization of HIF-1 $\alpha$  mRNA (Uchida *et al.*, 2004) upon long-term hypoxia.

In contrast, Net expression dramatically differs between 444 and CGL 3. As reported previously, tumorigenic CGL 3 depict reduced Net expression while high Net levels are observed in non-tumorigenic 444 (**Fig. 4.3 A and B**) (van Riggelen *et al.*, 2005). Moreover, Net protein levels in CGL 3 are further diminished upon 24 h hypoxia which is not observed in their non-tumorigenic counterparts 444 (**Fig. 4.3 B**). Hypoxia was suggested to inhibit hydroxylation of Net leading to its subsequent proteasomal degradation (Gross *et al.*, 2007). According to the absence of hypoxic Net regulation

in 444, this effect seems to be cell type dependent. In addition, hypoxic degradation of Net may rely on the severeness and duration of oxygen deprivation because the cited study of Gross *et al.* observed a complete loss of Net after 6 to 12 h at 1 % O<sub>2</sub>, whereas the present study applied 2 % O<sub>2</sub> for 24 h. Thus, decreasing oxygen pressure would correlate with a decrease in Net stability.

To monitor the hypoxic response on mRNA level, VEGF expression was analyzed (**Fig. 4.3 A**). As reported previously, the pro-angiogenic VEGF is transcriptionally upregulated under hypoxia in both non-tumorigenic 444 and tumorigenic CGL 3 (Forsythe *et al.*, 1996). The latter ones express higher basal levels of VEGF in comparison to their non-tumorigenic counterparts. Indeed, VEGF expression is frequently elevated in cancer cells and it correlates with high vascularization of cervical cancer (Kodama *et al.*, 1999).

In conclusion, the analyzed transcription factors Net, HIF-1 $\alpha$  and HIF-2 $\alpha$  are expressed in 444 and CGL 3, although in different levels depending on the cell line and the availability of oxygen.

### **5.1.3 Hypoxia differentially regulates Net and HIF-2 $\alpha$ binding to the GAPDH promoter in non-tumorigenic and tumorigenic cells**

The transcription factors Net and HIF-2 $\alpha$  mediate the cellular hypoxic response and were suggested to play a role in GAPDH transcriptional regulation. According to previous *in silico* analysis, the GAPDH promoter harbors multiple DNA binding motifs of Net. One of them (-248 to -227 bp upstream of the transcriptional start site) is located in close proximity to a previously described hypoxia response element (HRE) (-217 to -203 bp) (Hitschler, 2007; Lu *et al.*, 2002) (**Fig. 4.2**).

To shed light on the binding of Net and HIF-2 $\alpha$  at the GAPDH promoter under normoxia and 24 h hypoxia, a chromatin immunoprecipitation (ChIP) was performed (**Fig. 4.4 A and B**). The ChIP assay comprised two IP steps, a first one directed against RNA Pol II and a second one targeting Net or HIF-2 $\alpha$ . This approach allowed fishing of actively transcribed promoters bound by the specific transcription factors. Especially in the case of Net this approach is interesting, as previous studies focused only on its repressive properties. However, it was suggested to act as a *trans*-activator for other genes, too (Giovane *et al.*, 1994). A drawback of the pre-selection

of RNA Pol II bound promoters is the potential correlation of RNA Pol II promoter binding with the rate of transcription and the resulting mRNA levels. According to a simplified model, the limiting factor of transcription is the recruitment of RNA Pol II to the promoter and not its time of retention at the transcriptional start site. In the first case, variations of RNA Pol II promoter binding would influence the result of the ChIP assay. However, as a constitutive and highly expressed gene, GAPDH is generally bound by RNA pol II. Differences in GAPDH mRNA levels rather result from the speed of RNA Pol II promoter clearance than the rate of RNA Pol II recruitment to the promoter (Rougvie *et al.*, 1990). The efficiency of RNA Pol II hyperphosphorylation, representative for active RNA Pol II in most cases, does not account for the rate of transcription of housekeeping genes neither, as hypophosphorylated forms of RNA Pol II were shown to participate in their transcription, too (Weeks *et al.*, 2010). In fact, there is supposedly a chain of differentially phosphorylated RNA Pol II along the transcriptional start site of GAPDH. Therefore, the amount of active RNA Pol II bound to the GAPDH promoter is considered to be constant and does not reflect changes in the transcriptional activity of the gene.

How do Net and HIF-2 $\alpha$  influence the transcription of GAPDH? According to their promoter binding under normoxia and hypoxia, there seems to be a differential response to hypoxia in non-tumorigenic 444 compared to tumorigenic CGL 3 (**Fig. 4.4 B**). While hypoxia increases Net and HIF-2 $\alpha$  binding at the GAPDH promoter in 444, only minor changes happen in the case of CGL 3 after 24 h at 2 % O<sub>2</sub>. Taking into consideration that the expression of GAPDH is increased under hypoxia exclusively 444 while it is even reduced in CGL 3 (**Fig. 4.1 B**), it could indicate a *trans*-activating role of Net and HIF-2 $\alpha$  in GAPDH transcription. Interestingly, the promoter binding of both transcription factors is not reflected by their absolute protein levels determined by Western Blot (**Fig. 4.3 B**). While Net expression in 444 is unchanged upon hypoxia, its promoter binding is increased. Similarly, the slight reduction of overall Net protein levels upon hypoxia in CGL 3 is not reflected by the the extent of promoter binding, which remains unchanged. In the case of HIF-2 $\alpha$ , the effect is even more striking: While the protein is undetectable under normoxia by Western Blot, ChIP assay determines substantial amounts of HIF-2 $\alpha$  at the GAPDH

promoter under these conditions. Thus, the promoter binding of Net and HIF-2 $\alpha$  is probably determined by additional mechanisms, which may influence their impact on GAPDH transcription.

#### **5.1.4 Net and HIF-2 $\alpha$ synergistically activate GAPDH transcription exclusively in non-tumorigenic cells**

A possible synergistic effect of Net and HIF-2 $\alpha$  on GAPDH transcription is supported by previous studies on Net related proteins: the transcription factors Elk-1 and Ets-1, which belong to the same protein family as Net, were shown to cooperate with HIF-2 $\alpha$  (Aprelikova *et al.*, 2006, Elvert *et al.*, 2002). In this case, the protein interaction is facilitated by their shared helix-loop-helix (HLH) domain which Elk-1 and Ets-1 have in common with Net. Moreover, the *in silico* predicted distance of Net and HIF-2 $\alpha$  *cis*-regulatory elements within the GAPDH promoter matches the maximal spacing of 60 bp, which was previously reported as a common feature of Ets factor and HIF-2 $\alpha$  mediated gene regulation.

To decipher the impact of Net and HIF-2 $\alpha$  on GAPDH transcription in more detail, Luciferase reporter assays were performed (**Fig. 4.5**). Here, three important characteristics of Net and HIF-2 $\alpha$  mediated GAPDH transcriptional regulation were revealed: First, Net and HIF-2 $\alpha$  exert a *trans*-activating synergism on GAPDH transcription. The effect is exclusive for both proteins as HIF-1 $\alpha$  cannot substitute for HIF-2 $\alpha$  (**Fig. 4.5 A**). Secondly, the synergism can be further augmented by hypoxia (**Fig. 4.5 B**). Thirdly, only non-tumorigenic 444 display the effect. Neither co-transfection of Net and HIF-2 $\alpha$  nor induction of hypoxia significantly changes the reporter activity in CGL 3 (**Fig. 4.5 A and B**). Nevertheless, the physiological relevance of reporter assays is limited, as they only analyze the regulatory effect of over-expressed transcription factors on an approximately 1000 bp promoter fragment, which neglects the endogenous genomic environment and the interplay with other endogenous transcription factors. To rule out artificial side effects of the co-transfected proteins, the activity of the GAPDH reporter was monitored in the absence of over-expressed Net and HIF-2 $\alpha$ . Strikingly, the reporter was induced upon hypoxia exclusively in 444 (**Fig. 4.5 B**) which reflects the hypoxic upregulation of endogenous GAPDH in the non-tumorigenic cell lines IMR 90 and 444 (**Fig. 4.1**)

and underlines the physiological relevance of these results. Over-expression of Net and HIF-2 $\alpha$  indeed further augments the reporter activity in 444 under both normoxic and hypoxic conditions. Accordingly, both proteins might exert a *trans*-activating synergism on physiological GAPDH transcription, which probably participates in the hypoxic regulation of GAPDH transcription.

However, the question remains whether different Ets factors might influence GAPDH transcription. Hollenhorst and colleagues propose a model, where Ets transcription factors facilitate both general high transcription and trigger specific regulation depending on the properties of the Ets DNA binding site (EBS) (Hollenhorst *et al.*, 2007). In the case of GAPDH transcriptional regulation, both features are possible. The above described Net-HIF-2 $\alpha$  synergism is likely to be a gene specific mechanism, leading to the recruitment of a specific Ets factor to the GAPDH promoter. However, it cannot be excluded that other Ets factors which are able to interact with HIF-2 $\alpha$ , too, could substitute for Net. Possibly, such a mechanism enables the integration of different signaling pathways by multiple Ets factors. Post-translational modifications might determine which Ets factor is preferentially bound by HIF-2 $\alpha$  and concomitantly which Ets factor mainly regulates gene transcription. In the context of the present study, it remains to be elucidated, how different Ets factors regulate the GAPDH transcription in tumorigenic and non-tumorigenic cells and whether they are able to synergize with HIF-2 $\alpha$  in that context.

In contrast to Ets factors, HIFs mediate the hypoxic response in a highly gene-specific manner. Both, HIF-1 $\alpha$  and HIF-2 $\alpha$  are involved in the transcriptional regulation of GAPDH in a context dependent manner (Graven *et al.*, 1999 and 2003). Under long-term, chronic hypoxia, HIF-1 $\alpha$  protein levels decrease while HIF-2 $\alpha$  is constantly expressed (Uchida *et al.*, 2004) enabling a differential activation of HIF target genes according to the extent and duration of hypoxia. The observed Net-HIF-2 $\alpha$  synergistic activation of GAPDH (**Fig. 4.5**) might therefore predominantly play a role under long-term hypoxia. In addition, a transcriptional regulation of GAPDH by both HIFs would partially explain the different extent of hypoxic GAPDH activation on mRNA level (**Fig. 4.1**) and in the luciferase-reporter assay (**Fig. 4.5**). The latter one monitors the effect of over-expressed HIF-2 $\alpha$  on the reporter construct, representative for chronic hypoxia and independent of endogenous factors. In

contrast, the GAPDH qPCR reflects the *in vivo* transcriptional regulation influenced by the duration and extent of hypoxia, the genomic environment and post-transcriptional mechanisms. Future studies will be required to figure out the specific contribution of the Net and HIF-2 $\alpha$  synergism on GAPDH transcription. To decipher whether the duration of hypoxia influences the synergism, additional GAPDH-reporter assays may be required.

Overall, the important cellular functions of GAPDH require both a high but tightly regulated transcription. Thus, it is not surprising that HIFs and Ets factors themselves are able to integrate different context dependent signals on GAPDH transcription. The specific contribution of the Net-HIF-2 $\alpha$  synergism requires further investigation. There is evidence, that it might be involved in the GAPDH transcriptional regulation during chronic hypoxia.

### **5.1.5 Possible mechanisms determining the Net-HIF-2 $\alpha$ synergism**

As discussed above, the reporter assay depicts striking differences in the GAPDH transcriptional regulation between 444 and CGL 3. This observation is supported by the results of the ChIP assay (**Fig. 4.4**) and the endogenous GAPDH expression (**Fig. 4.1**) which display as well a different response to hypoxia in 444 and CGL 3. What could be causative for this observation? Mutations of the GAPDH promoter in either CGL 3 or 444 might alter the affinity of the *cis*-regulatory element to the respective transcription factor. However, this possibility can probably be excluded, as the data from ChIP and qPCR experiments corresponds to the reporter assay data. Furthermore, the post-translational modifications of either Net or HIF-2 $\alpha$  may be different in the cell lines which influences the recruitment of co-activators. In addition, a third co-factor could be required for the synergistic transcriptional activation by Net and HIF-2 $\alpha$ , which might be differentially regulated in both cell lines.

Possible factors which might play a role in the Net and HIF-2 $\alpha$  synergism are the “seven in absentia” homologues Siah 1 and Siah2. They are involved in the HIF-mediated hypoxic response and were recently suggested to participate in Net regulation (Nakayama *et al.*, 2004; Gross *et al.*, 2008). Therefore, overexpression studies including Net, Siah 1 and HIPK2 as a positive control for Siah 1 E3-ligase activity were performed (**Fig. 4.6 A**). It was shown that co-expression of Siah 1

reduced Net phosphorylation (**Fig. 4.6 A and B**). Possibly, Siah 1 targets an upstream kinase of Net for proteasomal degradation, which would explain the lack of Net phosphorylation in the presence of over-expressed wild-type Siah 1. However, this model does not apply to the fact, that co-expression of the E3-ligase-deficient Siah 1<sup>C44S</sup> mutant as well reduces the phosphorylation of Net, although to a lesser extent than the wild-type Siah 1 does. Alternatively, Siah 1 could act as an adapter protein for the recruitment of Net regulators. According to that model, the activity of Siah 1 would only be partially dependent on its E3-ligase function, as the putative adapter properties of the ligase-deficient Siah 1<sup>C44S</sup> seem to be slightly impaired. In addition, it cannot be excluded that the C44S amino acid substitution affects the protein conformation of the Siah 1 mutant. E3-ligase independent functions of Siah 1 are indeed described in the literature. It has been reported to act as a transporter, facilitating nuclear translocation of proteins (Hara *et al.*, 2005).

To clarify the role of Siah 1 in the Net-HIF-2 $\alpha$  *trans*-activating synergism, GAPDH-reporter assays were performed. However, Siah 1 did not significantly effect the activity of the Luciferase-reporter (**Fig. 4.7**). Thus, Siah 1 is probably not involved in the Net-HIF-2 $\alpha$  mediated transcriptional regulation of GAPDH.

According to the over-expression studies described above (**Fig. 4.6 A**), another explanation may be possible. As a positive control for the E3-ligase function of Siah 1, the kinase HIPK2 was included in the co-transfections and it cannot be ruled out that HIPK2 directly phosphorylates Net. High levels of HIPK2 correlate with an accumulation of phospho-Net. In contrast, overexpressed wild-type Siah 1 facilitates proteasomal degradation of HIPK2, which results in a reduction of the phosphorylated Net species. To date, an interaction of Net and HIPK2 has not yet been described. However, a possible link may be their common involvement in the SUMO-pathway. Net was previously described to interact with the SUMO E3-ligase PIAS1, which enhances the repressive properties of Net (Wasylyk *et al.*, 2005). In turn, HIPK 2 has been shown to be SUMOylated itself, which potentiates its *trans*-repressive properties (Roscic *et al.*, 2006). Future experiments are required to clarify the possible role of HIPK2 in Net regulation. A GAPDH-luciferase reporter assay including Net, HIF-2 $\alpha$ , HIPK2 and a kinase-deficient HIPK2 mutant might give further insights.

Additionally, certain post-translational modifications of Net and HIF-2 $\alpha$  may be required for the synergistic transcriptional activation of GAPDH. These modifications could be different in 444 and CGL 3 which may explain the absence of the synergism in the latter cell line. However, protein analysis of Net and HIF-2 $\alpha$  by Western Blot did not point to differences in post-translational modifications, as the migration pattern of both factors was similar in both cell lines (**Fig. 4.3 B**) and did not change upon hypoxia neither. Considering that certain post-translational protein modifications might interfere with the binding of Net and HIF-2 $\alpha$  specific antibodies applied in Western Blot, cell line dependent differences in the post-translational modifications of these factors cannot be excluded.

Overall, there is no evidence for a role of Siah 1 in the Net-HIF-2 $\alpha$  synergistic regulation of GAPDH expression. Possibly certain post-translational modifications of Net and HIF-2 $\alpha$  are prerequisite for their impact on GAPDH transcription. Moreover, a role of HIPK2 seems to be possible and may be subject for future investigations.

## **5.2 The role of GAPDH in the survival of chronic hypoxia**

### **5.2.1 Chronic hypoxia sensitizes tumorigenic cells to oxidative and energetic stress**

GAPDH exerts a number of pro- and anti-apoptotic functions and was suggested to act as a checkpoint control, which initiates cell death upon aberrant signaling (Colell *et al.*, 2009). As expected, GAPDH transcription is deregulated in the cervical cancer cell line HeLa and the HeLa x fibroblast hybrid CGL 3. To investigate whether this observation correlates with an altered cellular survival under long-term hypoxia, cell viability was monitored by FACS (**Fig. 4.9**) and ATP (**Fig. 4.8**) assay. Whereas the viability of non-tumorigenic IMR 90 and 444 is only minimally altered, the cell integrity is severely compromised in the tumorigenic CGL 3 and HeLa. According to the FACS assay, more than 40 % of the monitored HeLa cells undergo apoptosis during 24 h hypoxia (**Fig. 4.9**). Although the percentage of apoptotic cells is also increased in CGL 3 upon hypoxia, it does not exceed 10 %. This is in contrast to the cell viability determined by the ATP assay (**Fig. 4.8**), which indicates the most prominent drop for CGL 3. This obvious discrepancy could be explained by other forms of cell death like

necrosis. Indeed, hypoxic cell death can result both in apoptosis and necrosis, dependent on the cellular ATP content (Shimizu *et al.*, 1996). Execution of apoptosis requires sufficient amounts of ATP for chromatin laddering, membrane blebbing and nuclear condensation. Therefore it is normally induced, when ATP levels are within a certain range (Smets *et al.*, 1994). However, when apoptosis is inhibited or in case of severe ATP depletion, necrosis occurs. As mentioned above, CGL 3 cells display a more prominent drop of ATP compared to HeLa while there is only a minor increase of apoptosis. According to the ATP dependent cell death model, a sub-population of CGL 3 might retain sufficient amounts of ATP to undergo apoptosis, while the other ones blunted from ATP die of necrosis. This may explain why there is only a small increase of apoptotic cells under hypoxia (**Fig. 4.9**) despite the most prominent drop of cell viability (**Fig. 4.8**). Interestingly, Steinbach and colleagues report hypoxia-induced necrosis in an apoptosis competent cell model which implicates that there exist anti-apoptotic mechanisms preventing cell death as long as mitochondrial integrity and energy homeostasis are maintained and may describe the situation in CGL 3 (Nafz *et al.*, 2007). Indeed, necrosis is the predominant cell death within poorly vascularized tumor areas (al-Nafussi and Hughes, 1994).

However, the question remains why cancer cells are more susceptible to the energy stress of chronic hypoxia. In untransformed cells, an increased AMP/ATP ratio, indicating a low energy state, causes activation of AMP-activated protein kinase (AMPK). The kinase subsequently induces downstream pathways like mTOR required to restore energy homeostasis. Essential for activation of AMPK is its phosphorylation by the upstream kinase LKB1 which is not expressed in the tumorigenic cells HeLa and CGL 3 (Shaw *et al.*, 2004; Nafz *et al.*, 2007). Loss of LKB 1 causes aberrant elevated mTOR signalling which may promote tumorigenesis by facilitating cell proliferation independent of cellular energy levels. In contrast to HeLa and CGL 3, the non-tumorigenic 444 express LKB 1. Under chronic hypoxia they are therefore able to adapt to the falling energy levels which ensures cellular survival. HeLa and CGL 3 do not sense these alterations, driving them into metabolic catastrophe and cell death. Interestingly, oxygen deprivation itself is able to induce AMPK which is, however, not mediated by LKB1. Rather, CAMKK2, another

upstream kinase of AMPK, seems to facilitate the oxygen dependent activation (Høyer-Hansen and Jäätelä, 2007). Notably, differences in the CAMKK2 function between 444, CGL 3 and HeLa remain elusive.

Other explanations may apply for the mainly apoptotic cell death observed in HeLa (**Fig. 4.9**). Their apoptosis is probably triggered by other mechanisms than ATP depletion. Alternative causes would be accumulating cell damage due to reactive oxygen species, which are excessively released from the mitochondria upon hypoxia. Indeed, hypoxia triggers an increased ROS production in 444 and CGL 3 (**Fig. 4.10 lower line**). In HeLa, however, ROS levels of the viable cells remain unchanged. Thus, it is possible that HeLa is not able to tolerate an hypoxic elevation of ROS which correlates with the observed increase of apoptosis under hypoxia (**Fig. 4.9**). Furthermore, excessive ROS levels are known inducers of apoptosis in HeLa cells (Singh *et al.*, 2007). In turn, the elevated ROS levels displayed in viable CGL 3 contradict a causative role of oxidants in their sensitivity to chronic hypoxia (**Fig. 4.10 lower line**). The underlying mechanisms for the different tolerance of oxygen radicals in CGL 3 and HeLa remain elusive. Several explanations are possible: Minor differences in their p53 levels may be causative as low p53 levels sensitize cells for ROS induced DNA damage (Kim *et al.*, 1997). Moreover, the quality of their DNA repair (Bindra *et al.*, 2007), the regulation of anti-oxidant enzymes (Pelicano *et al.*, 2004) and the tolerance of misfolded proteins due to oxidative damage (Wouters and Koritzinsky, 2008) are further aspects influencing the susceptibility to ROS.

Taken together, the survival of chronic hypoxia differs in non-tumorigenic and tumorigenic cells. While 444 and IMR 90 display only a minor drop of cell viability and no significant increase in apoptosis upon hypoxia, the tumorigenic counterparts CGL 3 and HeLa seem to be severely impaired. However, they themselves show a different biological outcome: while HeLa massively undergo apoptosis, CGL 3 may turn into necrosis. As the cell death observed upon chronic hypoxia is probably caused by ATP depletion or excessive ROS levels rather than prolonged oxygen deprivation *per se*, these findings do not contradict the hallmark that cancer cells have acquired an increased resistance to hypoxic pressure (Graeber *et al.*, 1996).

Notably, after 24 h *in vitro* hypoxia, a subpopulation of CGL 3 and HeLa was still viable in this study. Thus, repeated cycles of chronic hypoxia could promote the selection of a hyper-resistant and more aggressive cancer phenotype.

### 5.2.2 The role of GAPDH in chronic hypoxia

Taken together, the results of the present study indicate a reduced survival of the tumorigenic cell lines CGL 3 and HeLa under *in vitro* long-term hypoxia correlating with the absence of the hypoxic upregulation of GAPDH by Net and HIF-2 $\alpha$ . In contrast, the hypoxic GAPDH response in the non-tumorigenic 444 and IMR 90 is accompanied by a non-impaired cell viability. Considering the role of GAPDH as a trigger in cell death and survival, it is tempting to speculate about its crucial functions under chronic hypoxia.

The hypoxic upregulation of GAPDH in the non-tumorigenic cells may certainly be required for the maintenance of the energy homeostasis as hypoxic upregulation of glycolytic enzymes is a well-described phenomenon and renders cells independent of the oxygen supply (Semenza *et al.*, 1994). In addition, GAPDH can itself regulate metabolism. Recently, the enzyme was shown to inactivate the mTOR pathway under low-glucose conditions which might be important for the adaption to chronic hypoxia as well (Lee *et al.*, 2009). Considering the hypoxic elevation of ROS levels observed in 444, GAPDH could be oxidized at its catalytic site, leading to a partial block of glycolysis and a subsequent rerouting of the glycolytic intermediates into the pentose phosphate pathway which fuels the antioxidant response (Ralsler *et al.*, 2007). Moreover, a translocation of GAPDH to the nucleus could be required to stabilize mediators of the DNA damage response and thus prevent genomic instability (Azam *et al.*, 2008). According to this versatile role, it is evident why GAPDH upregulation may be beneficial to survive chronic hypoxia: energy depletion, massive ROS accumulation and DNA damage are well-known triggers for cell death.

In contrast, the tumorigenic cell line CGL 3 and HeLa display an altered GAPDH response to chronic hypoxia. The data of the GAPDH-luciferase reporter assays suggest that CGL 3 and HeLa are not able to upregulate GAPDH under hypoxia at all (**Fig. 4.1** and **Fig. 4.5**). This inability may render CGL 3 unable to increase glycolysis upon hypoxia and could thus be causative for the ATP depletion observed in CGL 3

(**Fig. 4.8**). As cancer cells, HeLa and CGL 3 are optimized for proliferation even under harsh environmental conditions, when nutrients and oxygen are scarce. Indeed, the ability of cancer cells to sense declining ATP levels is often impaired (Nafz *et al.*, 2007; Shaw *et al.*, 2004). As mentioned above, GAPDH itself is able to trigger shut-down of the mTOR pathway. Under conditions of transient energy depletion, maintaining the mTOR signaling might provide a growth advantage. Considering, that the mTOR inhibition and the glycolytic function of GAPDH are exclusive, keeping GAPDH in glycolysis may promote cell growth under conditions of limited energy (Lee *et al.*, 2009).

Taken together, hypoxic GAPDH upregulation is a normal part of the hypoxic response in the non-tumorigenic cell lines and ensures survival under these conditions. On the other hand, the lack of the GAPDH response to hypoxia may contribute to the sensitivity of tumorigenic cell lines to *in vitro* chronic hypoxia. However, it cannot be excluded, that the maintenance of basal GAPDH levels in the cytosol may serve as a growth advantage under transient oxygen deprivation.

### 5.3 Hypoxic regulation of HPV18

Hypoxia is a poor prognostic factor for the therapy of cervical cancer patients which underlines the necessity to understand the contributing molecular mechanisms (Höckel *et al.*, 1993). In the case of cervical carcinomas, this includes knowledge about the hypoxic regulation of HPV expression, as the viral oncoproteins are causative for these tumors. Hypoxic incubation for 24 h resulted in a complete silencing of the viral early genes in non-tumorigenic and tumorigenic HPV-positive cells (**Fig. 4.11**). Assuming a transcriptional mechanism for HPV down-regulation, the impact of its main transcriptional regulator AP-1 was studied. However, the composition of the AP-1 complexes did not change which is in agreement with the expression of the cell line specific subunits. Rather the overall amount of AP-1 complexes was altered (**Fig. 4.12**). In conclusion, AP-1 does not seem to mediate the hypoxic response of HPV 18, raising the question for other factors which might be involved.

A potent inhibitor of HPV transcription under these circumstances could be NFκB. Binding of NFκB to the viral URR was shown to block HPV 16 early gene

transcription (Fontaine *et al.* 2000). However it is not clear whether NFκB exerts a similar effect on HPV 18 as some aspects of the transcriptional regulation of HPV 16 do not apply for HPV 18. To date it is unclear what determines the repressive properties of NFκB in the case of HPV as NFκB is mainly reported to act as a *trans*-activator and both ROS and hypoxia are potent activators of NFκB (Taylor and Cummins, 2009).

Energy depletion, caused by treatment with 2-deoxyglucose or low glucose conditions was previously reported to cause a down-regulation of HPV mediated by different alterations of the *trans*-activator Sp-1 (Maehama *et al.*, 1998; Kang *et al.*, 2003). Indeed, Kang and colleagues show absence of Sp-1 binding at the HPV 18 URR upon hypoxia. However, it is still unclear, whether absence of Sp-1 is the primary cause for the efficient down-regulation of HPV expression or whether other transcription factors participate in this action. It is possible, that hypoxia induces DNA binding of a potent transcriptional repressor of the viral URR which blocks the GC box (the Sp-1 DNA motif). Similar mechanisms lead to the inhibition of HPV transcription by its viral inhibitor E2 (Tan *et al.*, 1994).

Hypoxic silencing of HPV seems to be a general effect irrespective of tumorigenicity. Hypoxic loss of the viral oncoproteins might lead to a restabilization of pRB and p53. Indeed, hypoxia was shown to cause an accumulation of p53 in E6-positive keratinocytes (Kim *et al.*, 1997). p53 may subsequently facilitate the DNA damage response triggered by hypoxic ROS. However, stabilization of p53 upon oxygen deprivation increases the cellular susceptibility to apoptosis. Hypoxic loss of viral E7 expression allows reactivation of pRB which in turn attenuates cell cycle progression upon hypoxia. Considering that cell proliferation requires stable energy supply and abolishes fixation of hypoxic DNA damage, reactivation of pRB may prevent cell death. However, E6/E7 expressing keratinocytes display an increased sensitivity to hypoxia which implies the necessity for further transforming events during cervical carcinogenesis (Kim *et al.*, 1997). Indeed, the anti-apoptotic factor Bcl-2 is over-expressed in advanced cervical lesions (Saegusa *et al.*, 1996) and mediates cellular resistance to hypoxia (Liang *et al.*, 1995).

Taken together, it is unclear which transcription factors are involved in the hypoxic silencing of HPV early gene expression. Yet, different compositions of the AP-1

complex do not seem to play a role. Sp-1 and NF $\kappa$ B are possible candidates which could be involved. Further research will be required to clarify their impact on hypoxic HPV silencing and how these events influence the hypoxic phenotype of cervical tumors.

## 5.4 Conclusion

GAPDH was suggested to act as a trigger between cell death and survival and its deregulation was proposed to play an important role during tumorigenesis. Indeed, this study depicts a deregulation of both basal and hypoxic GAPDH transcription in tumorigenic CGL 3 and HeLa. In non-tumorigenic cells, the hypoxic increase of GAPDH expression was synergistically mediated by Net and HIF-2 $\alpha$  which correlates with their increased hypoxic binding at the GAPDH promoter. Either a modified post-translational regulation of Net and HIF-2 $\alpha$  or the requirement of a third factor which is deregulated in cancers might disable the synergistic activation of GAPDH in tumorigenic cells. Future studies will be required to clarify this issue.

The differential transcriptional regulation of GAPDH under chronic hypoxia in non-tumorigenic and tumorigenic cells may have an impact on their survival under these conditions. Hypoxic upregulation of GAPDH in non-tumorigenic cells correlates with a better cell viability. In turn, the tumorigenic cell lines HeLa and CGL 3 which do not increase their GAPDH expression, display a highly impaired cell viability upon hypoxia. However, high levels of apoptosis are exclusively observed in HeLa while CGL 3 depict only a small increase of apoptotic cells upon hypoxia despite poor cell viability. Thus, other forms of cell death like necrosis may be involved. Overall, the data supports a general sensitivity of cervical cancer cells to energy depleting conditions. The fact that nutrient depletion might contribute to the observed sensitivity of tumorigenic cells to chronic hypoxia resolves the apparent contradiction to the hallmark that cancer cells are more resistant to oxygen deprivation than non-tumorigenic cells. In the studied cell lines, GAPDH might therefore act as a sensor of oxygen and nutrient depletion which maintains energy homeostasis by its glycolytic properties, by counteracting cell proliferation and by promoting energy preserving mechanisms like autophagy.

Another determinant of cellular survival are the viral oncogenes E6 and E7. Their

expression is silenced upon hypoxia in all tested HPV-positive cell lines irrespective of their tumorigenicity. Although AP-1 could be excluded as causative for these events, other transcription factors like NFκB and Sp-1 might be involved. How the hypoxic loss of viral oncogene expression influences hypoxic resistance of cervical cancer requires further investigation.

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## 6. Literature

Aguilera A, Gómez-González B. (2008) Genome instability: a mechanistic view of its causes and consequences. *Nat Rev Genet.* 9(3):204-17.

al-Nafussi AI, Hughes DE. (1994) Histological features of CIN3 and their value in predicting invasive microinvasive squamous carcinoma. *J Clin Pathol.* 47(9):799-804.

Alqawi O, Wang HP, Espiritu M, Singh G. (2007) Chronic hypoxia promotes an aggressive phenotype in rat prostate cancer cells. *Free Radic Res.* 41(7):788-97.

Ameri K, Luong R, Zhang H, Powell AA, Montgomery KD, Espinosa I, Bouley DM, Harris AL, Jeffrey SS. (2010) Circulating tumour cells demonstrate an altered response to hypoxia and an aggressive phenotype. *Br J Cancer.* 102(3):561-9.

Appelhoff RJ, Tian YM, Raval RR, Turley H, Harris AL, Pugh CW, Ratcliffe PJ, Gleadow JM. (2004) Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J Biol Chem.* 279(37):38458-65.

Aprelikova O, Wood M, Tackett S, Chandramouli GV, Barrett JC. (2006) Role of ETS transcription factors in the hypoxia-inducible factor-2 target gene selection. *Cancer Res.* 66(11):5641-7.

Ayadi A, Zheng H, Sobieszczuk P, Buchwalter G, Moerman P, Alitalo K, Wasylyk B. (2001) Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1. *EMBO J.* 20(18):5139-52.

Azam S, Jouvét N, Jilani A, Vongsamphanh R, Yang X, Yang S, Ramotar D. (2008) Human glyceraldehyde-3-phosphate dehydrogenase plays a direct role in reactivating oxidized forms of the DNA repair enzyme APE1. *J Biol Chem.* 283(45):30632-41.

Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, Vousden KH. (2006) TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell.* 126(1):107-20.

Bernard HU, Burk RD, Chen Z, van Doorslaer K, Hausen H, de Villiers EM. (2010) Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology.* 401(1):70-9.

Bertout JA, Patel SA, Simon MC. (2008) The impact of O<sub>2</sub> availability on human cancer. *Nat Rev Cancer.* 8(12):967-75.

Bindra RS, Crosby ME, Glazer PM. (2007) Regulation of DNA repair in hypoxic cancer cells. *Cancer Metastasis Rev.* 26(2):249-60.

Bodily JM, Meyers C. (2005) Genetic analysis of the human papillomavirus type 31 differentiation-dependent late promoter. *J Virol.* 79(6):3309-21.

- Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H. (1984) A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J.* 3(5):1151-7.
- Bouallaga I, Massicard S, Yaniv M, Thierry F. (2000) An enhanceosome containing the Jun B/Fra-2 heterodimer and the HMG-I(Y) architectural protein controls HPV 18 transcription. *EMBO Rep.* 1(5):422-7.
- Bouvard V, Storey A, Pim D, Banks L. (1994) Characterization of the human papillomavirus E2 protein: evidence of trans-activation and trans-repression in cervical keratinocytes. *EMBO J.* 13(22):5451-9.
- Boyer SN, Wazer DE, Band V. (1996) E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res.* 56(20):4620-4.
- Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976 72:248-54.
- Bristow RG, Hill RP. (2008) Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. *Nat Rev Cancer.* 8(3):180-92.
- Brown JM. (1979) Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. *Br J Radiol.* 52(620):650-6.
- Bucana C, Saiki I, Nayar R. (1986) Uptake and accumulation of the vital dye hydroethidine in neoplastic cells. *J Histochem Cytochem.* 34(9):1109-15.
- Buchwalter G, Gross C, Wasyluk B. (2005) The ternary complex factor Net regulates cell migration through inhibition of PAI-1 expression. *Mol Cell Biol.* 25(24):10853-62.
- Butz K, Hoppe-Seyler F. (1993) Transcriptional control of human papillomavirus (HPV) oncogene expression: composition of the HPV type 18 upstream regulatory region. *J Virol.* 67(11):6476-86.
- Cairns RA, Kalliomaki T, Hill RP. (2001) Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. *Cancer Res.* 61(24):8903-8.
- Carter WO, Narayanan PK, Robinson JP. (1994) Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leukoc Biol.* 55(2):253-8.
- Cesari F, Brecht S, Vintersten K, Vuong LG, Hofmann M, Klingel K, Schnorr JJ, Arsenian S, Schild H, Herdegen T, Wiebel FF, Nordheim A. (2004) Mice deficient for the ets transcription factor elk-1 show normal immune responses and mildly impaired neuronal gene activation. *Mol Cell Biol.* 24(1):294-305.

- 
- Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc Natl Acad Sci U S A*. 95(20):11715-20.
- Chen YH, Huang LH, Chen TM. (1996) Differential effects of progestins and estrogens on long control regions of human papillomavirus types 16 and 18. *Biochem Biophys Res Commun*. 224(3):651-9.
- Chen YH, Layne MD, Chung SW, Ejima K, Baron RM, Yet SF, Perrella MA. (2003) Elk-3 is a transcriptional repressor of nitric-oxide synthase 2. *J Biol Chem*. 278(41):39572-7.
- Chen W, Li F, Mead L, White H, Walker J, Ingram DA, Roman A. (2007) Human papillomavirus causes an angiogenic switch in keratinocytes which is sufficient to alter endothelial cell behavior. *Virology*. 367(1):168-74.
- Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, Fleming MD, Schreiber SL, Cantley LC. (2008) The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature*. 452(7184):230-3.
- Chung SW, Chen YH, Yet SF, Layne MD, Perrella MA. (2006) Endotoxin-induced down-regulation of Elk-3 facilitates heme oxygenase-1 induction in macrophages. *J Immunol*. 176(4):2414-20.
- Colell A, Ricci JE, Tait S, Milasta S, Maurer U, Bouchier-Hayes L, Fitzgerald P, Guio-Carrion A, Waterhouse NJ, Li CW, Mari B, Barbry P, Newmeyer DD, Beere HM, Green DR (2007) GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. *Cell*. 129(5):983-97.
- Colell A, Green DR, Ricci JE. (2009) Novel roles for GAPDH in cell death and carcinogenesis. *Cell Death Differ*. 16(12):1573-81.
- Costello P, Nicolas R, Willoughby J, Wasylyk B, Nordheim A, Treisman R. (2010) Ternary Complex Factors SAP-1 and Elk-1, but Not Net, Are Functionally Equivalent in Thymocyte Development. *J Immunol*. 185(2):1082-92.
- Covello KL, Kehler J, Yu H, Gordan JD, Arsham AM, Hu CJ, Labosky PA, Simon MC, Keith B. (2006) HIF-2 $\alpha$  regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes Dev*. 20(5):557-70.
- Dalton S, Treisman R. (1992) Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. *Cell*. 68(3):597-612.
- Dastoor Z, Dreyer JL. (2001) Potential role of nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase in apoptosis and oxidative stress. *J Cell Sci*. 114(Pt 9):1643-53.
-

- 
- Day PM, Lowy DR, Schiller JT. (2003) Papillomaviruses infect cells via a clathrin-dependent pathway. *Virology*. 307(1):1-11.
- Day PM, Baker CC, Lowy DR, Schiller JT. (2004) Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. *Proc Natl Acad Sci U S A*. 101(39):14252-7.
- Dean PN, Jett JH. (1974) Mathematical analysis of DNA distributions derived from flow microfluorometry. *J Cell Biol*. 60(2):523-7.
- De Castro Arce J, Soto U, van Riggelen J, Schwarz E, Hausen HZ, Rösl F. (2004) Ectopic expression of nonliganded retinoic acid receptor beta abrogates AP-1 activity by selective degradation of c-Jun in cervical carcinoma cells. *J Biol Chem*. 279(44):45408-16.
- de Wilde J, De-Castro Arce J, Snijders PJ, Meijer CJ, Rösl F, Steenbergen RD. (2008) Alterations in AP-1 and AP-1 regulatory genes during HPV-induced carcinogenesis. *Cell Oncol*. 30(1):77-87.
- Del Vecchio AM, Romanczuk H, Howley PM, Baker CC. (1992) Transient replication of human papillomavirus DNAs. *J Virol*. 66(10):5949-58.
- Doorbar J, Ely S, Sterling J, McLean C, Crawford L. (1991) Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature*. 352(6338):824-7.
- Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, Acker T, Rauter M, Plate K, Sieweke M, Breier G, Flamme I. (2003) Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2 $\alpha$ ) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *J Biol Chem*. 278(9):7520-30.
- Epner DE, Partin AW, Schalken JA, Isaacs JT, Coffey DS. (1993) Association of glyceraldehyde-3-phosphate dehydrogenase expression with cell motility and metastatic potential of rat prostatic adenocarcinoma. *Cancer Res*. 53(9):1995-7.
- Evander M, Frazer IH, Payne E, Qi YM, Hengst K, McMillan NA. (1997) Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. *J Virol*. 71(3):2449-56.
- Fontaine V, van der Meijden E, de Graaf J, ter Schegget J, Struyk L. (2000) A functional NF-kappaB binding site in the human papillomavirus type 16 long control region. *Virology*. 272(1):40-9.
- Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*. 16(9):4604-13.

- 
- Gallagher S, Winston SE, Fuller SA and Hurrell JGR. (1997) Immunoblotting and Immunodetection In: *Current Protocols in Molecular Biology* (Ed.) Asube F, Brendt R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K., JohnWiley & Sons, Inc. 10.8.1-10.8.21.
- Garcia-Carranca A, Thierry F, Yaniv M. (1988) Interplay of viral and cellular proteins along the long control region of human papillomavirus type 18. *J Virol.* 62(11):4321-30.
- Gilbert DM, Cohen SN. (1987) Bovine papilloma virus plasmids replicate randomly in mouse fibroblasts throughout S phase of the cell cycle. *Cell.* 50(1):59-68.
- Giovane A, Pintzas A, Maira SM, Sobieszczuk P, Wasyluk B. (1994) Net, a new ets transcription factor that is activated by Ras. *Genes Dev.* 8(13):1502-13.
- Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature.* 379(6560):88-91.
- Graham FL, van der Eb AJ. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52(2):456-67.
- Graham FL, Smiley J, Russell WC, Nairn R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol.* 36(1):59-74.
- Graven KK, McDonald RJ, Farber HW. (1998) Hypoxic regulation of endothelial glyceraldehyde-3-phosphate dehydrogenase. *Am J Physiol.* 274(2 Pt 1):C347-55.
- Graven KK, Yu Q, Pan D, Roncarati JS, Farber HW. (1999) Identification of an oxygen responsive enhancer element in the glyceraldehyde-3-phosphate dehydrogenase gene. *Biochim Biophys Acta.* 1447(2-3):208-18.
- Graven KK, Bellur D, Klahn BD, Lowrey SL, Amberger E. (2003) HIF-2alpha regulates glyceraldehyde-3-phosphate dehydrogenase expression in endothelial cells. *Biochim Biophys Acta.* 1626(1-3):10-8.
- Graves BJ, Petersen JM. (1998) Specificity within the ets family of transcription factors. *Adv Cancer Res.* 1998;75:1-55.
- Grinstein E, Wernet P, Snijders PJ, Rösl F, Weinert I, Jia W, Kraft R, Schewe C, Schwabe M, Hauptmann S, Dietel M, Meijer CJ, Royer HD. (2002) Nucleolin as activator of human papillomavirus type 18 oncogene transcription in cervical cancer. *J Exp Med.* 196(8):1067-78.
- Gross C, Buchwalter G, Dubois-Pot H, Cler E, Zheng H, Wasyluk B. (2007) The ternary complex factor net is downregulated by hypoxia and regulates hypoxia-responsive genes. *Mol Cell Biol.* 27(11):4133-41.
-

---

Gross C, Dubois-Pot H, Wasylyk B. (2008) The ternary complex factor Net/Elk-3 participates in the transcriptional response to hypoxia and regulates HIF-1 alpha. *Oncogene*. 27(9):1333-41.

Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, Simon MC, Hammerling U, Schumacker PT. (2005) Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell Metab*. 1(6):401-8.

Hames BD, Rickwood D. (1990) Gel electrophoresis of proteins: A practical approach. *Oxford Univ. Press*.

Hanahan D, Weinberg RA. (2000) The hallmarks of cancer. *Cell*. 2000 Jan 7;100(1):57-70.

Hara MR, Agrawal N, Kim SF, Cascio MB, Fujimuro M, Ozeki Y, Takahashi M, Cheah JH, Tankou SK, Hester LD, Ferris CD, Hayward SD, Snyder SH, Sawa A. (2005) S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nat Cell Biol*. 7(7):665-74.

Hara S, Hamada J, Kobayashi C, Kondo Y, Imura N. (2001) Expression and characterization of hypoxia-inducible factor (HIF)-3alpha in human kidney: suppression of HIF-mediated gene expression by HIF-3alpha. *Biochem Biophys Res Commun*. 287(4):808-13.

Helmlinger G, Yuan F, Dellian M, Jain RK. (1997) Interstitial pH and pO<sub>2</sub> gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. *Nat Med*. 3(2):177-82.

Hitschler A (2007) Use of Chromatin Immunoprecipitation to target transcription factor controlled promoters. *Diploma thesis*. Bielefeld University.

Höckel M, Vorndran B, Schlenger K, Baussmann E, Knapstein PG. (1993) Tumor oxygenation: a new predictive parameter in locally advanced cancer of the uterine cervix. *Gynecol Oncol*. 51(2):141-9.

Hollenhorst PC, Shah AA, Hopkins C, Graves BJ. (2007) Genome-wide analyses reveal properties of redundant and specific promoter occupancy within the ETS gene family. *Genes Dev*. 21(15):1882-94.

Hollenhorst PC, Chandler KJ, Poulsen RL, Johnson WE, Speck NA, Graves BJ. (2009) DNA specificity determinants associate with distinct transcription factor functions. *PLoS Genet*. 2009 Dec;5(12):1-12.

Holmquist-Mengelbier L, Fredlund E, Löfstedt T, Noguera R, Navarro S, Nilsson H, Pietras A, Vallon-Christersson J, Borg A, Gradin K, Poellinger L, Pahlman S. (2006) Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. *Cancer Cell*. 10(5):413-23.

- 
- Høyer-Hansen M, Jäättelä M. (2007) Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ.* 14(9):1576-82.
- Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC. (2003) Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol.* 23(24):9361-74.
- Hu CJ, Sataur A, Wang L, Chen H, Simon MC. (2007) The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. *Mol Biol Cell.* 18(11):4528-42.
- Huitorel P, Pantaloni D. (1985) Bundling of microtubules by glyceraldehyde-3-phosphate dehydrogenase and its modulation by ATP. *Eur J Biochem.* 150(2):265-9.
- Hur MW, Edenberg HJ. (1992) Cloning and characterization of the ADH5 gene encoding human alcohol dehydrogenase 5, formaldehyde dehydrogenase. *Gene.* 121(2):305-11.
- Hussein D, Estlin EJ, Dive C, Makin GW. (2006) Chronic hypoxia promotes hypoxia-inducible factor-1alpha-dependent resistance to etoposide and vincristine in neuroblastoma cells. *Mol Cancer Ther.* 5(9):2241-50.
- Hwang NR, Yim SH, Kim YM, Jeong J, Song EJ, Lee Y, Lee JH, Choi S, Lee KJ. (2009) Oxidative modifications of glyceraldehyde-3-phosphate dehydrogenase play a key role in its multiple cellular functions. *Biochem J.* 423(2):253-64.
- Imamura T, Kikuchi H, Herraiz MT, Park DY, Mizukami Y, Mino-Kenduson M, Lynch MP, Rueda BR, Benita Y, Xavier RJ, Chung DC. (2009) HIF-1alpha and HIF-2alpha have divergent roles in colon cancer. *Int J Cancer.* 124(4):763-71.
- Ishiji T, Lace MJ, Parkkinen S, Anderson RD, Haugen TH, Cripe TP, Xiao JH, Davidson I, Chambon P, Turek LP. (1992) Transcriptional enhancer factor (TEF)-1 and its cell-specific co-activator activate human papillomavirus-16 E6 and E7 oncogene transcription in keratinocytes and cervical carcinoma cells. *EMBO J.* 11(6):2271-81.
- Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim Av, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science.* 292(5516):468-72.
- Kang HT, Ju JW, Cho JW, Hwang ES. (2003) Down-regulation of Sp1 activity through modulation of O-glycosylation by treatment with a low glucose mimetic, 2-deoxyglucose. *J Biol Chem.* 278(51):51223-31.

Kappel A, Röncke V, Damert A, Flamme I, Risau W, Breier G. (1999) Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice. *Blood*. 93(12):4284-92.

Kim CY, Tsai MH, Osmanian C, Graeber TG, Lee JE, Giffard RG, DiPaolo JA, Peehl DM, Giaccia AJ. (1997) Selection of human cervical epithelial cells that possess reduced apoptotic potential to low-oxygen conditions. *Cancer Res*. 57(19):4200-4.

Kim JW, Kim SJ, Han SM, Paik SY, Hur SY, Kim YW, Lee JM, Namkoong SE. (1998) Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in human cervical cancers. *Gynecol Oncol*. 71(2):266-9.

Klotz LO, Pellieux C, Briviba K, Pierlot C, Aubry JM, Sies H. (1999) Mitogen-activated protein kinase (p38-, JNK-, ERK-) activation pattern induced by extracellular and intracellular singlet oxygen and UVA. *Eur J Biochem*. 260(3):917-22.

Kodama J, Seki N, Tokumo K, Hongo A, Miyagi Y, Yoshinouchi M, Okuda H, Kudo T. (1999) Vascular endothelial growth factor is implicated in early invasion in cervical cancer. *Eur J Cancer*. 35(3):485-9.

Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227(5259):680-5.

Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev*. 16(12):1466-71.

Lavallard VJ, Pradelli LA, Paul A, Bénétteau M, Jacquelin A, Auberger P, Ricci JE. (2009) Modulation of caspase-independent cell death leads to resensitization of imatinib mesylate-resistant cells. *Cancer Res*. 69(7):3013-20.

Lee MN, Ha SH, Kim J, Koh A, Lee CS, Kim JH, Jeon H, Kim DH, Suh PG, Ryu SH. (2009) Glycolytic flux signals to mTOR through glyceraldehyde-3-phosphate dehydrogenase-mediated regulation of Rheb. *Mol Cell Biol*. 29(14):3991-4001.

Lee W, Mitchell P, Tjian R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell*. 49(6):741-5

Lehninger AL., Nelson DL., Cox MM. (1993) *Principles of Biochemistry*, Worth, New York, ed. 2.

Leprince D, Gégonne A, Coll J, de Taisne C, Schneeberger A, Lagrou C, Stehelin D. (1983) A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. *Nature*. 306(5941):395-7.

- 
- Li B, Ni P, Zhu Q, Cao H, Xu H, Zhang S, Au C, Zhang Y. (2008) Growth inhibitory effect of the ternary complex factor Net on human pancreatic carcinoma cell lines. *Tohoku J Exp Med.* 216(2):139-47.
- Liang XH, Mungal S, Ayscue A, Meissner JD, Wodnicki P, Hockenbery D, Lockett S, Herman B. (1995) Bcl-2 protooncogene expression in cervical carcinoma cell lines containing inactive p53. *J Cell Biochem.* 57(3):509-21.
- Liao H, Hyman MC, Lawrence DA, Pinsky DJ. (2007) Molecular regulation of the PAI-1 gene by hypoxia: contributions of Egr-1, HIF-1alpha, and C/EBPalpha. *FASEB J.* 21(3):935-49.
- Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM. (1999) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci U S A.* 96(20):11364-9.
- Lu S, Gu X, Hoestje S, Epner DE. (2002) Identification of an additional hypoxia responsive element in the glyceraldehyde-3-phosphate dehydrogenase gene promoter. *Biochim Biophys Acta.* 1574(2):152-6.
- Lucerna M, Pomyje J, Mechtcheriakova D, Kadl A, Gruber F, Bilban M, Sobanov Y, Schabbauer G, Breuss J, Wagner O, Bischoff M, Clauss M, Binder BR, Hofer E. (2006) Sustained expression of early growth response protein-1 blocks angiogenesis and tumor growth. *Cancer Res.* 66(13):6708-13.
- Maehama T, Patzelt A, Lengert M, Hutter KJ, Kanazawa K, Hausen H, Rösl F. (1998) Selective down-regulation of human papillomavirus transcription by 2-deoxyglucose. *Int J Cancer.* 76(5):639-46.
- Maruyama W, Akao Y, Youdim MB, Davis BA, Naoi M. (2001) Transfection-enforced Bcl-2 overexpression and an anti-Parkinson drug, rasagiline, prevent nuclear accumulation of glyceraldehyde-3-phosphate dehydrogenase induced by an endogenous dopaminergic neurotoxin, N-methyl(R)salsolinol. *J Neurochem.* 78(4):727-35.
- Milde-Langosch K. (2005) The Fos family of transcription factors and their role in tumorigenesis. *Eur J Cancer.* 41(16):2449-61.
- Muller FL, Liu Y, Van Remmen H. (2004) Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem.* 279(47):49064-73.
- Münger K, Phelps WC, Bubb V, Howley PM, Schlegel R. (1989) The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol.* 63(10):4417-21.

- 
- Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med.* 348(6):518-27.
- Murdoch C, Muthana M, Lewis CE. (2005) Hypoxia regulates macrophage functions in inflammation. *J Immunol.* 175(10):6257-63.
- Murphy MP. (2009) How mitochondria produce reactive oxygen species. *Biochem J.* 417(1):1-13.
- Nafz J, De-Castro Arce J, Fleig V, Patzelt A, Mazurek S, Rösl F. (2007) Interference with energy metabolism by 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside induces HPV suppression in cervical carcinoma cells and apoptosis in the absence of LKB1. *Biochem J.* 403(3):501-10.
- Nakamura M, Bodily JM, Beglin M, Kyo S, Inoue M, Laimins LA. (2009) Hypoxia-specific stabilization of HIF-1alpha by human papillomaviruses. *Virology.* 387(2):442-8.
- Nakayama K, Frew IJ, Hagensen M, Skals M, Habelhah H, Bhoumik A, Kadoya T, Erdjument-Bromage H, Tempst P, Frappell PB, Bowtell DD, Ronai Z. (2004) Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia. *Cell.* 117(7):941-52.
- Nasrin N, Ercolani L, Denaro M, Kong XF, Kang I, Alexander M. (1990) An insulin response element in the glyceraldehyde-3-phosphate dehydrogenase gene binds a nuclear protein induced by insulin in cultured cells and by nutritional manipulations in vivo. *Proc Natl Acad Sci U S A.* 87(14):5273-7.
- Nichols WW, Murphy DG, Cristofalo VJ, Toji LH, Greene AE, Dwight SA. (1977) Characterization of a new human diploid cell strain, IMR-90. *Science.* 196(4285):60-3.
- Nordsmark M, Alsner J, Keller J, Nielsen OS, Jensen OM, Horsman MR, Overgaard J. (2001) Hypoxia in human soft tissue sarcomas: adverse impact on survival and no association with p53 mutations. *Br J Cancer.* 84(8):1070-5.
- Nordsmark M, Bentzen SM, Rudat V, Brizel D, Lartigau E, Stadler P, Becker A, Adam M, Molls M, Dunst J, Terris DJ, Overgaard J. (2005) Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. *Radiother Oncol.* 77(1):18-24.
- O'Connor M, Bernard HU. (1995) Oct-1 activates the epithelial-specific enhancer of human papillomavirus type 16 via a synergistic interaction with NFI at a conserved composite regulatory element. *Virology.* 207(1):77-88.

---

O'Connor MJ, Tan SH, Tan CH, Bernard HU. (1996) YY1 represses human papillomavirus type 16 transcription by quenching AP-1 activity. *J Virol.* 70(10):6529-39.

Oikawa T, Yamada T. (2003) Molecular biology of the Ets family of transcription factors. *Gene.* 303:11-34.

O'Rourke JF, Tian YM, Ratcliffe PJ, Pugh CW. (1999) Oxygen-regulated and transactivating domains in endothelial PAS protein 1: comparison with hypoxia-inducible factor-1alpha. *J Biol Chem.* 274(4):2060-71.

Papp-Szabó E, Josephy PD, Coomber BL. (2005) Microenvironmental influences on mutagenesis in mammary epithelial cells. *Int J Cancer.* 116(5):679-85.

Pelicano H, Carney D, Huang P. (2004) ROS stress in cancer cells and therapeutic implications. *Drug Resist Updat.* 7(2):97-110.

Pires IM, Bencokova Z, Milani M, Folkes LK, Li JL, Stratford MR, Harris AL, Hammond EM. (2010) Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. *Cancer Res.* 70(3):925-35.

Pollak N, Dölle C, Ziegler M. (2007) The power to reduce: pyridine nucleotides - small molecules with a multitude of functions. *Biochem J.* 402(2):205-18.

Prusty BK, Das BC. (2005) Constitutive activation of transcription factor AP-1 in cervical cancer and suppression of human papillomavirus (HPV) transcription and AP-1 activity in HeLa cells by curcumin. *Int J Cancer.* 113(6):951-60.

Racker E, Krimsky I. (1952) Mechanism of action of glyceraldehyde-3-phosphate dehydrogenase. *Nature.* 169(4312):1043-5.

Rao VN, Huebner K, Isobe M, ar-Rushdi A, Croce CM, Reddy ES. (1989) elk, tissue-specific ets-related genes on chromosomes X and 14 near translocation breakpoints. *Science.* 244(4900):66-70.

Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. (2005) Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Mol Cell Biol.* 25(13):5675-86.

Révillion F, Pawlowski V, Hornez L, Peyrat JP. (2000) Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. *Eur J Cancer.* 36(8):1038-42.

Rösl F, Das BC, Lengert M, Geletneky K, zur Hausen H. (1997) Antioxidant-induced changes of the AP-1 transcription complex are paralleled by a selective suppression of human papillomavirus transcription. *J Virol.* 71(1):362-70.

- 
- Roscic A, Möller A, Calzado MA, Renner F, Wimmer VC, Gresko E, Lüdi KS, Schmitz ML. (2006) Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. *Mol Cell*. 24(1):77-89.
- Rougvie AE, Lis JT. (1990) Postinitiation transcriptional control in *Drosophila melanogaster*. *Mol Cell Biol*. 10(11):6041-5.
- Sadakata H, Okazawa H, Sato T, Supriatna Y, Ohnishi H, Kusakari S, Murata Y, Ito T, Nishiyama U, Minegishi T, Harada A, Matozaki T. (2009) SAP-1 is a microvillus-specific protein tyrosine phosphatase that modulates intestinal tumorigenesis. *Genes Cells*. 14(3):295-308.
- Saegusa M, Takano Y, Hashimura M, Shoji Y, Okayasu I. (1995) The possible role of bcl-2 expression in the progression of tumors of the uterine cervix. *Cancer*. 76(11):2297-303.
- Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*. 63(6):1129-36.
- Schreiber E, Matthias P, Müller MM, Schaffner W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res*. 17(15):6419.
- Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, zur Hausen H. (1985) Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature*. 314(6006):111-4.
- Semenza GL, Roth PH, Fang HM, Wang GL. (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem*. 269(38):23757-63.
- Sen N, Hara MR, Kornberg MD, Cascio MB, Bae BI, Shahani N, Thomas B, Dawson TM, Dawson VL, Snyder SH, Sawa A. (2008) Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. *Nat Cell Biol*. 10(7):866-73.
- Shafti-Keramat S, Handisurya A, Kriehuber E, Meneguzzi G, Slupetzky K, Kirnbauer R. (2003) Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. *J Virol*. 77(24):13125-35.
- Sharrocks AD. (2001) The ETS-domain transcription factor family. *Nat Rev Mol Cell Biol*. 2(11):827-37.
- Shaulian E. (2010) AP-1--The Jun proteins: Oncogenes or tumor suppressors in disguise? *Cell Signal*. 22(6):894-9
-

- 
- Shaw PE, Schröter H, Nordheim A. (1989) The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. *Cell*. 56(4):563-72.
- Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC. (2004) The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci U S A*. 101(10):3329-35.
- Sherman L, Jackman A, Itzhaki H, Stöppler MC, Koval D, Schlegel R. (1997) Inhibition of serum- and calcium-induced differentiation of human keratinocytes by HPV16 E6 oncoprotein: role of p53 inactivation. *Virology*. 237(2):296-306.
- Shimizu S, Eguchi Y, Kamiike W, Itoh Y, Hasegawa J, Yamabe K, Otsuki Y, Matsuda H, Tsujimoto Y. (1996) Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL. *Cancer Res*. 56(9):2161-6.
- Shweiki D, Itin A, Soffer D, Keshet E. (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*. 359(6398):843-5.
- Singh M, Sharma H, Singh N. (2007) Hydrogen peroxide induces apoptosis in HeLa cells through mitochondrial pathway. *Mitochondrion*. 7(6):367-73.
- Smets LA, Van den Berg J, Acton D, Top B, Van Rooij H, Verwijs-Janssen M. (1994) mBCL-2 expression and mitochondrial activity in leukemic cells with different sensitivity to glucocorticoid-induced apoptosis. *Blood*. 84(5):1613-9.
- Soto U, Das BC, Lengert M, Finzer P, zur Hausen H, Rosl F. (1999) Conversion of HPV18 positive non-tumorigenic HeLa-fibroblast hybrids to invasive growth involves loss of TNF-alpha mediated repression of viral transcription and modification of the AP-1 transcription complex. *Oncogene*. 18:3187-98.
- Stanbridge, E. (1984) Genetic analysis of tumorigenicity in human cell hybrids. *Cancer Surv*. 3:335-350
- Stanbridge EJ, Wilkinson J. (1980) Dissociation of anchorage independence from tumorigenicity in human cell hybrids. *Int J Cancer*. 26(1):1-8.
- Steinbach JP, Wolburg H, Klumpp A, Probst H, Weller M. (2003) Hypoxia-induced cell death in human malignant glioma cells: energy deprivation promotes decoupling of mitochondrial cytochrome c release from caspase processing and necrotic cell death. *Cell Death Differ*. 10(7):823-32.
- Stöhr M, Goertler K. (1979) The Heidelberg flow analyzer and sorter (HEIFAS) approach on the prescreening of uterine cancer. *J Histochem Cytochem*. 27(1):564-6.
-

---

Straight SW, Hinkle PM, Jewers RJ, McCance DJ. (1993) The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes. *J Virol.* 67(8):4521-32.

Sulter G, Elting JW, Stewart R, den Arend A, De Keyser J. (2000) Continuous pulse oximetry in acute hemiparetic stroke. *J Neurol Sci.* 179(S 1-2):65-9.

Sunamura M, Duda DG, Ghattas MH, Lozonschi L, Motoi F, Yamauchi J, Matsuno S, Shibahara S, Abraham NG. (2003) Heme oxygenase-1 accelerates tumor angiogenesis of human pancreatic cancer. *Angiogenesis.* 6(1):15-24.

Sundararaj KP, Wood RE, Ponnusamy S, Salas AM, Szulc Z, Bielawska A, Obeid LM, Hannun YA, Ogretmen B. (2004) Rapid shortening of telomere length in response to ceramide involves the inhibition of telomere binding activity of nuclear glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem.* 279(7):6152-62.

Tan SH, Leong LE, Walker PA, Bernard HU. (1994) The human papillomavirus type 16 E2 transcription factor binds with low cooperativity to two flanking sites and represses the E6 promoter through displacement of Sp1 and TFIID. *J Virol.* 68(10):6411-20

Tang X, Zhang Q, Nishitani J, Brown J, Shi S, Le AD. (2007) Overexpression of human papillomavirus type 16 oncoproteins enhances hypoxia-inducible factor 1 alpha protein accumulation and vascular endothelial growth factor expression in human cervical carcinoma cells. *Clin Cancer Res.* 13(9):2568-76.

Taylor CT, Cummins EP. (2009) The role of NF-kappaB in hypoxia-induced gene expression. *Ann N Y Acad Sci.* 1177:178-84.

Thierry F, Heard JM, Dartmann K, Yaniv M. (1987) Characterization of a transcriptional promoter of human papillomavirus 18 and modulation of its expression by simian virus 40 and adenovirus early antigens. *J Virol.* 61(1):134-4

Thomlinson RH, Gray LH. (1955) The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br J Cancer.* 9(4):539-49.

Toussaint-Smith E, Donner DB, Roman A. (2004) Expression of human papillomavirus type 16 E6 and E7 oncoproteins in primary foreskin keratinocytes is sufficient to alter the expression of angiogenic factors. *Oncogene.* 23(17):2988-95.

Thrash-Bingham CA, Tartof KD. (1999) aHIF: a natural antisense transcript overexpressed in human renal cancer and during hypoxia. *J Natl Cancer Inst.* 91(2):143-51.

Triantafyllou A, Liakos P, Tsakalof A, Georgatsou E, Simos G, Bonanou S. (2006) Cobalt induces hypoxia-inducible factor-1alpha (HIF-1alpha) in HeLa cells by an iron-independent, but ROS-, PI-3K- and MAPK-dependent mechanism. *Free Radic Res.* 40(8):847-56.

Turrens JF, Alexandre A, Lehninger AL. (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys.* 237(2):408-14.

Uchida T, Rossignol F, Matthay MA, Mounier R, Couette S, Clottes E, Clerici C. (2004) Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. *J Biol Chem.* 2004 Apr 9;279(15):14871-8.

van Riggelen J, Buchwalter G, Soto U, De-Castro Arce J, Hausen HZ, Wasyluk B, Rösl F. (2005) Loss of net as repressor leads to constitutive increased c-fos transcription in cervical cancer cells. *J Biol Chem.* 280(5):3286-94.

Vander Heiden MG, Cantley LC, Thompson CB. (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science.* 324(5930):1029-33.

Wang GL, Semenza GL. (1993a) Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J Biol Chem.* 268(29):21513-8.

Wang GL, Semenza GL. (1993b) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A.* 90(9):4304-8.

Wang GL, Jiang BH, Rue EA, Semenza GL. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O<sub>2</sub> tension. *Proc Natl Acad Sci U S A.* 92(12):5510-4.

Warburg O., Posener K., Negelein E.. (1925) Ueber den Stoffwechsel der Carcinomzelle; *Biochemische Zeitschrift.* 152:319-344.

Wasyluk C, Criqui-Filipe P, Wasyluk B. (2005) Sumoylation of the net inhibitory domain (NID) is stimulated by PIAS1 and has a negative effect on the transcriptional activity of Net. *Oncogene.* 24(5):820-8.

Weeks JR, Hardin SE, Shen J, Lee JM, Greenleaf AL. (2010) Locus-specific variation in phosphorylation state of RNA polymerase II in vivo: correlations with gene activity and transcript processing. *Genes Dev.* 7(12A):2329-44.

Weinmann AS, Bartley SM, Zhang T, Zhang MQ, Farnham PJ. (2001) Use of chromatin immunoprecipitation to clone novel E2F target promoters. *Mol Cell Biol.* 21(20):6820-32.

Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, Maxwell PH. (1998) Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood.* 92(7):2260-8.

Wiesener MS, Jürgensen JS, Rosenberger C, Scholze CK, Hörstrup JH, Warnecke

C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU. (2003) Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. *FASEB J.* 17(2):271-3.

Wouters BG, Koritzinsky M. (2008) Hypoxia signalling through mTOR and the unfolded protein response in cancer. *Nat Rev Cancer.* 8(11):851-64.

Yamashita K, Discher DJ, Hu J, Bishopric NH, Webster KA. (2001) Molecular regulation of the endothelin-1 gene by hypoxia. Contributions of hypoxia-inducible factor-1, activator protein-1, GATA-2, AND p300/CBP. *J Biol Chem.* 76(16):12645-53.

Yan Q, Bartz S, Mao M, Li L, Kaelin WG Jr (2007) The hypoxia-inducible factor 2alpha N-terminal and C-terminal transactivation domains cooperate to promote renal tumorigenesis in vivo. *Mol Cell Biol.* 27(6):2092-102.

Yuneva M, Zamboni N, Oefner P, Sachidanandam R, Lazebnik Y. (2007) Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J Cell Biol.* 178(1):93-105.

Zheng H, Wasylyk C, Ayadi A, Abecassis J, Schalken JA, Rogatsch H, Wernert N, Maira SM, Multon MC, Wasylyk B. (2003) The transcription factor Net regulates the angiogenic switch. *Genes Dev.* 17(18):2283-97.

Zheng L, Roeder RG, Luo Y. (2003) S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. *Cell.* 114(2):255-66.

Zhou J, Sun XY, Stenzel DJ, Frazer IH. (1991) Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology.* 185(1):251-7.

Zhou Y, Yi X, Stoffer JB, Bonafe N, Gilmore-Hebert M, McAlpine J, Chambers SK. (2008) The multifunctional protein glyceraldehyde-3-phosphate dehydrogenase is both regulated and controls colony-stimulating factor-1 messenger RNA stability in ovarian cancer. *Mol Cancer Res.* 6(8):1375-84.

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