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Role of hypoxia and hypoxia inducible factors (HIFs) in cells of Ewing's Sarcoma Family Tumors

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Abbreviations

ALDOC	Aldolase C
ARNT	aryl hydrocarbon receptor nuclear translocator
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
CAIX	carbonic anhydrase IX
CTAD	C-terminal activation domain
DFX	desferrioxamine
DNA-BD	DNA binding domain
ESFT	Ewing Sarcoma Family Tumors
ETV1	ETS variant gene 1
ETV4	ETS variant gene 4
FEV	Fifth Ewing sarcoma Variant
FIH-1	factor inhibiting hypoxia inducible factor-1
Fli1	friend leukemia integration site 1
GLUT1/3	glucose transporter 1/3
HIF	hypoxia inducible factor
HRE	hypoxia responsive element
Id2	inhibitor of DNA binding 2
IGFBP3	insulin-like growth factor binding protein-3
LZIP	leucine zipper
MMP	matrix metalloproteinase
NLS	nuclear localization signal
NTAD	N-terminal activation domain
ODD	oxygen degradation domain
PAC	PAS-associated C-terminal domain
PAS	PER/ARNT/Sim domain
PDGF	platelet-derived growth factor
PDK1	pyruvate dehydrogenase kinase 1
PHD	prolyl hydroxylase domain protein
pVHL	von Hippel Lindau tumor suppressor protein
TBP	TATA box binding protein
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor

Abstract

Ewing's Sarcoma Family Tumors (ESFT) are the second most common malignancy of children and young adults that affect mostly soft tissue and solid bone. ESFT are very aggressive and highly metastatic, and are characterized by chromosomal translocations involving the *EWSR*1 gene and several ETS transcription factor genes. The most frequently observed translocation, t(11;22)(q24;q12), results in the fusion of portions of the *EWSR1* gene with the *Fli1* (Friend leukemia integration site 1) transcription factor gene, and is observed in 80-85% of all cases.

Interestingly, many attributes of ESFT are typically associated with hypoxia in many tumor entities and linked to high metastatic potential and bad prognosis. Solid tumors have been shown to respond to hypoxia mainly via hypoxia inducible factor- 1α (HIF-1α). Since the role of hypoxia and especially the contribution of HIF-1α to the aggressiveness of ESFT remain unknown, we aimed at clarifying the role of hypoxia inducible factors in ESFT cell lines.

For that purpose, we first checked whether HIF-1α can be induced in the ESFT cell lines TC252 and SK-N-MC by using the canonical hypoxia mimetics cobalt chloride ($CoCl_2$) and Desferrioxamine (DFX). We showed that HIF-1α was induced both in a dose- and time-dependent manner. Notably, increased HIF-1α protein levels were accompanied by elevated EWS-Fli1 levels, indicating that hypoxia might regulate EWS-Fli1 which is discussed as a possible therapeutic target in ESFT. To reveal, whether this finding was HIF-1α dependent or independent, we first over-expressed either a wild-type version of HIF-1α or a mutant version, baring the P564A and N803A mutations within the oxygen degradation domain. Secondly, we performed knockdown studies using shRNA targeting HIF-1α. Both experiments revealed that EWS-Fli1 protein is regulated in a HIF-1α dependent manner, whereas EWS-Fli1 mRNA levels remained unchanged. To analyse whether hypoxia has any functional consequence on ESFT cell lines in vitro we further performed proliferation, migration and invasion assays. The latter clearly showed that hypoxia enhances the invasive capability of ESFT cell lines whereas no significant effect on proliferation and migration was observed.

Zusammenfassung

Ewing's Sarcoma Family Tumors (ESFT) sind die am zweithäufigsten auftretenden Knochen- und Weichteil-tumore bei Kindern und Jugendlichen. ESFT sind äußerst aggressive und stark metastasierende Tumore, welche durch chromosomale Translokationen zwischen dem *EWSR1* Gen sowie Genen der Familie der ETS Transkriptions-Faktoren charakterisiert sind. Die am häufigsten auftretende Translokation t(11;22)(q24;q12) umfasst die Fusion des *EWSR1* Gens mit dem *Fli1* Gen und tritt in 80-85% der Fälle auf. Interessanterweise sind viele Eigenschaften der ESFT generell typisch für hypoxische Tumoren. Dazu zählt eine erhöhte Tumor-Aggressivität, die mit hohem Metastasierungspotential und einer damit verbundenen schlechten Prognose einhergeht. Viele solide Tumore sind hypoxisch und reagieren auf diese Bedingungen hauptsächlich mit der Regulation des hypoxia inducible factors-1α (HIF-1α). Da die Rolle von Hypoxie und im Speziellen von HIF-1α in der Bösartigkeit von Ewing Sarkomen noch weitestgehend unbekannt ist, wurde die Rolle der hypoxia inducible factors in ESFT Zelllinien untersucht. Um HIF-1α zu induzieren wurden die Zelllinien TC252 und SK-N-MC sowohl mit Cobalt Chlorid als auch mit Desferrioxamin behandelt, welche anerkannter Weise den Zustand der Hypoxie imitieren. Tatsächlich konnte HIF-1α, sowohl in einer Konzentrations- als auch in einer Zeit-abhängigen Art und Weise, nachgewiesen werden. Interessanterweise, wurde nicht nur HIF-1α, sondern auch EWS-Fli1 induziert. Da EWS-Fli1 als möglicher therapeutischer Angriffspunkt in ESFT gilt, wurde im Folgenden untersucht, ob der erhöhte EWS-Fli1 Level von HIF-1α abhängig oder unabhängig ist. Hierfür wurden einerseits sowohl Wildtyp HIF-1α als auch mutiertes HIF-1α, welches eine P564A und N803A Mutation in der (*oxygen degradation domain*) trägt, über- exprimiert, andererseits wurde HIF-1α durch Verwendung von shRNA gezielt ausgeschaltet. Beide Experimente zeigten, dass EWS-Fli1 Protein in einer HIF-1α abhängigen Art und Weise reguliert wurde, die EWS-Fli1-mRNA hingegen blieb unverändert. Um festzustellen, ob Hypoxie funktionelle Konsequenzen zur Folge hat, wurde sowohl Proliferation, Migration, als auch das Invasions-Potential von ESFT Zelllinien untersucht. Tatsächlich konnte gezeigt werden, dass das Invasions-Potential durch Hypoxie erhöht wurde, während keine Unterschiede im Proliferation- und Migration- Verhalten nachweisbar waren.

1 Introduction

1.1 The role of transcription factors in regulating gene expression

Since the human genome was sequenced first in 2001 by Celera Genomics [1] and IHGSC [2] the near-complete sequence (99% accuracy) was only obtained three years later in 2004. The current estimation of protein-coding genes is in the range of 20,000-25,000 [3]. Additionally, there are many non-coding RNAs that affect a great variety of cellular processes like transcriptional regulation, mRNA stability and translation as well as RNA processing and modification [4].

The ability to express these genes under different environmental conditions, and at different time points during cell differentiation and development, reflects the great dynamics in the regulation of gene expression. Gene expression of eukaryotic protein-coding genes is a multi-step process that is mainly regulated at the level of transcription initiation as well as transcription elongation, mRNA processing, transport and translation [5]. However, it is important to emphasize that the chromatin-state decides whether the basal transcription machinery gets access to the promoter-region of a specific gene or not [6]. Transcription of eukaryotic protein-coding genes is performed by RNA Polymerase II: (1)The promoter region is composed of the core promoter, which is recognized by the general transcription factors in order to form a transcription pre-initiations complex (PIC) that directs the RNA polymerase II to the transcription start site [5] (Fig.1).

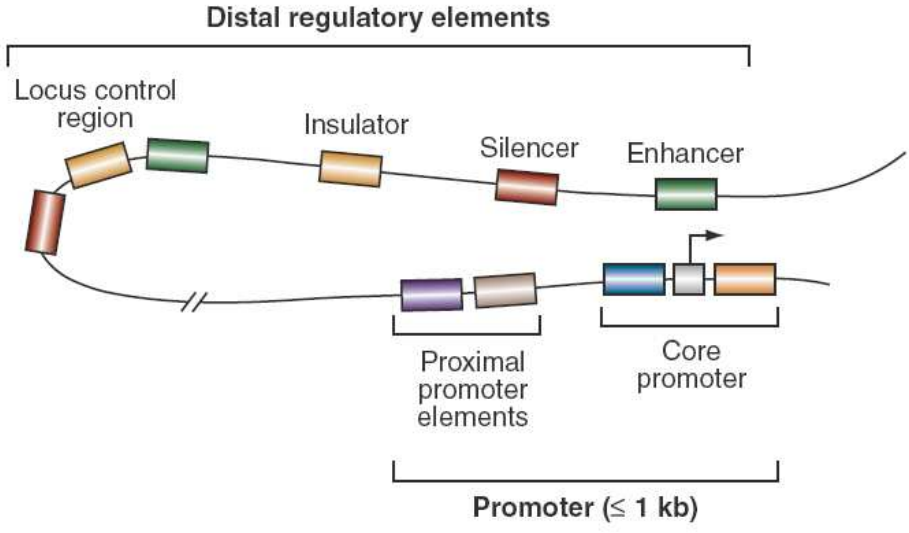

Fig. 1: Transcriptional regulatory elements [5].

Proximal promoter elements as well as the core promoter build up the promoter region which is typically ≤1kb. (2) Enhancers, silencers, insulators or locus control regions belong to the so called cis-acting distal regulatory DNA elements, which contain recognition sites for trans-acting DNA-binding transcription factors that either enhance or repress transcription [5].

Protein-coding genes that are transcribed by RNA polymerase II involve transcription factors that can be classified into three groups: general transcription factors (GTFs) that facilitate the PIC assembly, promoter-specific activator proteins (activators) consisting of a DNA-binding domain and an activation domain, as well as coactivators, e.g. TATA-box binding protein (TBP) [7].

Since the focus of my thesis is on a promoter specific transcription factor, I will concentrate mainly on its molecular and structural features as well as the principles of DNA recognition.

1.1.1 Sequence-specific DNA binding factors

Sequence-specific DNA binding proteins affect the various gene specific programmes of transcriptional control by binding to the proximal promoter and distal regulatory elements (e.g. enhancer, silencer). These factors therefore play a key role in mediating the genetic regulatory information to the transcription system. Transcription is a complex multi-step process where sequence specific factors act together with the core RNA Polymerase II transcriptional machinery as well as co-regulators, chromatin remodelling factors and enzymes that catalyze covalent modifications of histones. All sequence specific transcription factors exhibit common properties (Fig.2)[8]: (A) they consist of several modules (e.g. DNA-binding module, activation or repression module) [9], (B) chromatin is the integral component in regulating the function of sequence specific transcription factors [10, 11], (C) they typically bind the DNA in clusters in order to overcome the low binding-specificity of single factors [12] and thereby function synergistically in the activation of transcription [13].

Specific transcription factors recognize transcription factor binding sites (TFBSs) that are typically small (in the range between 6-12 bp) and described by a consensus sequence. Transcription factors, especially sequence specific activators, frequently form heterodimers and/or homodimers [5].

The special subunit composition of a transcription factor may highly influence the binding specificity and therefore alter its regulatory function [14].

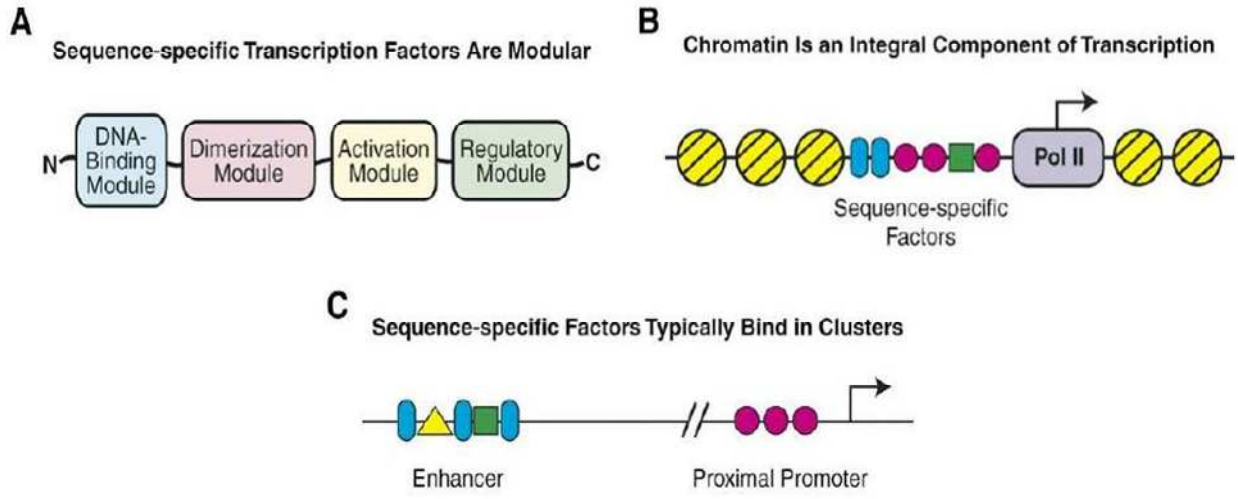

Fig. 2: Schematic representation of typically sequence specific transcription factors and their properties [8].
(A) Sequence specific factors are composed of several modules. (B) Chromatin functions as integral regulator of sequence specific transcription factors. (C) They typically bind the DNA in clusters.

When the sequence specific transcription factors (activators or repressors) bind to their consensus sequence within a proximal promoter, they mainly work by recruitment of either transcriptional coactivators or corepressors to the DNA template via direct protein-protein interactions [15].

1.2 Transcription factor families

Transcription factor families can be classified due to their structural properties and the way they recognize and bind to their specific DNA template [16].

1. *Helix-turn-Helix (HTH) structure*

The helix-turn-helix motif is a DNA-recognition motif whose crystal structure (e.g. λ Cro protein [17, 18]) has been shown to be a well conserved recognition motif consisting of an α-helix, a turn and a second α-helix [19, 20]. It is important to emphasize that HTH motifs can not fold or function by themselves thus they are always part of bigger DNA-binding domains [16].

The winged helix-turn-helix motif consists of three α-helices and four β-sheets. The third α-helix, the ‘wing’ region between β-strands 3 and 4 as well as the loop between α-helices 2 and 3 contain residues that build up the main protein-DNA contacts [21].

2. Homeodomain structure

Initially, the homeodomain structure was analysed by 2D NMR studies in Drosophila [22, 23] and further analysis revealed that the homeodomain structure contains a HTH motif as well [24]. The homeodomain is based on an extended N-terminal arm and three α-helices as well as a fourth α-helix which is composed of the C-terminal residues [22].

3. Zinc finger motif

The zinc finger family of proteins include proteins which are involved in differentiation, development (e.g. Drosophila) regulation of the basal transcription machinery as well as various regulatory functions in eukaryotic organisms [25, 26]. A typical zinc finger motif consists of a specific sequence pattern: Cys-X_{2or4}-Cys-X_{12}-His-X_{3-5}-His[16].

4. Steroid receptors

Steroid receptors are very important regulatory proteins which contain separate domains for hormone binding, DNA binding as well as transcriptional activation [27, 28]. Structural analysis of glucocorticoid and estrogen receptors revealed that each of these peptides fold into a globular domain with a pair of α-helices [29, 30].

5. Leucine Zipper motif

Leucine zipper motif was found to be a well conserved sequence pattern in different eukaryotic transcription factors and they characteristically bear a hepta repeat of leucines over a region of 30-40 residues [31]. Leucine zipper motifs tend to form two parallel α-helices in a coiled-coiled manner [32]. The ability to form heterodimers enables leucin zipper- motif carrying transcription factors (e.g. AP-1 consisting of Fos- and Jun-protein) [33] to perform different combinations of either activation or repression thus covering a great range of regulatory properties [34].

6. Helix-loop-helix motif

Helix-loop-helix transcription factors (HLH TFs), as well as leucin zipper proteins, play fundamental roles in differentiation and development [16]. HLH-proteins have a basic region that leads to DNA binding and a neighbouring region that allows heterodimerisation [35]. Heterodimerisation allows mixing of activators, negative

regulators or ubiquitously expressed proteins to modulate gene expression in this family of proteins [36].

In the following sections I will focus on bHLH- and ETS- transcription factors based on their important roles in the topic of my thesis.

1.2.1 Basic helix-loop-helix/PAS family of transcription factors

The basic helix-loop-helix transcriptional regulators function in critical fundamental biological processes, such as cell differentiation, regulation of homeostasis as well as stress response and are found in organisms from yeast to humans [37].

Basic helix-loop-helix proteins can be categorized into three main sub-families (Fig 3.): (a) transcriptional regulator proteins that contain only the bHLH dimerisation domain (b) proteins that either bear a second leucin zipper (Zip) dimerisation domain or (c) an additional PAS dimerisation domain [38].

Typical members of group (a) belong to the proteins that are involved in myogenesis (e.g. myoD) and neurogenesis [37], whereas proteins of group (b) are involved in the Myc/Max/Mad network of transcription factors [39]. Both bHLH transcription factor family members bind to specific DNA regions that contain the G (or A) CAXXTGG (or A) E-box consensus sequence [40].

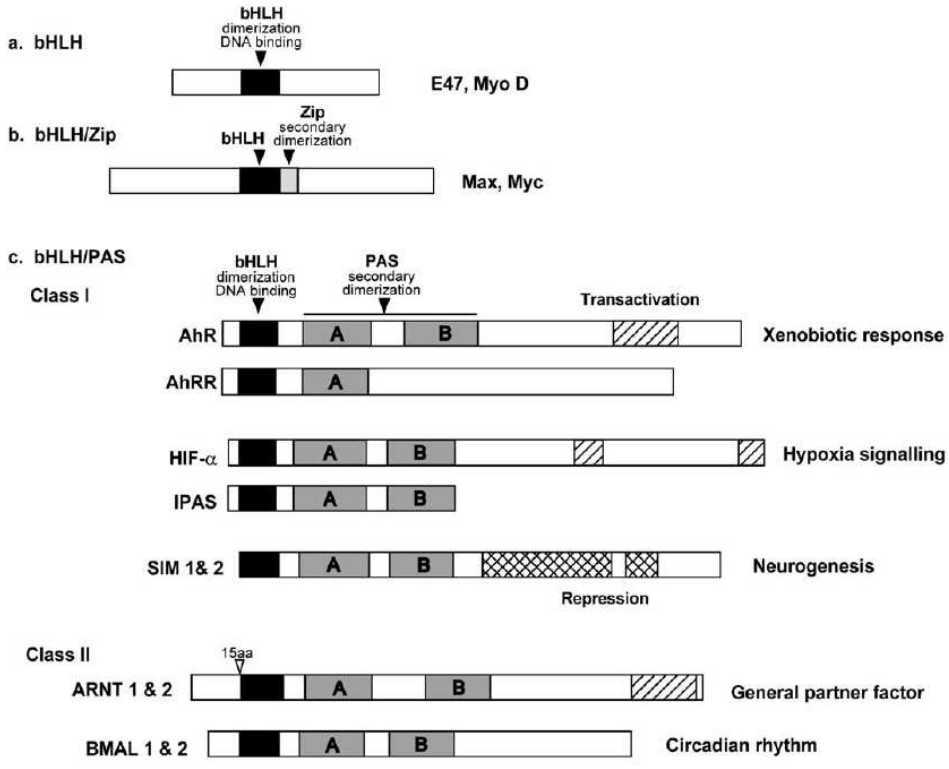

Fig. 3: Domain structure of bHLH transcription factor family members [38]

In contrast, basic helix-loop-helix/PAS proteins tend to be factors whose activity is signal-regulated and often recognize DNA sequences that diverge from the classical E-box consensus sequence [41, 42]. Since bHLH/PAS transcription factors differ in their dimerisation behaviour they have been grouped into two classes. Class I factors neither homodimerise nor heterodimerise with other class I factors (e.g. HIF-α factors) [43] but need to dimerise with class II factors (e.g. ARNT) to form functional active transcription factor complexes [44]. Once a bHLH/Pas protein dimerises with a second bHLH/PAS protein in order to form a functional transcription factor within the nucleus, the basic region of the bHLH domain binds the specific DNA consensus sequence thus interacting with the transcriptional machinery to enhance or repress expression of the target gene [45].

The dimerisation reaction is highly specific and regulated through the PAS domain, which acts as a secondary dimerisation interface whereas the N-terminal bHLH-domain functions as the primary dimerisation interface [42]. Notably, many bHLH/PAS transcription factors like the HIFα proteins, that play an essential role in oxygen sensing, or SIM proteins which control neural development, seem to be biologically essential, as revealed in knock-out studies [46-48].

1.2.2 The ETS-domain transcription factor family

ETS transcription factors (e.g. Ets1/2, Fli-1, Erg, PU.1, TEL) are characterized by their specific DNA-binding structure (ETS domain) [21]. Structural studies on the ETS-domain transcription factor Fli-1 revealed that the ETS domain belongs to the family of winged helix-turn-helix motif carrying transcription factors [49]. Further structural analysis on Ets-1 [50] , PU.1 [51], SAP-1 [52] and Elk1 [53] revealed structural conservations and showed that each of these proteins contained three α-helices and four β-sheets (Fig. 4). In addition, protein-protein interactions are mediated intramolecularly or by co-regulatory proteins [21].

Some members of the ETS-domain family can be classified by further conserved domains, such as the pointed domain (Pnt) [54] which has been shown to function in homo-oligomerization [55], heterodimerisation [56] and transcriptional repression [57].

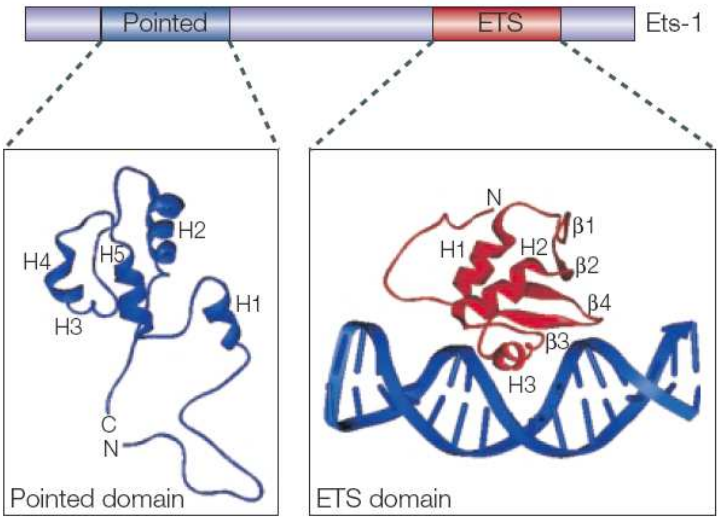

Fig. 4: Structure of an ETS-domain transcription factor (Ets-1)[21].

ETS-domain transcription factors bind to a common GGAA/T motif. This motif is sufficient for individual ETS-domain proteins, to maintain specific DNA-binding up to an 11 base-pair sequence [58].

Notably, the alteration of a single amino acid within the C-terminus of the DNA-recognition helix in the ETS domain is enough to change the DNA-binding specificity [59] and of protein-protein interactions [60]. Since most of the ETS-domain TFs are autoregulated, their DNA-binding activity is on hold till an adequate signal, such as phosphorylation or co-regulator- binding occurs [21].

1.3 Hypoxia and the HIF-system

Cells, tissues and organs need to maintain appropriate oxygen levels in order to survive and ensure a proper cellular function. Maintaining the oxygen homeostasis, therefore, is key to all oxygen-dependent processes and crucial to minimize production of reactive oxygen species (ROS) that are able to cause oxidative damage to DNA, proteins and lipids [61].

Once the oxygen levels decrease to a certain value between 5-0.5%, this state is designated as hypoxia (Fig.5). Hypoxia is known to induce certain response mechanisms such as placental and vascular development, but it also plays a causal role in ischemic-related diseases and cancer [62]. Notably, when oxygen is completely absent (anoxia), cells stop their ATP-synthesis and undergo apoptosis soon after the anoxia exposure [63, 64]. On the other hand, cells that are exposed to hypoxia convert to a more anaerobic glycolytic metabolism to sustain ATP-synthesis

and therefore escape apoptosis [65]. However, hypoxic cells undergo rapid proliferation thereby generating levels of local anoxia concomitantly. For that reason, cells must quickly respond to this oxygen stress in order to survive [62].

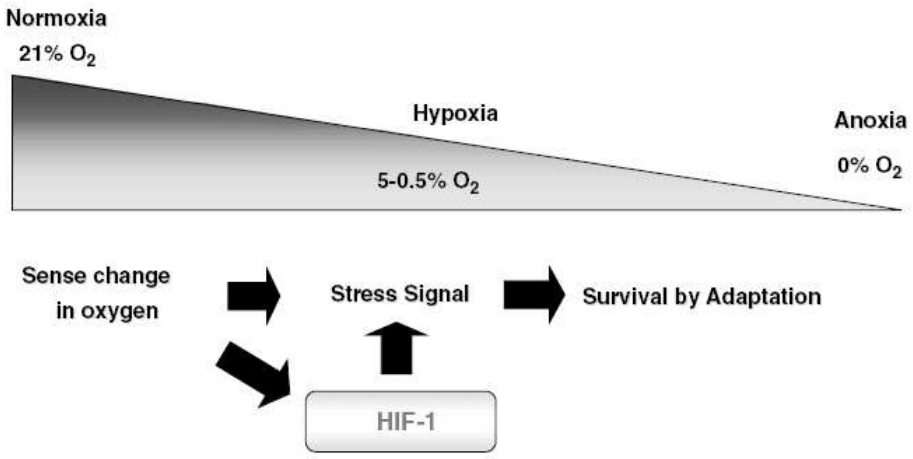

Fig. 5: Reduced oxygen levels activate response mechanisms via the HIF-system [62].

The main transcriptional regulators of this hypoxic response are named hypoxia inducible factors and were first discovered in 1992 by Semenza et.al. [66]. This broad-action transcription factors are expressed in virtually all mammalian cells of the body [66-68].

1.3.1 The HIF-family

The HIF family of transcription factors are basic helix-loop-helix/PAS domain transcription factors (Fig.6) that are composed of a heterodimeric α and β subunit [69]. This transcription factor family consists of three known HIF-α isoforms: HIF-1α, HIF-2α and HIF-3α, each consisting of various splice variants [70]. In contrast to the HIF-α proteins the HIF-1β protein, also known as ARNT (aryl hydrocarbon receptor nuclear translocator) possesses several splice variants and is constitutively expressed [71, 72].

In addition to the PAS (Per/ARNT/Sim) domain, the three HIF-α isoforms possess an oxygen degradation domain (ODD) that subjects them to oxygen-dependent regulation by hydroxylases [70]

As mentioned in section 1.1.1, sequence specific transcription factors (activators or repressors) are modular and usually contain a DNA-binding domain as well as an activation or repression domain.

Both HIF-1α and HIF-2α contain a C-terminal activation domain, whereas HIF-3α lacks this specific domain on its C-terminus and is thereby thought to act as an inhibitor of these two hypoxia inducible factors [71]. Furthermore, HIF-1α and HIF-2α contain a nuclear localization signal (NLS) and an N-terminal activation domain, whereas HIF-3α possesses a leucine-zipper domain instead of a C-terminal activation domain [70].

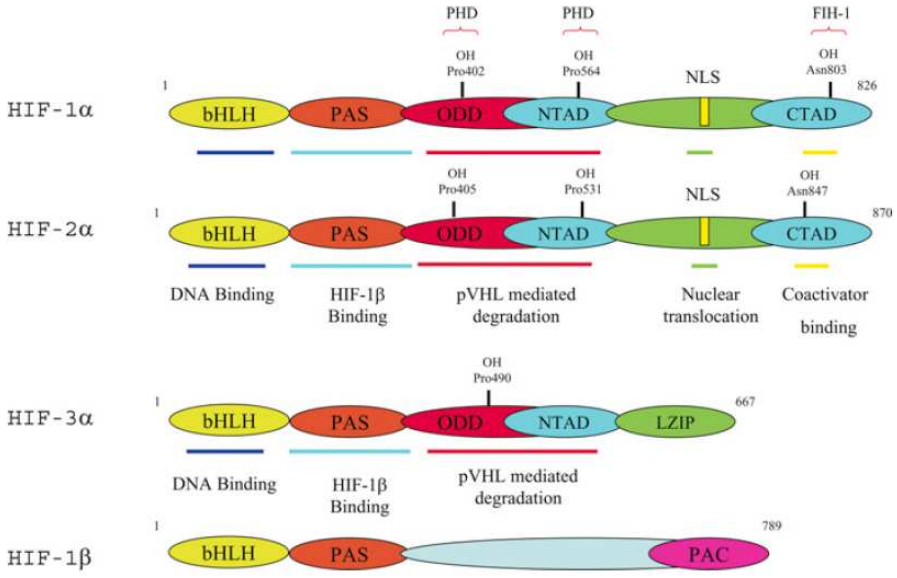

Fig. 6: Domain structure of hypoxia inducible transcription factors [70].
Abbreviations: CTAD, C-terminal activation domain; LZIP, leucine zipper ; NLS, nuclear localization signal ; NTAD, N-terminal activation domain ; PAS, PER/ARNT/Sim domain; PAC, PAS-associated C-terminal domain.

HIF-1α is the best understood isoform which functions within the cell in a non-redundant way together with HIF-2α [70], which was shown to be involved in important cellular functions [73, 74].

Once HIF-α and HIF-β heterodimerize within the nucleus, they bind together with the transcriptional coactivators CBP/p300 to hypoxia responsive elements (HREs), which consist of the core motif G/ACGTG [75].

1.3.2 Molecular regulation of hypoxia inducible factors

The identifications of a novel class of dioxygenases made HIF proteins prime candidates for oxygen sensing proteins [70].

The hydroxylation reactions can be performed by either PHDs (prolyl hydroxylase domain proteins), that recognize prolyl residues or by FIH-1s (factor inhibiting hypoxia inducible factor-1) that recognize asparaginyl residues (Fig.7) [75].

1.3.2.1 Prolyl hydroxylation

Hydroxylation of prolyl residues is catalyzed by the PHDs that recognize the ODD (oxygen degradation domain) within the C- or N-terminus of the hypoxia inducible transcription factors [76]. PHDs require specific co-factors to catalyze this reaction, such as oxygen, iron and 2-oxoglutarate [77]. The hydroxylation of HIF-α's mediates the interaction with the von Hippel-Lindau tumor suppressor (pVHL), which acts as an E3 ligase, promoting the ubiquitination-mediated proteasomal degradation [78, 79]. Four different PHD isoforms are known: PHD1-4 but only PHD1-3 have been shown to hydroxylate HIF [76]. PHD1 and PHD3 show higher affinity towards HIF-2α, whereas PHD2 favours HIF-1α [80].

1.3.2.2 Asparaginyl hydroxylation

Asparaginyl hydroxylation is catalyzed by FIH-1s [81]. The FIH catalyzed hydroxylation event prevents binding of the CBP/p300 co-activator to the HIFs thus impeding the target gene activation [70].

Notably, studies on FIH-1 and p300 could show that even though these proteins were absent, some HIF-target genes were still HIF-inducible [82, 83].

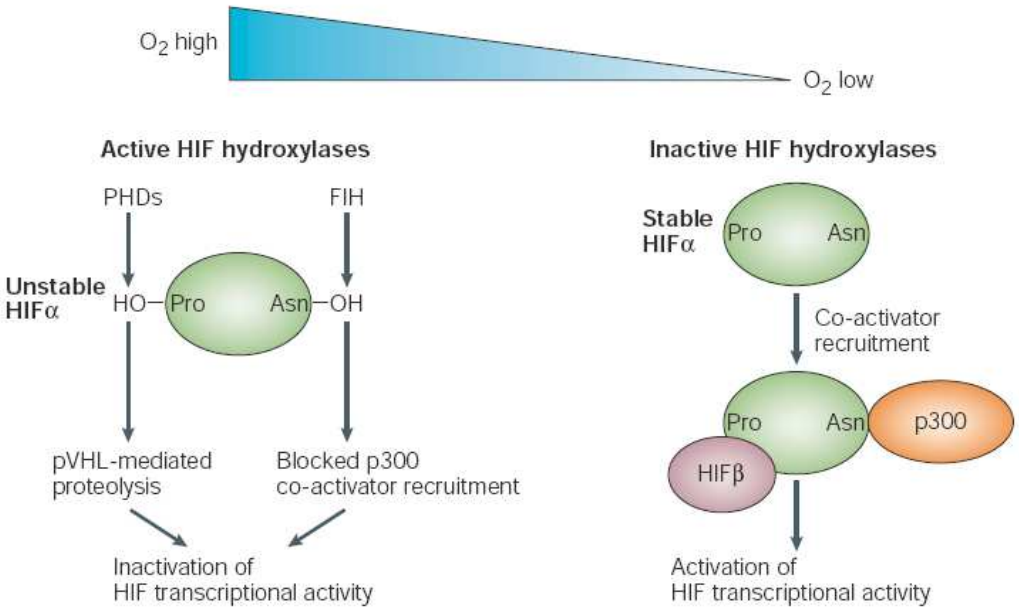

Fig. 7: Molecular regulation of HIF by prolyl and asparaginyl hydroxylation [75].

Under normoxic conditions, both PHDs and FIHs can hydroxylate their specific target residues within the oxygen degradation domains (ODD) of HIF-α subunits.

This leads to an E3 ligase (von Hippel-Lindau tumor suppressor complex) mediated ubiquitinylation of HIF-α, that triggers proteasomal degradation, or to a blocked p300 co-activator recruitment, thus preventing HIF from activating specific target genes. When the oxygen levels decrease, HIF α subunits get stabilized due to the inactive hydroxylases. Stabilized HIF-α subunits can dimerise with HIF-1β within the nucleus, bind the co-activator CBP/p300 and turn on target gene transcription by binding to hypoxia responsive elements (HREs) [61].

1.3.3 Cross-talk between hypoxia responsive transcription factors

Hypoxia activates HIF as well as other hypoxia responsive transcription factors. Taken together, all of these factors mediate the hypoxia response in a collective manner to alter the gene expression profile of the cell (Fig. 8) [70].
This section should reveal the diversity of transcription factors that are activated during hypoxia and how these factors might function in order to ensure a co-ordinate cellular response.

The NF-κB family of transcription factors

The NF-κB family is composed of seven proteins which are encoded by five genes (RelA, RelB, c-Rel, NF-κB1(p105/p50), NF-κB2 (p100/p52)) [84]. The NF-κB transcription factors are known to play important roles in the immune system and inflammatory responses but recent studies revealed that NF-κB plays a role in disorders such as cancer [85]. It was already known that hypoxia induces NF-κB [86], but the underlying molecular mechanisms were not so clear. It had been shown that NF-κB acts as both a survival signal and as a pro-death factor and therefore shows a dual nature of regulation [70]. Furthermore, it was shown that NF-κB directly modulates HIF-1 transcriptionally [87] , which might reveal interesting mechanisms of how these factors work together in order to respond to low oxygen levels.

AP-1 transcription factors

AP-1 transcription factors are formed by combination of dimers between Jun, Fos and ATF (activating transcription factor) transcription factors and therefore regulate highly complex biological functions, such as proliferation, apoptosis and tumorigenesis [70]. Knock-out studies revealed the high importance of some AP-1 members in developmental processes [88]. Since AP-1 generally acts by co-operation with other transcription factors in order to modulate their activity, this is also

thought to occur under hypoxia due to clear evidence of co-operation between these two transcription factors [89]. Additionally, it was observed that NF-κB co-operates with AP-1 [90].

p53

Since p53 is one of the most important tumor suppressors, activated by a great variety of cellular stress signals, it is not surprising that p53 is activated by hypoxia as well. Even though hypoxia does not induce any detectable DNA damage, it induces p53 but surprisingly both in an HIF-1 dependent and independent manner [91]. Studies with focus on the transcriptional activity of p53 revealed that p53 did not induce the same sets of genes under hypoxia compared to typical p53-activating stimuli, such as UV-light [91, 92]. Beside these controversial findings, it is clear that p53 plays a role in hypoxia- induced apoptosis [70].

The Myc family of transcription factors

The Myc family is composed of four members: c-Myc, N-Myc, L-Myc, and S-Myc [93]. Since the Myc family of transcription factors play important roles in various diverse biological processes, such as cell growth, cell proliferation, inhibition of cell differentiation, angiogenesis as well as genomic instability, they are tightly regulated [93, 94]. Under hypoxic conditions, cells usually stop to proliferate and undergo cell cycle arrest via induction of cyclin-dependent kinase inhibitors, which are normally repressed under normoxia [95, 96].

Interestingly, it has been shown that Myc and HIF can compete for promoter binding sites in order to activate target gene transcription. Normally, the cyclin-dependent kinase inhibitor p21 is repressed under normoxia by c-Myc which binds to its promoter, whereas, under hypoxia c-Myc is replaced by HIF, thus active p21 leads to cell cycle arrest [97]. Surprisingly, neither HIF transcriptional activity, nor its DNA binding is essential for induction of cell cycle arrest [96].

There are many processes where Myc and HIF have contrasting effects, but both transcription factors are up-regulated in various tumor cells where they promote the same biological processes, such as angiogenesis [98].

However, both HIF as well as Myc can co-operate in order to respond to hypoxic stress by inducing shared target genes like VEGF (vascular endothelial growth factor) or PDK1 (pyruvate dehydrogenase kinase 1) [99].

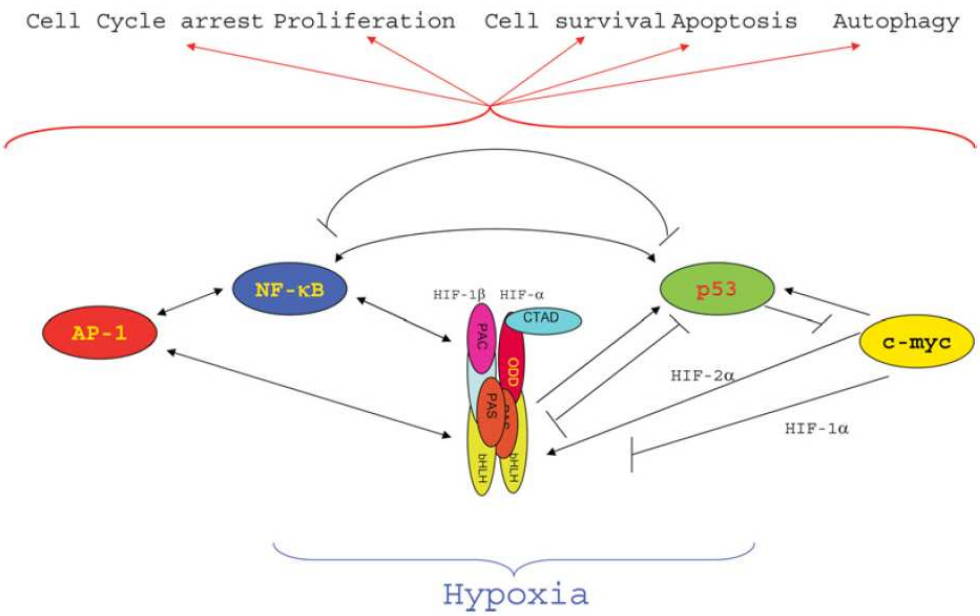

Fig. 8: Cross-talk between hypoxia responsive transcription factors [70].
Even though the hypoxia inducible factors are the main regulators of hypoxia, they co-operate with other hypoxia responsive transcription factors in order to ensure a co-ordinate cellular response.

1.4 HIF target genes

Functional HIF complexes (HIF-α/HIF-β) bind to specific hypoxia responsive elements (HREs) within regulatory regions of their target genes to modulate gene expression. These transcriptional targets play different roles in the complex regulation of oxygen homeostasis and are involved in cell migration, hormonal regulation, energy metabolism, angiogenic signalling as well as cell growth and apoptosis (Fig.9) [75].

For example, erythropoietin and various iron-metabolising genes are direct targets of HIF-1α which lead to increased capacity of red blood cells to transport oxygen during erythropoiesis [61, 100, 101]. Other prominent HIF targets are; vascular endothelial growth factor (VEGF) [102], Glucose transporter 1/3 (GLUT1/3) [103], or Aldolase-A/C (ALDO A/C) [68], all of which are involved in angiogenesis, glucose uptake or glycolysis.

Large-scale gene-expression arrays revealed that in any given cell, hundreds of genes are either up- or down-regulated by hypoxia [104-106]. Notably, the set of genes that are regulated by hypoxia greatly differ between different cell types [75].

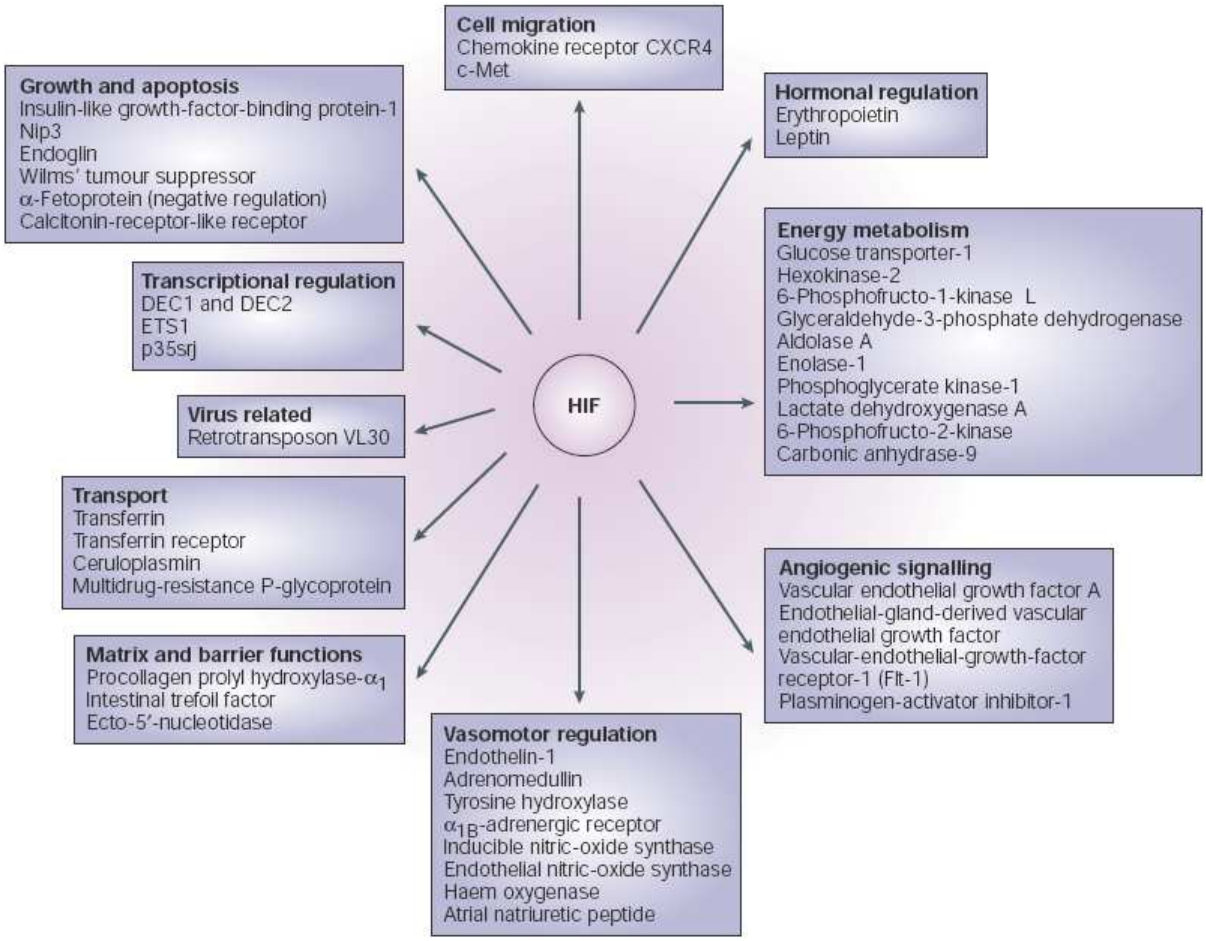

Fig. 9: HIF target genes, an overview [75].

Genetic studies revealed that defective HIF-α-subunit-cells, as well as defective von Hippel Lindau tumor suppressor-cells, in combination with non-specific hydroxylase inhibitors, mostly need a functional HIF/pVHL/hydroxylase system to respond to hypoxia [75].

1.5 Hypoxia and cancer

The majority of solid tumors show expression of HIF-1α. These tumors, involving brain, bladder, breast, colon, ovarian, pancreatic, renal as well as prostate [107, 108], must increase their oxygen supply due to hypoxic areas that evolve during tumor-growth via angiogenesis as well as switch to glycolytic ATP-production, which is known as the Warburg effect [109].

However, the important role of hypoxia inducible factors in these adaptive processes leave no doubt that both HIF-1α and HIF-2α play fundamental roles in tumor progression and grade, in order to provide a selective advantage to tumor cells [61].

Characteristically, hypoxic tumors show common features:

(1) They cannot grow beyond a certain size (mm^3) without turning on angiogenesis to maintain their oxygen- and nutrient- supply [110, 111]. (2) Increased vascularisation in tumors is often correlated with reduced survival rates in patients [112]. (3) Hypoxic levels within tumors are positively correlated with increased invasion capability as well as metastasis and death [113]. (4) The proliferation rate of cancer cells are enhanced in comparison to the angiogenic rates [110]. (5) Hypoxic tumors are resistant to chemotherapy, immunotherapy and radiotherapy [114].

It is important to emphasize that beside HIF-1α, whose function in hypoxic tumor growth is of main interest, HIF-2 α is also up-regulated in human cancers [115].

Since over-expression of HIF-1α correlates with high aggressiveness as well as poor prognosis and treatment failure [65], it could be advantageous to develop agents that inhibit HIF activation.

1.6 Cancer classification

Cancers have been classified due to the tissues and specific cell types from which they originate [116]. In contrast to this type of classification, the WHO (World Health Organisation) utilizes an organ system approach that is based on the different body sites at which the tumors occur [117].

The organ system approach of tumor classification is problematic since nearly all organs are comprised of various organ-specific as well as organ non-specific cell types. A complete list of tumors that occur in one specific organ could therefore be shared with another organ that possibly consists of an analogous tissue-composition. Therefore, the same tumors appear in every site-specific classification over and over again [117].

The histological based cancer classification categorises the various types of cancers as follows:

(1) Carcinomas

Carcinomas arise from epithelial cells and tend to infiltrate the surrounding tissue and therefore have a high metastatic potential [118]. 90% of all human cancers are carcinomas, which could be due to the high proliferation rate within the epithelia [116].

(2) Lymphoma

Lymphoma is the general term that characterizes various neoplastic diseases derived from lymphoid tissues. They are further sub-divided into Hodgkin disease, immunoproliferative small intestinal disease and non- Hodgkin's disease [118].

(3) Leukemia

Leukemia is a malignant disease of the blood-forming organs and characterized through the malfunctioning proliferation and differentiation of leukocytes, as well as their precursors within the bone marrow and blood. Originally, leukemias were divided into either acute or chronic leukemias, which reflected the life expectancies of patients. Additionally, leukemias can be further classified into myeloid and lymphoid leukemias [118].

(4) Melanoma:

Melanomas derive from melanin forming cells and therefore affect the skin of nearly every site in the body. This malignant neoplasm is highly metastatic and therefore accompanied by poor prognosis [118].

(5) Sarcoma

Sarcoma is a highly malignant neoplasm of connective tissue which is formed by proliferation of mesodermal cells [118].

Compared to this historical type of classification, there is currently no modern cancer classification existing but the technological progress makes it possible to develop new classification methods, based on molecular gene expression profiles using microarrays [117, 119].

1.7 Ewing's Sarcoma

Sarcomas are one of the most aggressive and frequently metastatic malignancies of children and adults, which mostly originate from mesenchymal stem/progenitor cells [120, 121].

Ewing's sarcoma is the second most common malignancy of children and young adults that affects mostly soft tissue and solid bone [122]. There are 1-3 million incidences per year in the Western hemisphere with a slightly higher frequency in males than in females [123].

Although Ewing's sarcomas may arise in any bone and from soft tissue, the most common sites of these tumors are the pelvic bones (Fig. 10), followed by the long bones of the lower extremities and the bones of the chest wall. Metastasis occur mainly in the bone marrow, bones and lungs in about 25% of all patients [124].

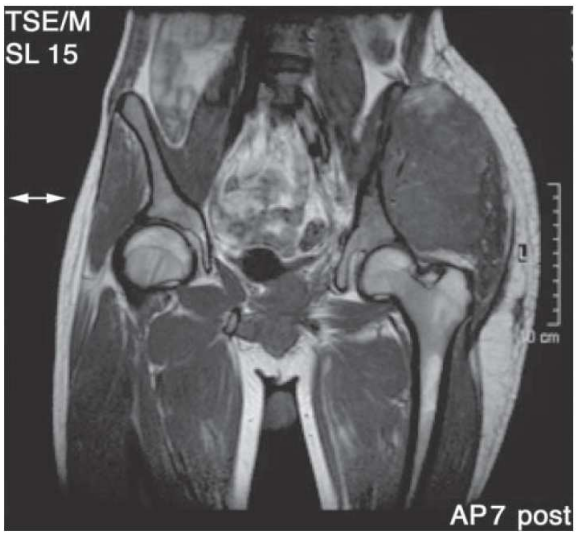

Fig. 10: Ewing's sarcoma of the pelvis, magnetic resonance image [124].

Ewing's sarcoma belongs to a group of small blue round cell tumors that exhibit a poorly differentiated cell phenotype (Fig.11). This histological group consists of neuroblastoma, alveolar rhabdomyosarcoma, lymphoblastic lymphoma and Ewing's Sarcoma family tumors (ESFT) [123]. Since all of these tumors share common morphological features, it has been difficult to diagnose Ewing's sarcoma due to the lack of specific molecular markers [122].

For instance, Ewing's sarcomas express high levels of the transmembrane glycoprotein CD99 [125], which is expressed by other small blue round cell tumors as well.

However, intense immunohistochemistry of small blue round cell tumors is frequently required to ensure appropriate diagnosis. As already mentioned, ESFT, just like lymphoblastic lymphomas, express CD99 but only lymphoblastic lymphoma expresses CD45 whereas Ewing's sarcomas do not. Furthermore, alveolar rhabdomyosarcoma may express CD99 but the difference to Ewing's sarcomas is expression of specific markers like desmin, myogenin and MyoD1 that are lacking in ESFT cells. Neuroblastomas as well as ESFT tend to express neural specific enolase (NSE) and S-100 but, additionally, neuroblastomas are vimentin-negative and neurofilament-positive whereas Ewing's sarcomas are not [123].

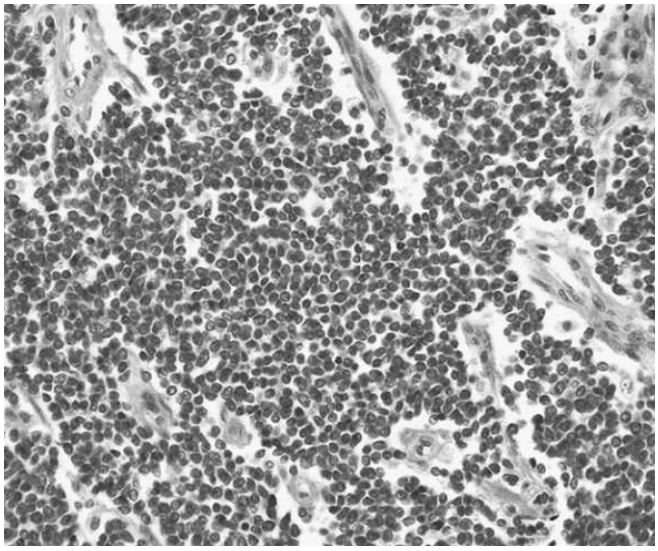

Fig. 11: Typical small blue round cell phenotype of Ewing's sarcoma [123].

In the early 1990s, ESFT were characterized by a chromosomal translocation that gives rise to a functional fusion protein called EWS-Fli1 [126]. This discovery led to improved diagnosis due to the usage of fluorescence in situ hybridization (FISH), and/or reverse transcriptase polymerase chain reaction (RT-PCR) [122].

1.8 EWS-Fli1

ESFT are characterized by chromosomal translocations involving the *EWSR1* gene and several ETS transcription factor genes, giving rise to EWS-ETS oncoproteins. The most frequently observed translocation, t(11;22)(q24;q12), results in the fusion of portions of the *EWSR1* gene with the *Fli1* (Friend leukemia integration site 1) transcription factor gene and is observed in 80-85% of all cases [122].

The *EWSR1* gene, which encodes for the EWS protein (Fig.12), is composed of an N-terminal serine-tyrosine-glutamine-glycine-rich (SYQG) region, that has a high transactivation potential [127, 128], and of a C-terminal RNA recognition motif. Additionally, this RNA-binding protein contains three arginine-glycine-glycine-rich (RGG) regions which have been shown to interact with RNA as well [126].

On the other hand, Fli1 belongs to the ETS transcription factor family, as mentioned in section 1.2.2, that recognizes a conserved DNA sequence [129].

The most common EWS-Fli1 fusion involves the first 264 amino-acid EWS portion and the C-terminal Fli1 portion, consisting of 233 amino-acids. This gene fusion is due to a specific rearrangement which affects intron 7 of *EWSR1* and intron 5 of *Fli1* [122]. However, there are alternative EWS-Fli1 fusion proteins that arise from other breakpoints, but all bear the transactivation domain of EWS and the DNA-binding domain of Fli1. The resulting chimeric transcription factor is aberrantly active and capable of binding DNA [130, 131].

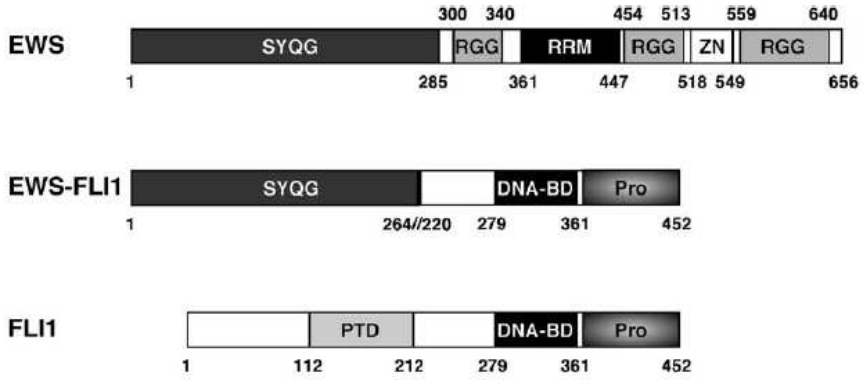

Fig. 12: Fusion of the RNA-binding protein EWS with the DNA-binding protein FLI 1, resulting in a chimeric, aberrantly active transcription factor which is able to bind DNA [122].

Abbreviations: DNA-BD, DNA binding domain; Pro, proline-rich activation domain; PTD, pointed domain; RGG, arginine-glycine-glycine-rich region; RRM, RNA recognition motif; SYQG, serine-tyrosine-glutamine-glycine-rich region; ZN, zinc finger.

Notably, it is widely accepted that EWS-Fli1 fusion proteins are oncogenic due to clear evidence demonstrating that they can transform NIH3T3 mouse fibroblasts in vitro [132]. On the other hand, introduction of siRNA or EWS-Fli1 antisense constructs into Ewing's sarcoma cells resulted in increased apoptosis, growth inhibition as well as prevention of tumor formation in nude mice [133-135]. Interestingly, no single Ewing tumor has been identified to contain a EWS-ETS protein with a defective DNA-binding domain, indicating that this ability is essential for the oncogenic potential of Ewing tumors [122]. Additionally, the fusion mode of EWS and Fli1 determines the transactivation potential of the resulting oncoprotein [136].

Beside the EWS-Fli1 fusion, there are around 15% of Ewing tumors that do not show a t(11;22)(q24;q12) translocation. The most common translocation, among these 15 % of tumors, involves the EWS-ERG fusion between the *EWSR1* gene and the *ERG* (ETS-related gene) gene that makes up ~ 10% of all cases [137]. Other members of the ETS-family of transcription factors that are known to fuse with the EWSR1 gene are: ETV1 (ETS variant gene 1) [138], ETV4 (ETS variant gene 4, also known as E1AF) [139], and FEV (fifth Ewing sarcoma variant) [140]. The latter are fairly rare and make up <1% of all ESFT cases [122].

Since EWS-ETS fusion proteins operate as transcription factors with a highly potent transactivation- as well as DNA-binding-domain, they may up- or down-regulate many target genes in ESFT [122]. It has been shown that these chimeric transcription factors affect fundamental biological processes, such as stimulation of cell proliferation (e.g. PDGF, platelete-derived growth factor) [141], evading growth inhibition (e.g. TGF-β, transforming growth factor-β; Id2, inhibitor of DNA binding 2) [142, 143], escape from apoptosis (e.g. IGFBP-3, insulin-like growth factor binding protein-3) [144] or invasion and formation of metastasis (e.g. MMPs, matrix metalloproteinases) [122].

1.9 Aim of the thesis

Since ESFT are highly malignant and often associated with poor prognosis, it is of great interest to identify new prognostic markers in order to enable risk-adapted therapy.

Hypoxia is known to stimulate invasion and metastasis in many tumors resulting in adverse prognosis. Solid tumors that have been shown to contain hypoxic areas respond mainly via HIF-1α to promote further tumor development.

So far, the role of hypoxia and especially the contribution of HIF-1α to the aggressiveness of ESFT remain unknown. The aim of this thesis, therefore, is to investigate the role of hypoxia and HIFs in ESFT cell lines.

2 Materials and Methods

2.1 Materials

2.1.1 Media

RPMI 1640 with GlutaMAX^{Tm}-I

Invitrogen, Groningen, Netherlands

Add 10% fetal calf serum (FCS Gold, PAA Laboratories, Linz, Austria) and 100.000 Units/l penicillin / streptomycin (PAA Laboratories, Linz, Austria)

DMEM

Invitrogen, Groningen, Netherlands

1000 mg/L glucose, 4mM L-glutamine and 110 mg/L sodium pyruvate

Add 10% fetal calf serum (FCS Gold, PAA Laboratories, Linz, Austria) and 100.000 Units/l penicillin / streptomycin (PAA Laboratories, Linz, Austria)

Luria Broth (LB)

1% Trypton,

1% NaCl,

0,5% Yeast-extract; LB was autoclaved

Opti-MEM: Invitrogen, Groningen, Netherlands

Trypsin / EDTA: PAA Laboratories, Linz, Austria

Accutase: PAA Laboratories, Linz, Austria

2.1.2 Buffers

TBS-T: 50mM Tris, 150mM NaCl, 0,1% Tween 20; pH 7,5

TBS: 50mM Tris, 150mM NaCl, pH 7,5

PBS: 137mM NaCl; 3mM KCl; 6,5mM Na_2HPO_4-$2H_2O$; 1,5mM KH_2PO_4

Laemmli buffer

15,1g Tris

72g glycine

25ml 20% SDS

per 1 liter

Transfer buffer

14g glycine

3g Tris

20% methanol

per 1 liter

2x sample buffer

20% (v/v) glycerol

6% ß-mercaptoethanol

3% SDS

125mM Tris-Cl pH 6,8

small amount of bromphenol blue crystals

Ponceau S staining solution (10x stock)

2g Ponceau S

30g trichloroacetic acid

30g 5-sulfosalicylic acid

fill up to 100ml with dH_20

Loading Dye

4M Urea

80mM EDTA

10% Saccharose

0,25% BPB

TBE

5,4g Tris Base

2,75g Boric Acid

2ml 0,5M EDTA/pH8

per 1 liter

Blocking solution

10% (v/v) blocking reagent (Roche, Basel, Switzerland) in maleic acid buffer (100mM Maleic Acid, 150 mM NaCl, pH= 7.5, sterile).

2.1.3 Chemicals

Desferrioxamine (DFX): Compound that mimics hypoxia by chelating iron, therefore impeding appropriate prolyl hydroxylase domain protein (PHD) function, resulting in stabilization of HIF-1α. (D 9533-1G, Sigma, St. Louis, USA)

Cobalt (II) chloride ($CoCl_2$): A transition metal that inhibits prolyl hydroxylase domain proteins (PHDs) by depleting the cells of ascorbic acid, which is a co-factor of prolyl hydroxylases, with concomitant HIF-1α stabilization. (C 8661-25G, Sigma, St.Louis, USA)

Propidium Iodide (PI): (P4170) Sigma, St. Louis, USA

Doxycycline: Sigma, St. Louis, USA

Puromycin : Sigma, St. Louis, USA

Zeocin: Cayla, Toulouse, France

Blasticidin: Invitrogen, Groningen, Netherlands

Ampicillin: Biomol, Hamburg, Germany

Trypan blue: Sigma, St. Louis, USA

2.1.4 Ewing tumor cell lines

TC252

Established by T. Triche (Dep. of Pathology, Children's hospital, Los Angeles, USA); p53 wild type, expresses the type I (Exon 7 [EWS]/ Exon 6 [Fli1]) EWS-Fli1 fusion.

SK-N-MC

Established by J. Biedler (Memorial Sloan Ketternig Cancer Center, New York, USA); truncated p53, expresses the type I EWS-Fli1 fusion, derived from pPNET localized within the rib.

STA-ET-7.2

Cell lines that are designated 'STA-ET' were established at the CCRI (Children's Cancer Research Institute, Vienna, Austria). STA.ET7.2 was established from a pleural effusion; p53 mutant (R273C) [145]; expresses type II (Exon 7 [EWS]/ Exon 5 [Fli1]) EWS-Fli1 fusion.

STA-ET-1

Established at the CCRI, Vienna, Austria, expresses type I EWS-Fli1 fusion and harbours wild type p53.

ASP14

Established from the A673 parental ESFT cell line by Javier Alonso (Laboratorio de Patología Molecular de Tumores Sólidos Infantiles, Departamento de Biología Molecular y Celular del Cáncer, Instituto de Investigaciones Biomédicas, Madrid, Spain); p53 mutant (2BP-INS 118/119) [145], expresses type I EWS-Fli1 fusion and doxycycline-inducible small hairpin RNA against EWS-Fli1.

VH64

Established by F. Van Valen (Dep. of Paediatrics, University of Münster, Germany); p53 wild type, expresses type II EWS-Fli1 fusion.

WE68

Established by F. Van Valen (Dep. of Paediatrics, University of Münster, Germany); wild type p53 and expresses type I EWS-Fli1 fusion.

2.1.5 Antibodies

Anti-HIF-1α: Mouse monoclonal antibody against amino acids 610-727 of human HIF-1α. (Becton Dickinson transduction laboratories, USA, 610959) **Dilution: 1:200; 1:100**

Anti-HIF-2α: Mouse monoclonal antibody (ab8356), clone number-[ep190b], human HIF-2α. (Abcam, Cambridge Science Park, Cambridge, UK) **Dilution 1:500**

Anti-β-actin: Mouse monoclonal [ab8226] to beta Actin, clone number [mAbcam 8226]. (Abcam, Cambridge Science Park, Cambridge, UK) **Dilution 1:10000**

Anti-Fli-1 (C-19): Rabbit polyclonal antibody against the C-terminus of Fli1 (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-356). **Dilution: 1:500**

Anti-Fli-1(Hybridoma): Supernatant of Hybridoma cell line 7.3, producing monoclonal antibody against the C-terminus of Fli1, was derived from Olivier Delattre (Institut Curie, Paris, France) **use: undiluted**

Anti-mouse POD: Anti-Mouse IgG, (H+L), Peroxidase conjugated secondary antibody using chemiluminescence. (product no. 31430, Pierce, Rockford Illinois, USA) **Dilution: 1:10000**

Anti-rabbit mouse POD: Anti-Rabbit IgG, (H+L), Peroxidase conjugated secondary antibody using chemiluminescence (product no. 31460, Pierce, Rockford Illinois, USA) **Dilution: 1:10000**

2.1.6 Plasmids

pEF-Bos-cs-ΔHIF-1α: EF1 promoter based mammalian expression vector, bearing a non-degradable version of HIF-1α (P564A and N803A within the ODD of HIF-1α). (Gift from Dr. Murray Whitelaw, School of Molecular and Biomedical Sciences, The University of Adelaide, Adelaide, Australia) [146]

pEF-Bos-cs-HIF-1α: EF1 promoter based mammalian expression vector bearing wild type HIF-1α. (Gift from Dr. Murray Whitelaw, School of Molecular and Biomedical Sciences, The University of Adelaide, Adelaide, Australia) [146]

pRS-puro-shHIF-1α: Retroviral vector containing shRNA oligo that targets HIF-1α. (Gift from Dr. M. Vooijs, Department of Pathology, University Medical Centre Utrecht, Netherlands) [147]

pSuperΔRVsh30: mammalian expression vector encoding shRNA against Ews-Fli type I. (constructed by Jozef Ban, CCRI, Vienna) [148]

pCMV-GFP-shHIF-2α: CMV promoter based mammalian expression vector, containing shRNA against HIF-2α. (Gift from Prof. Hsu, Department of Pathology and Laboratory Medicine, Hollings Cancer Center, Medical University of South Carolina, South Carolina, USA) [149]

pSuperΔRV: pSUPER-based retroviral mammalian expression vector. (Gift from Reuven Agami, Division of Tumor Biology, Netherlands Cancer Institute, Amsterdam, Netherlands) [150]

2.1.7 Oligonucleotides

Aldolase C (ALDOC):	forward primer: ACTCCATACCACAGCCCTTG reverse primer: GCAATTTCTGCCCTCAG **product size: 211bp**
Bnip3:	forward primer: CTGGACGGAGTAGCTCCAAG reverse primer: AGCAGCAGAGATGGAAGGAA **product size: 351bp**
Carbon anhydrase IX: (CAIX)	forward primer: ATCTGCCCAGTGAAGAGGATT reverse primer: TCTCCAGGAGCCTCAACAGTA **product size: 151bp**
EWS-Fli1:	forward primer: TCCTACAGCCAAGCTCCAAGTC reverse primer: ACTCCCCGTTGGTCCCCTCC **product size: 328bp**
Glucose transporter 1: (GLUT1)	forward primer: CTTCACTGTCGTGTCGCTGT reverse primer: TGAAGAGTTCAGCCACGATG **product size: 229bp**
Glucose transporter 3: (GLUT3)	forward primer: TGGGGCTATCTTGGTCTTTG reverse primer: GTAATGAGGAAGCCGGTGAA **product size; 221bp**
HIF-1α:	forward primer: CTCAAAGTCGGACAGCCTCA reverse primer: CCCTGCAGTAGGTTTCTGCT **product size: 440bp**
HIF-2 α:	forward primer: AGGGGACGGTCATCTACAACC reverse primer: ATGGCCTTGCCATAGGCTGAG **product size: 307bp**

Insulin growth factor binding protein (Igfbp3):	forward primer: CAGAGACTCGAGCACAGCAC reverse primer: GATGACCGGGGTTTAAAGGT **product size: 194bp**
Vascular endothelial Growth factor (VEGF):	forward primer: CCTCCGAAACCATGAACTTT reverse primer: AGAGATCTGGTTCCCGAAAC **product size: 740bp**
β-Actin:	forward primer: GCCGGGAAATCGTGCGTG reverse primer: GGGTACATGGTGGTGCCG **product size: 305bp**

2.1.8 Kits

BD Cycletest™ Plus:

BD cycletest was used to estimate the cell-cycle phase distributions of differently treated ESFT cell lines via FACS analysis. (BD Biosciences, San Jose, USA, 340242)

Transwell® permeable inserts, Corning:

Transwell inserts (8µm pore size, polycarbonate (PC) coated) were used for invasion assay with various ESFT cell lines. (Corning Incorporated, Life Sciences, NY, USA)

Culture-Insert µ-Dish $^{35mm,\ low}$, Ibidi treat:

Culture-Inserts ready to use in a µ-Dish 35 mm ibiTreat, tissue culture treated, sterile, low walls, were used for 2D migration assays (Scratch Assay) with various ESFT cell lines. (no. 80206, ibidi GmBH, Martinsried, Germany)

SuperSignal® West Femto Maximum Sensitivity Substrate Kit:

SuperSignal® West Femto Maximum Sensitivity Substrate Kit is an extremely sensitive enhanced chemiluminescent substrate for detecting horseradish peroxidase (HRP) on western blots. (34096, Thermo Fisher Scientific, USA)

2.2 Methods

2.2.1 DNA/RNA methods

2.2.1.1 RNA extraction

RNA extraction was performed using the RNAeasy Mini Kit (Qiagen, Austin, USA) according to the manufacturer's instructions.

2.2.1.2 cDNA synthesis

5µg of total RNA was denatured at 70℃ for 10 minutes. After 2 min on ice, master mix, containing MMLV reverse transcriptase (Promega, Madison, USA), random hexamer primers and dNTP's were incubated for 60 min at 37℃. Subsequent incubation for 30 min on 42℃ and addition of RNase free water was followed by incubation for 5 min at 70℃; cDNA was stored at -20℃.

2.2.1.3 RT-PCR

Standard RT-PCR was performed on 20-50ng cDNA template, mixed with a nucleotide mix, containing 2,5mM of dCTP, dATP, dTTP and dGTP (Promega, Madison, USA), 511 Reaction Buffer, (Finnzymes, Espoo, Finnland) containing 15mM $MgCl_2$, 0,4µM of each primer, and 0,5µl DyNAzyme DNA Polymerase (2U/µl, Finnzymes, Espoo, Finnland). The mix was filled up with ddH_2O to a total volume of 50µl per PCR reaction.

The polymerase chain reaction was performed by using the Dyad-Disciple thermal cycler (Biorad, California, USA) under following conditions:

Denaturation at 95℃ for 2 min, 95℃ for 1 min, specific annealing temperature, between 50-70℃, for 30 sec, elongation at 72℃ for 1 min, all for the first 10 x cycles. This programme was slightly modified for the next 25 x cycles: 95℃ for 30 sec, annealing temperature for 15 sec, elongation at 72℃ for 1 min 30 sec, 72℃ for 10 min and 15℃ forever.

The following optimized PCR conditions were obtained:

HIF1alpha
[20ng] cDNA; 10pmol/µl primer; annealing: 58.4℃; 2.0mM MgCl2; x35cycles, elongation 1'-1'30''

HIF2alpha
[20ng] cDNA; 10pmol/µl primer; annealing: 66.8℃; 3.0mM MgCl2; 1% DMSO; x35cycles; elongation 1'-1'30''

Igfbp3
[20ng] cDNA; 10pmol/µl primer; annealing: 55.5℃; 3.0mM MgCl2; x35cycles; elongation 1'-1'30''

AldolaseC
[20ng] cDNA; 10pmol/µl primer; annealing: 64.6℃; 3.0mM MgCl2; x35cycles; elongation
1'-1'30''

VEGF
[20ng] cDNA; 20pmol/µl primer; annealing: 55.5℃; 1.5mM MgCl2; x35cycles; elongation 1'-1'30''

EWS-Fli1
[20ng] cDNA; 20pmol/µl primer; annealing: 64.6℃; 1.5mM MgCl2; x35cycles; elongation 1'-1'30''

Bnip3
[20ng] cDNA; 20pmol/µl primer; annealing: 53.2℃; 1.5mM MgCl2; x35cycles; elongation 1'-1'30''

β-actin
elong. 1'-1'30''; annealing: 58.4℃ x 22cycles; 20pmol /µl; 1.5mM MgCl2;

Glut3
[20-50ng] cDNA; 20pmol/µl primer; annealing: 55.5℃; 0.8mM MgCl2; x35cycles; elongation 1'-1'30''

Glut1
[20-50ng]cDNA; 20pmol/µl primer; annealing: 64.6℃; 2.0mM MgCl2; x35cycles; elongation 1'-1'30''

CAIX
[20-50ng]cDNA; 20pmol/µl primer; annealing: 61.8℃; 2.0mM MgCl2; x35cycles; elongation 1'-1'30''

2.2.1.4 Quantitative RT-PCR

5µg of total RNA was denatured at 70℃ for 10 minutes. After 2 min on ice, master mix, containing MMLV reverse transcriptase (Promega, Madison, USA), random hexamer primers and dNTP's were incubated for 60 min at 37℃. Subsequent incubation for 30 min at 42℃ and addition of RNase-free water was followed by incubation for 5 min at 70℃; cDNA was stored at -20℃.

Reactions were set up in a total volume of 25µl containing 12,5µl 2x Universal PCR Master Mix, including uracil N'-glycosylase and AmpliTaq Gold DNA polymerase (Applied Biosystems, Vienna, Austria) and 1.5mM $MgCl_2$, 900nM (EWS-Fli1, EWS, β2-microglobulin) of each primer and 400nM (EWS-Fli1, EWS, β2-microglobulin) TaqMan probe, and 6µl of cDNA template. The mixtures were prepared in 96-well optical microtiter plates and amplified on the ABI 7900 Sequence Detection System using the following cycling parameters: 2 min at 50℃, 10 min a 95℃, and 50 cycles of 15s at 95℃ and 60s at 60℃. The beta-2-microglobulin values were used for normalization.

EWS-Fli1

(Type I Exon 7/6): forward primer: CAGCCAAGCTCCAAGTCAATATAG
reverse primer: GCTCCTCTTCTGACTGAGTCATAAGA
probe: CTGCCCGTAGCTGCTGCTCTGTTG

EWS: forward primer: ACAGCAGAGTAGCTATGGTCAACAA
reverse primer: ACTTGGAGCTTGGCTGTAGGAT
probe: AGCCTCCCACTAGTTACCCACCCCAAA

β2-microglobulin: forward primer: TGAGTATGCCTGCCGTGTGA
reverse primer: TGATGCTGCTTACATGTCTCGAT
probe: CCATGTGACTTTGTCACAGCCCAAGATAGTT

2.2.1.5 Maxi Prep

The day prior to preparation, 250ml of LB (containing 10mg/ml ampicillin) were inoculated with a pre-culture of the corresponding plasmid and incubated at 37℃ overnight. Qiagen Endotoxin free MaxiPrep kit (Qiagen, Austin, USA) was used for preparation according to the manufacturer's instructions.

2.2.2 Protein methods

2.2.2.1 SDS- Polyacrylamide Gel Electrophoresis (PAGE)

The SDS- polyacrylamide gel consists of a stacking and a separating gel:

Seperating gel:

	6%	**8,5%**	**12,5%**
30%Acrylamid / 0,8% Bis	1,05ml	1,4ml	2,1ml
H_2O	2,625ml	2,275ml	1,575ml
1,5M Tris pH8,8	1,25ml	1,25ml	1,25ml
20% SDS	25µl	25µl	25µl
10% APS	50µl	50µl	50µl
TEMED	6µl	6µl	6µl

Stacking gel:

30%Acrylamid / 0,8% Bis	415µl
H_2O	1,7ml
1M Tris pH6,8	315µl
20% SDS	12,5µl
10% APS	25µl
TEMED	2,5µl

Cells were counted and adjusted to a concentration of 30.000 cells/ µl with PBS and the same volume of 2x sample buffer. Samples were boiled for 10 min at 96℃, followed by centrifugation at top speed, and finally loaded on the SDS-gel. The gel was run at 40mA for ~ 60 min, till the bromphenol blue front began to phase out.

2.2.2.2 Western Blot

The transfer was started by assembling the transfer unit, consisting of the typical sandwich conformation (sponge, 3x Whatman paper, gel, nitrocellulose membrane, 3x Whatman paper, sponge), which was put in the blotting tank. The transfer occurred during 90 min at 400mA with the blotting tank cooled on ice.
The nitrocellulose membrane was stained with 1x PonceauS solution for several minutes, and was subsequently scanned. To avoid unspecific binding of the primary antibody, the membrane was incubated in 1% blocking solution for 60 minutes at room temperature. The primary antibody was diluted in 0.5% blocking solution, added to the membrane and incubated overnight at 4℃. On the next day, the membrane was washed three times with TBST and once with 0.5x blocking solution for 10 minutes at room temperature. Again, the secondary antibody was diluted in 0.5% blocking solution and incubated with the membrane for 1h at room temperature, followed by three times washing with TBST. The membrane was then carefully rinsed once with deionized water and incubated, with appropriate dilution of SuperSignal® West Femto Maximum Sensitivity Substrate Kit (Thermo Fisher Scientific, USA), for 3- 5 minutes in the dark. Films were developed using a standard radiograph processor (AGFA, CP-1000).

2.2.2.3 Cell culture techniques

ESFT cell lines were routinely cultured in RPMI 1640 with GlutaMAX^{Tm}-I (Invitrogen, Groningen, Netherlands), containing 10% fetal calf serum (FCS Gold, PAA Laboratories, Linz, Austria) and 100.000 Units/l penicillin / streptomycin (PAA Laboratories, Linz, Austria) in 5% CO_2 at 37℃.
For hypoxia studies, ESFT cell lines were cultured in RPMI 1640, containing 10% FCS, Pen/Strep and 25mM Hepes, placed in a hypoxia chamber at 1% O_2 and incubated in a humified atmosphere in 5% CO_2 at 37℃. To induce HIF-1α under normoxic conditions (21% O_2), 200µM $CoCl_2$ or 150µM DFX were used.

2.2.2.4 Transfection

Cells were split, according to the required amount, and cultured mainly in middle sized flasks (75cm^2). Transfection was performed, when cells reached 70-80% confluency, using Lipofectamine and Plus reagent (Invitrogen, Groningen, Netherlands) in serum-free OptiMEM I medium (Invitrogen, Groningen, Netherlands) according to the manufacturer's instructions.
Cells were incubated in OptiMEM I, including the transfection mix, for four hours at 37℃. Subsequently, the serum free medium was replaced by supplemented RPMI medium. Puromycin selection [1µg/ml] was initiated on the following day and cells were harvested after 72h.

2.2.3 Functional Assays

2.2.3.1 Scratch-Assay

Culture-Insert µ-Dish $^{35mm,\ low}$, Ibidi treat (ibidi GmBH, Martinsried, Germany) were used to perform 2D- migration assays. Cells were seeded, according to the cell type, between 4 x 10^4- 5 x 10^4 cells/segment and cultured in standard RPMI 1640 with GlutaMAX^{Tm}-I (containing Penc./Strep. and FCS). The following day, the supplemented RPMI was replaced with RPMI containing just Penicillin/Streptomycin but no FCS, in order to starve the cells for 18 hours. The culture insert was removed according to the manufacturer's instructions and migration was monitored with a standard inverted microscope after each day. Image analysis was carried out by S.CO LifeScience (S.CORE image analysis, Garching (Munich), Germany).

2.2.3.2 Proliferation Assay

Depending on the duration of the experiment, ESFT cell lines were seeded at a density between 2x10^5-5x10^5 cells/ well into 6-well plates, cultured in RPMI 1640 (10% FCS, Pen/Strep (PAA, Linz, Austria)) and incubated for 3-5 days in a humified atmosphere containing 5%CO_2. Cells were treated with Accutase (PAA Laboratories, Linz, Austria), for 10 minutes and 10µl of the cell suspension were mixed with the same volume of trypan blue (T-8154-100, Sigma, St. Louis, USA), and subsequently applied onto a Bürker counting chamber.

2.2.3.3 Cell cycle Assay

Cell cycle analysis of ESFT cell lines was performed using the BD Cycletest™ Plus (BD Biosciences, San Jose, USA, 340242) according to the manufacturer's instructions.

2.2.3.4 Invasion Assay

The invasive potential of ESFT cell lines was tested using 8µm, polycarbonate coated Transwell inserts (Corning Incorporated, Life Sciences, NY, USA). RPMI 1640 (Invitrogen, Groening, Netherlands) plus 10% FCS was filled into the lower compartment. A total of $5x10^5$ cells resuspended in RPMI 1640 serum free medium were seeded in the upper compartment and incubated overnight at 37℃ in 5% CO_2 atmosphere. Cells that migrated through the membrane were stained according to the manufacturer's instructions with 0,2% crystal violet and counted optically using a standard inverted microscope.

2.2.3.4.1 Matrigel Coating

Matrigel (BD Biosciences, 354248, San Jose, CA USA) was thawed overnight at 4℃ on ice and kept on ice before use. Pre-cooled pipettes, tubes and tips were used, since matrigel rapidly polymerizes at 22℃ to 35℃. Ma trigel was diluted in serum free medium (RPMI 1640 + Pen/Strep) to 1mg/ml final concentration and 1ml of diluted matrigel/6-well insert was used. Plates have been stored at 2-8℃ before use.

3 Results

3.1 Hypoxia mimetics induce HIF-1α in a dose dependent manner

Since hypoxia inducible factor 1-α is the main regulator of hypoxia, it was of great interest to detect this specific sensor-protein in ESFT cell lines. In the first approach we used canonical hypoxia mimetics, such as Cobalt-chloride ($CoCl_2$) or Desferrioxamine (DFX), to check whether HIF-1α protein is inducible under standard culture conditions (37℃ in 5% CO_2, 21% O_2 atmosphere) and how the protein levels change upon treatment with different concentrations of these mimetics.

$CoCl_2$ is a transition metal that depletes the cells from ascorbic acid, thus impeding appropriate PHD function. On the other hand, DFX is known to function as an iron-chelator, therefore inhibiting hydroxylation of HIF-1α. Both hypoxia mimetics were used in order to test for dose-dependent HIF-1α induction using 50, 100, 150 and 200µM $CoCl_2$ or DFX (Fig.13).

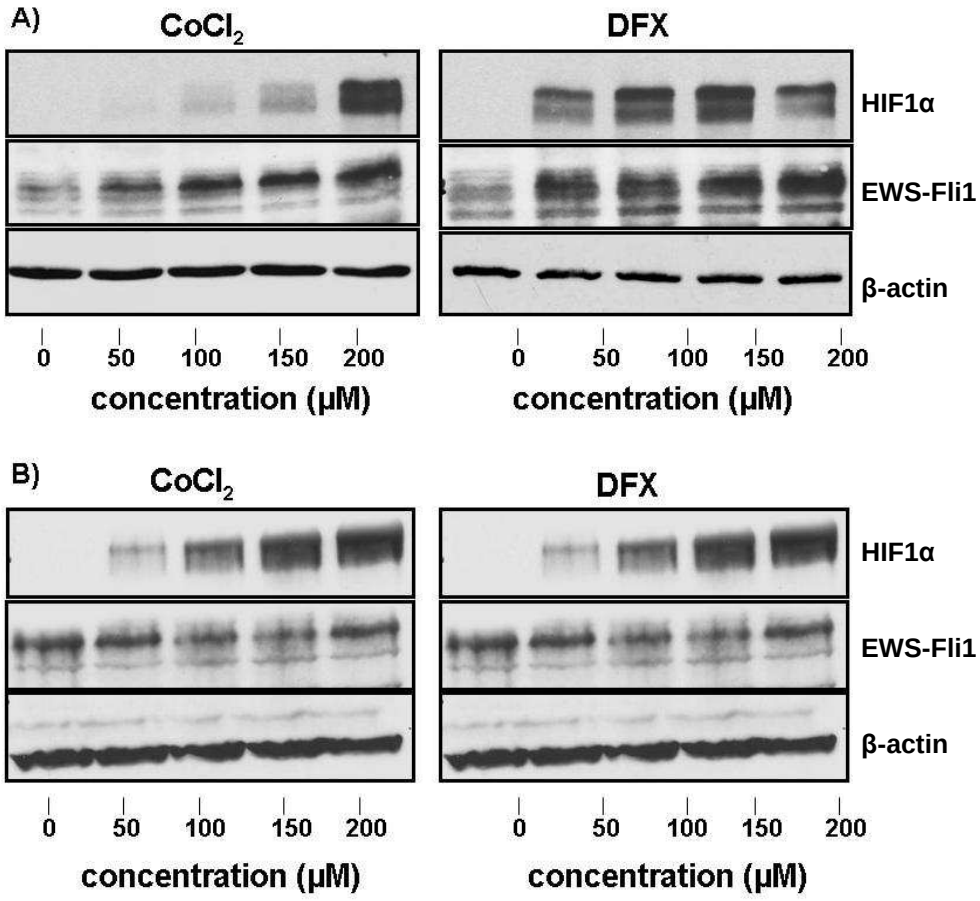

Fig. 13: HIF-1α induction using $CoCl_2$/DFX is dose-dependent. The ESFT cell lines A) TC252 B) SK-N-MC were utilized.

In the very first experiments, we used the ESFT cell lines SK-N-MC, TC252, STA-ET1, WE68, ASP14 and STA-ET-7.2. Although we initially performed studies on many different ESFT cell lines, we chose to subsequently focus on TC252 and SK-N-MC that have been extensively characterized. Importantly, these cell lines are known to differ in their p53 status (section 2.1.4), which may affect their response to hypoxia.

The ESFT cells were seeded in middle sized flasks (75cm^2), incubated under normoxic conditions (37℃, 5% CO_2, 21% O_2 in a humified atmosphere) and harvested by using trypsin, once the cells reached 70-80% confluence.

These experiments revealed that HIF-1α not only accumulates to high levels upon $CoCl_2$/DFX-treatment, but also reaches the peak of induction at 200µM $CoCl_2$ and 150-200µM DFX. Notably, the increased HIF-1α -levels in TC252 ESFT cells correlated proportionally with an increase of EWS-Fli1, indicating that hypoxia might influence EWS-Fli1 protein levels.

3.2 HIF-1α induction is time dependent

After having assessed the optimal $CoCl_2$-concentration, we established the kinetics of HIF-1α induction. For that purpose, we performed several time-course experiments using either 200µM $CoCl_2$ (Fig.14) or 1% O_2 (Fig.15) as a standard hypoxia condition.

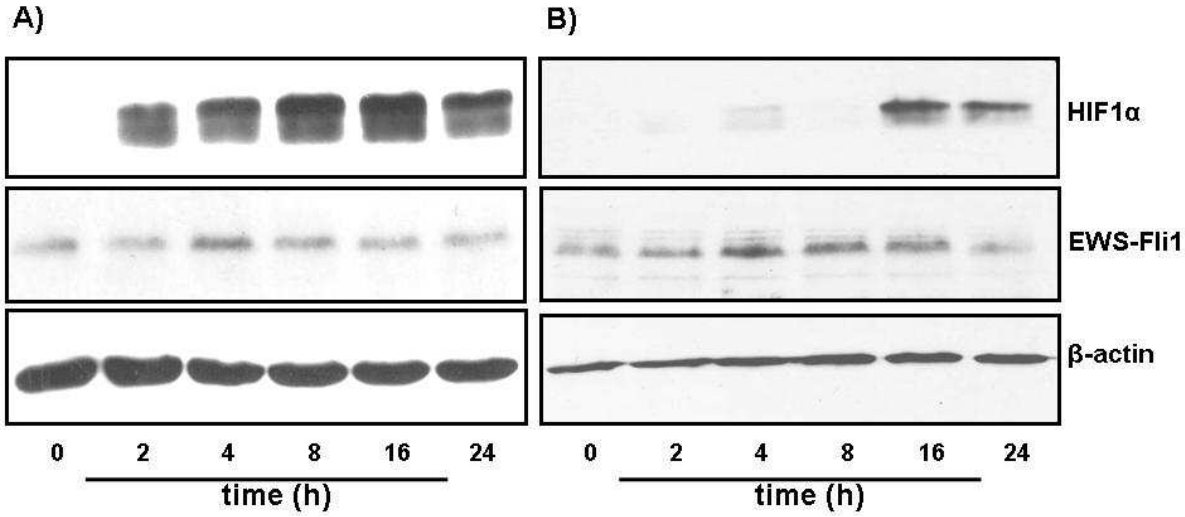

Fig. 14: Kinetics of HIF-1α and EWS-Fli1 expression upon $CoCl_2$ treatment.

A) TC252- and B) SK-N-MC- ESFT cell lines. Cells were incubated with 200µM $CoCl_2$ at 37℃ and 5% CO_2 in a humified atmosphere, containing 21% O_2, and harvested after 0, 2, 4, 8, 16, and 24 hours.

These time-course experiments showed again, that EWS-Fli1 levels are transiently elevated upon HIF-1α induction. Interestingly, EWS-Fli1 seems to reach its expression-peak between 8 and 16 h, followed by a decrease of the protein to basal expression levels. This might be explained by the fact that EWS-Fli1 levels are essential for ESFT cells in order to survive, but are toxic when expressed at very high levels. Therefore, ESFT cell lines might show an adaption response to sustain their viability by holding accurate EWS-Fli1 levels.

In addition we performed time-course experiments for 24, 48, 72, and 96 h under hypoxic conditions (1% O_2) in order to see how HIF-1α levels change with time (Fig. 15). Notably, hypoxia-treatment supported our previous observations with hypoxia mimetics, revealing maximum HIF-1α expression at 16 h and transiently increased EWS-Fli1 levels upon HIF-1α induction.

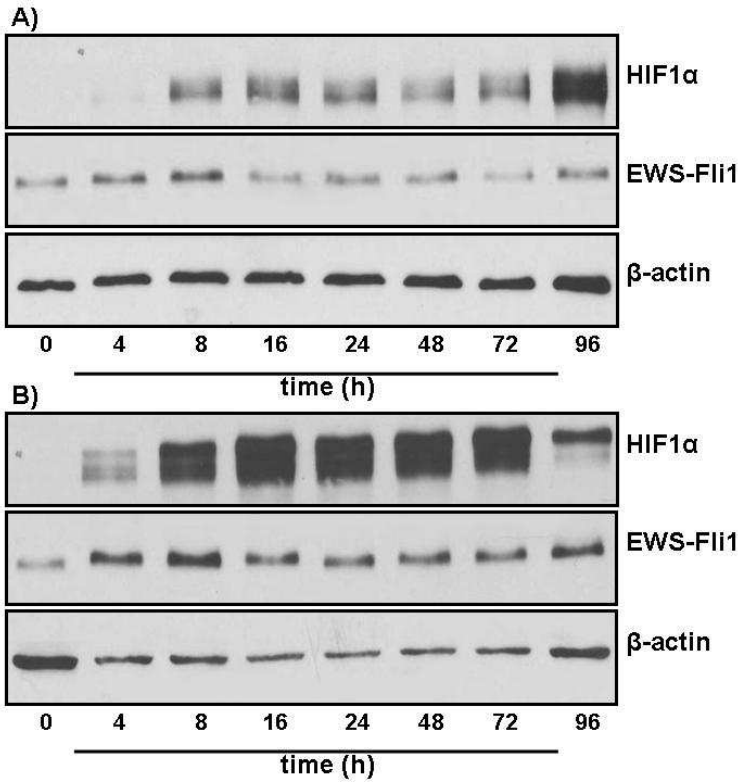

Fig. 15: Kinetics of HIF-1α and EWS-Fli1 expression upon hypoxia (1% O_2) treatment.
A) TC252- and B) SK-N-MC- ESFT cell lines. Cells were incubated in a humified atmosphere containing 1% O_2, 5% CO_2 at 37℃ and harvested after 0, 4, 8, 16, 24, 48, 7 2, and 96 hours.

In addition, these time-course experiments at 1% O_2 revealed that HIF-1α is already induced within the first four hours at low oxygen and stays fairly stable throughout a period of four days.

3.3 Hypoxia does not affect m-RNA levels of EWS-Fli1

The next step was to investigate whether, the increased EWS-Fli1 levels that have been monitored on protein level are reflected on the mRNA-level. Therefore, cDNAs from the time-course experiments were utilized to perform standard RT-PCR for HIF-1α, EWS-Fli1 and several hypoxia regulated genes. β-Actin was used for control.

Fig. 16: EWS-Fli1 mRNA levels did not change upon hypoxia treatment (1% O_2).
Samples corresponding to the time points 0, 4, 8 and 16h were chosen according to the clear EWS-Fli1 induction on protein level (Fig.15).

Abbreviations: HIF-1/HIF-2α, hypoxia inducible factor 1/2 α; EWS-Fli1, fusion of EWS and Fli1 giving rise to a chimeric transcription factor; GLUT1/3, glucose transporter 1/3; ALDOC, Aldolase C; Bnip3, BCL2/adenovirus E1B 19kDa interacting protein 3; VEGF, vascular endothelial growth factor; CAIX, carbonic anhydrase IX; IGFBP3, insulin-like growth factor binding protein 3; β-actin, house-keeping gene

We could clearly show that the mRNA levels of EWS-Fli1 were not affected upon hypoxia treatment, whereas the protein levels exhibited enhanced EWS-Fli1. These results were confirmed by utilizing qRT-PCR on the same samples (Fig.17).

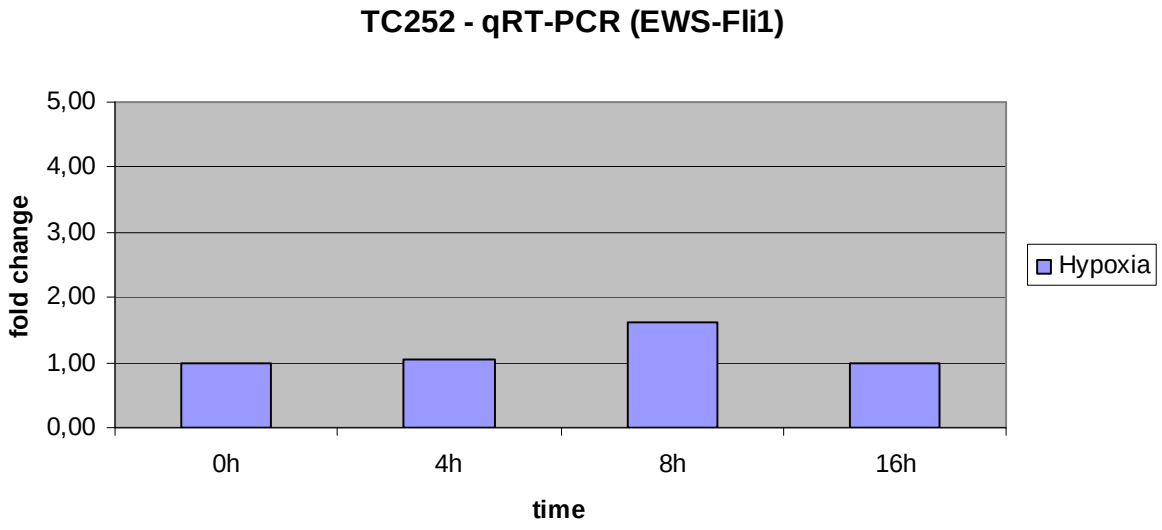

Fig. 17: Quantitative analysis of EWS-Fli1 mRNA derived from TC252 cells.
A representative qRT-PCR of cDNAs derived from TC252 cells that were utilized for the time-course experiment (Fig.15) as well as for RT-PCR (Fig.16). The experiment was carried out in triplicates and the mean values were used for the calculation of fold changes.

These results indicate that it is most likely a post transcriptional mechanism that regulates EWS-Fli1 enhancement.

Additionally, some prominent HIF-1α target genes were clearly induced under hypoxia, such as GLUT1, GLUT3, ALDOC, VEGF, CAIX and IGFBP3 whereas HIF-2α was constitutively expressed. Unfortunately, due to problems in the cDNA synthesis with samples derived from SK-N-MC, these results could only partially be reproduced, but are not shown here. Beside these problems, we reproduced that EWS-Fli1 mRNA levels were not enhanced under hypoxia at any time in both TC252 and SK-N-MC cell lines.

3.4 HIF-1α over-expression is accompanied by elevated EWS-Fli1 levels

To proof our previous observations, we over-expressed both a wild type HIF-1α and a non-degradable version of HIF-1α, carrying the P564A and N803A mutations within the ODD (ΔHIF-1 α), in ESFT cell lines and subsequently analyzed EWS-Fli1- and HIF-1α (Fig.18).

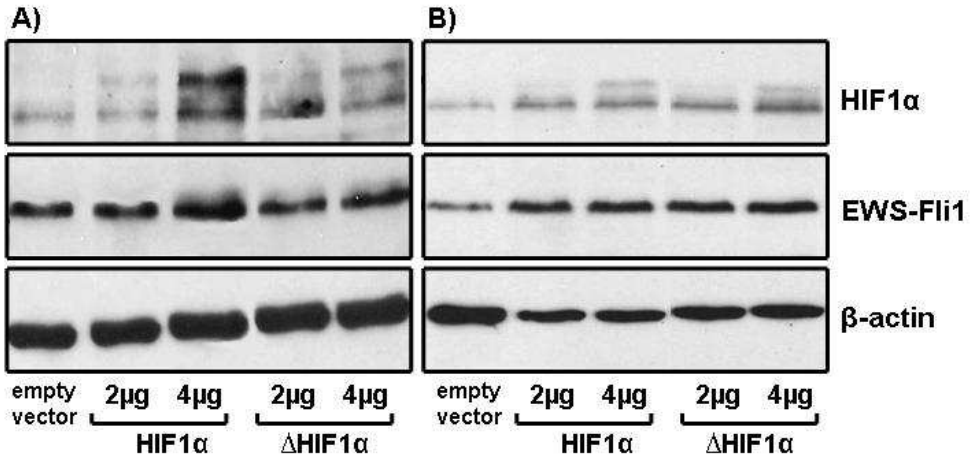

Fig. 18: Induction of EWS-Fli1 protein by over-expression of wild type and mutant HIF-1α. Transfection of A) TC252- and B) SK-N-MC- ESFT cell lines with either 2 or 4µg of pEF-Bos-cs-plasmid carrying HIF-1α wild type or a HIF-1α mutant version designated as Δ HIF-1α. Cells were incubated under normoxic conditions (21% O_2) in 5% CO_2 at 37℃.

For that purpose, ESFT cell lines, TC252 and SK-N-MC, were transfected with an EF1- driven mammalian expression vector (pEF-Bos-cs), bearing either the wild type- or mutant- version of HIF-1α. Puromycin selection was accomplished for three days and cells were harvested afterwards.

These experiments clearly showed that both, over-expression of wild type HIF-1α and mutant HIF-1α under normoxia, caused a noticeable increase of EWS-Fli1 levels.

3.5 EWS-Fli1 is regulated in an HIF-dependent manner

The ultimate experiment to investigate the role of HIF-1α for increased EWS-Fli1 levels was to knockdown HIF-1α and monitor EWS-Fli1 levels concomitantly (Fig.19). Again, TC252 and SK-N-MC were utilized as model ESFT-cell lines. Cells were transfected with shRNA targeting HIF-1α (section 2.1.6), puromycin selected and subsequently harvested.

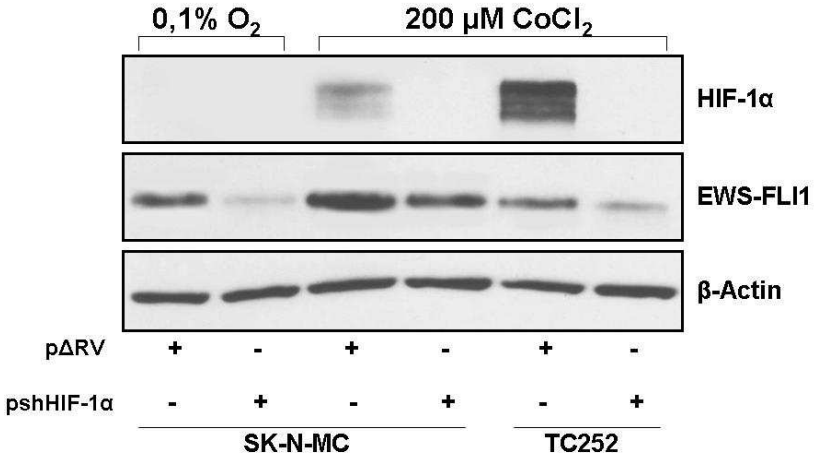

Fig. 19: Knockdown of HIF-1α using shRNA.
Both, SK-N-MC and TC252 ESFT cell lines were treated with 200µM $CoCl_2$ to mimic hypoxia under normal conditions (21% O_2) in order to definitely induce HIF-1α. In addition, SK-N-MC cells were incubated under 0,1% O_2 but unfortunately, no HIF-1α signal was obtained. Cells were treated with either empty vector (pΔRV) or shRNA against HIF-1α (psh HIF-1α) for 3 days.

This knockdown studies clearly showed that EWS-Fli1 levels decreased with HIF-1α knockdown, indicating that EWS-Fli1 is regulated in an HIF-1α dependent manner. This conclusion is supported by our time-course data as well as by the HIF-1α over-expression experiments.

Unfortunately, hypoxic treatment with 0,1% O_2 failed to reveal HIF-1α protein induction which might be explained by the very short half life of HIF-1α (< 5min) [151] during the harvesting period at normoxia.

3.6 EWS-Fli1 possibly represses HIF-2α in Asp14 cells

HIF-1, HIF-2α and EWS-Fli1 expression were also investigated in the A673 derived ESFT cell line ASP14, which allows for inducible knockdown of EWS-Fli1 via addition of doxycycline.

We over-expressed HIF-1α in ASP14 cell by transfecting with either pEF-Bos- HIF-1α or pEF-Bos-Δ HIF-1α in the presence or absence of doxycycline (Fig. 20).

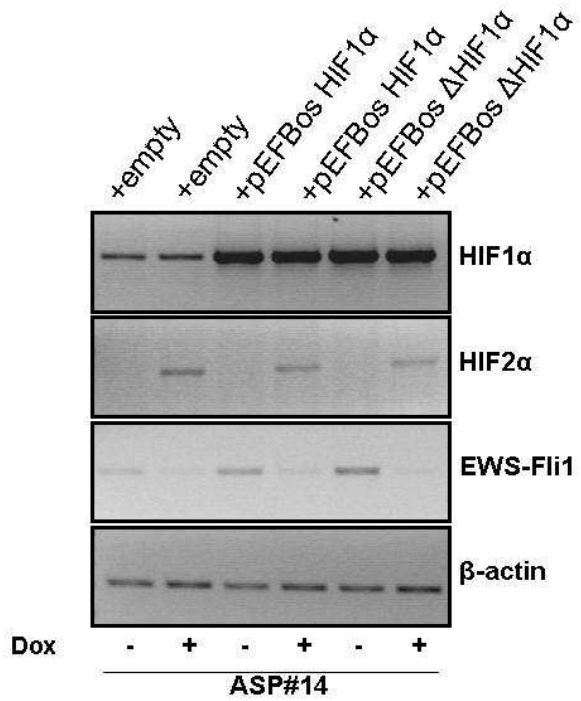

Fig. 20: HIF-2α mRNA levels were induced upon doxycycline-mediated EWS-Fli1 knockdown. RT-PCR of either wild type- or mutant- HIF-1α transfected ASP14 cells. Knock down of EWS-Fli1 via doxyycline [1µg/ml] did not have any affect on HIF-1α mRNA whereas, HIF-2α mRNA levels were induced. This finding suggests that HIF-2α might be a target of EWS-Fli1.

Surprisingly, RT-PCR of transfected ASP14 cells showed that even though HIF-2α mRNA was completely absent in the presence of EWS-Fli1, knockdown of EWS-Fli1 by adding doxycycline induced HIF-2α mRNA. This data suggests that HIF-2α might be a target of EWS-Fli1 in the ASP14 cell line.

3.7 Over-expression of HIF-1α in ASP14 cells leads to increased EWS-Fli1 mRNA levels

As shown in Figure 20, EWS-Fli1 levels were increased in ASP14 cells in the absence of doxycycline due to the over-expression of both wild-type- and mutant HIF-1α. To determine the magnitude of the elevated EWS-Fli1 mRNA levels in these cells we utilized qRT-PCR to quantify this increase properly.

qRT-PCR was carried out by using a probe against EWS-Fli1 (fusion type I), EWS and β2-microglobulin, which was utilized for normalization. The same cDNAs which were used in the previous experiment (Fig.20) served as template for the quantitative RT-PCR.

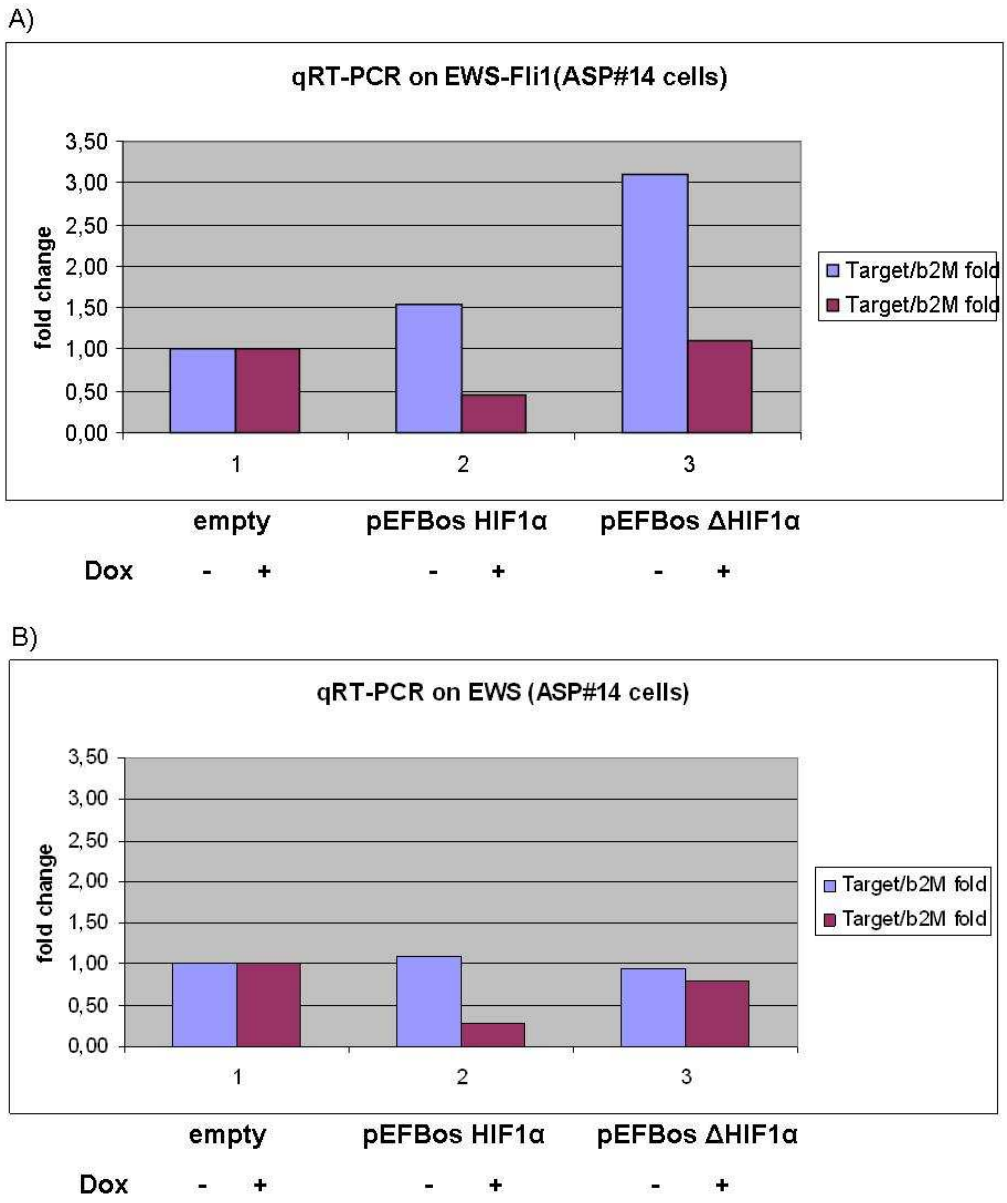

Fig. 21: Quantitative analysis of EWS-Fli1 mRNA levels after over-expression of wild type or mutant HIF-1α in ASP14 cells.

A qRT-PCR of cDNAs (cf. Fig.20) revealed that EWS-Fli1 mRNA was up-regulated due to over-expression of HIF-1α. The highest change, up to 2 fold, was accomplished by transfecting with pEFBos-ΔHIF-1α whereas wild type HIF-1α transfection resulted only in a 0,5 fold change. The experiment was carried out in triplicates and the mean values were used for calculation of fold changes.

In contrast to TC252 and SK-N-MC cells, EWS-Fli1 mRNA levels were slightly up-regulated in ASP14 cells upon HIF-1α/Δ HIF-1α over-expression up to two fold, whereas EWS mRNA levels did not change.

Even though this qRT-PCR analysis revealed that EWS-Fli1 mRNA levels were up-regulated, these findings could not be reproduced in other ESFT cell lines.

3.8 Hypoxia does not enhance proliferation in vitro

Our previous data showed that hypoxia leads to increased EWS-Fli1 levels in a HIF-1α dependent manner, but we further wanted to know if hypoxia causes any functional consequences in vitro.

First, we studied proliferation of ESFT cell lines which have been cultured under hypoxic conditions compared to normoxia treated cells (Fig. 22).

For these studies, 5 x 10^4 cells of the ESFT cell lines TC252 and SK-N-MC were seeded in 6-well plates. One day after seeding, the experiment was started by exposing the cells to either normoxic (21% O_2) or hypoxic conditions (1% O_2; 200µM $CoCl_2$) for three days. The experiment was performed in triplicates and cells were counted every 24 hours.

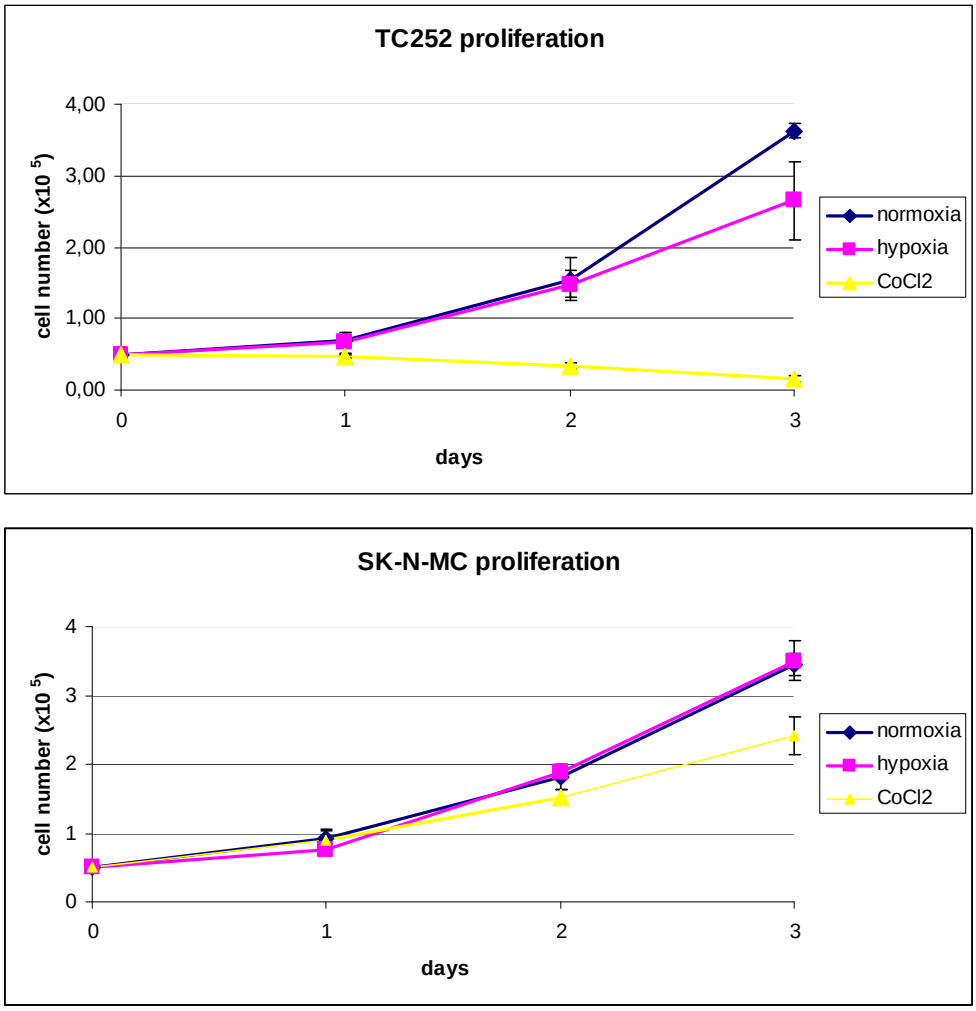

Fig. 22: Proliferation of TC252 and SK-N-MC ESFT cell lines within a period of three days. Experiments were carried out in triplicates and discrimination between living and dead cells was accomplished by Trypan Blue exclusion.

Although hypoxia did not enhance proliferation, neither in TC252 nor in SK-N-MC, clear differences in the response to hypoxic conditions between the cell lines were observed. SK-N-MC cells proliferated fairly consistent both under normoxia and hypoxia and were only slightly affected by $CoCl_2$ treatment. . In contrast, TC252 cells clearly showed decreased proliferation rates and increased apoptosis, suggesting a p53 response under hypoxia.

3.9 Hypoxia mediates a G1-arrest in the ESFT cell line TC252

To study the cell cycle during incubation at hypoxic (1% O_2; 200µM $CoCl_2$) versus normoxic conditions (21% O_2) in more detail, we seeded 2 x 10^4 cells of both TC252 and SK-N-MC in 6-well plates and monitored cell cycle distribution over a period of 5 days by using BD Cycletest™ Plus followed by FACS analysis. Propidium Iodide (PI), added 10 minutes prior to the FACS measurement, was used to determine the amount of dead cells. All experiments were carried out in triplicates.

The cell cycle distribution of SK-N-MC cells, which were incubated for five days either under normoxic or hypoxic conditions, did not reveal outstanding differences. If there was any difference at all, hypoxia treated SK-N-MC cells exhibited a slight increase in G2-phase in the first two days of treatment which adjusted to normal levels already on day 3 (Fig. 23).

Notably, TC252 cells that were cultured under hypoxic conditions, showed a marked G1-arrest compared to the normoxia control. Both, hypoxia (1% O_2) and $CoCl_2$-treatment consistently induced a G1-arrest throughout the first four days. $CoCl_2$ caused the strongest G1-arrest accompanied by reduced cell numbers and apoptosis on days four and five (Fig 24).

Taken together, these results indicate that hypoxia, or treatment with hypoxia mimetics, does not lead to increased S-phases corroborating our previous proliferation data. Therefore hypoxia does not enhance proliferation but rather may induce a p53 dependent cell cycle arrest.

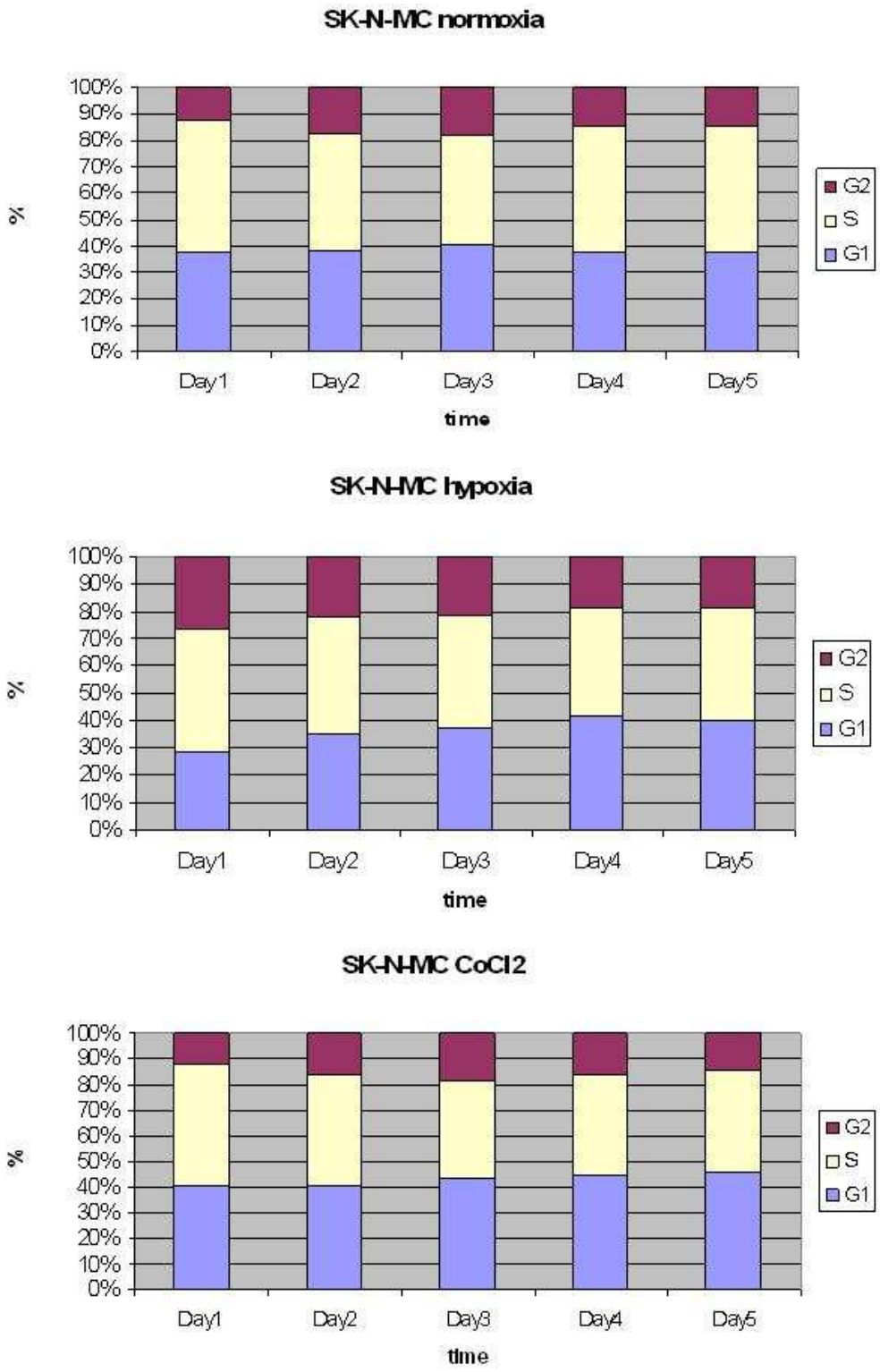

Fig. 23: Cell cycle analysis of the SK-N-MC ESFT cell line.

Cells were cultured under normoxic (21% O_2) and hypoxic conditions (1% O_2; 200µM $CoCl_2$), and DNA content was analyzed by FACS. For this long term study, 2 x 10^4 cells were seeded in 6-well plates; experiments were carried out in triplicates.

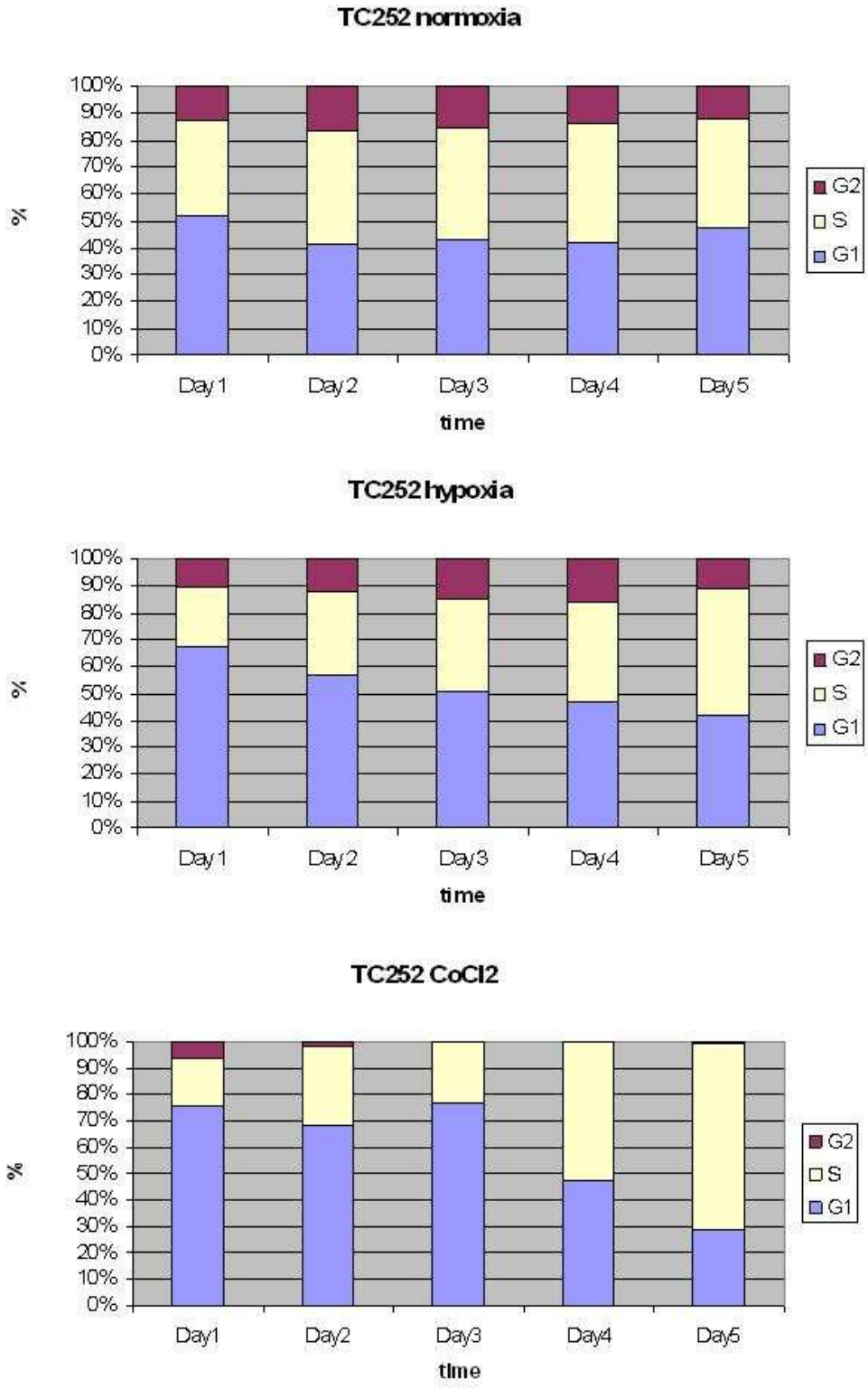

Fig. 24: Cell cycle analysis of the TC252 ESFT cell line.

The same culturing conditions and cell numbers were used as in Fig. 23. TC252 hypoxia and $CoCl_2$ treated cells clearly exhibit a strong G1 arrest, indicating that p53 might affect the response of ESFT cell lines in vitro.

3.10 Hypoxia does not enhance migration in vitro

Since our previous experiment revealed, that there was no proliferation advantage for TC252 and SK-N-MC cell lines that were cultured under hypoxic conditions, we further wanted to know if hypoxia causes any functional consequence on the migration ability of ESFT cell lines using the wound healing (scratch) assay (Fig. 25).

To assure comparable conditions during the wound healing assay, we utilized standardized dishes (ibidi GmBH, Martinsried, Germany) bearing inserts that gave rise to a 400µm ± 50µm scratch area. The insert was composed of two segments that were seeded with either 5 x 10^4 TC252 or 4 x 10^4 SK-N-MC cells. The cells were starved for 18h in serum free medium and inserts were removed on the next day. Pictures were taken 24, 48 and 72 h after removing the insert by using a standard inverted microscope (magnification 5 x).

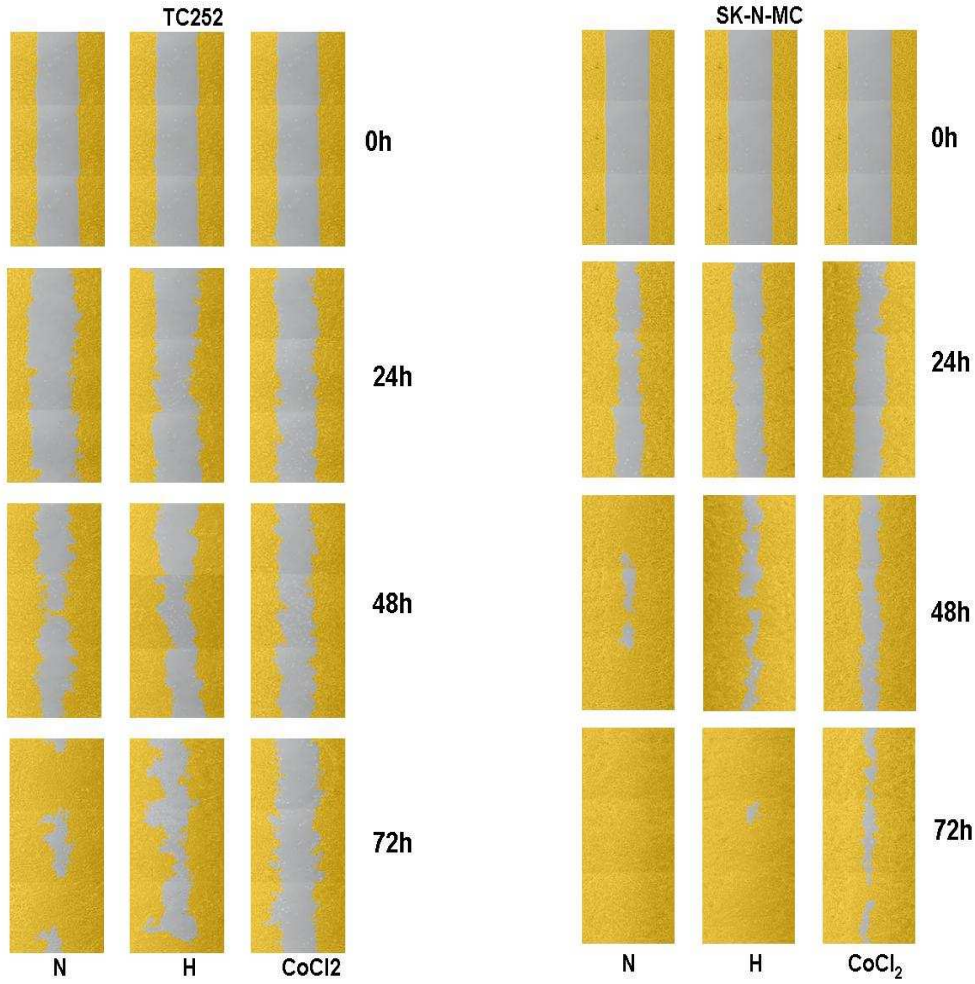

Fig. 25: Migration ability of TC252 and SK-N-MC cells to close a distinct scratch region of 400µm ± 50µm.

Both cell lines were incubated either under hypoxic (H) (1%O_2) or normoxic (N) conditions (21% O_2), or under 200µM $CoCl_2$.

As shown in Fig.26, TC252 cells that were cultured either with 200µM $CoCl_2$ or 1% O_2 revealed almost no migration which corresponded to our previous findings of a hypoxia induced G1 arrest. By contrast, SK-N-MC cells were just slightly affected by hypoxia and migrated fairly consistent.

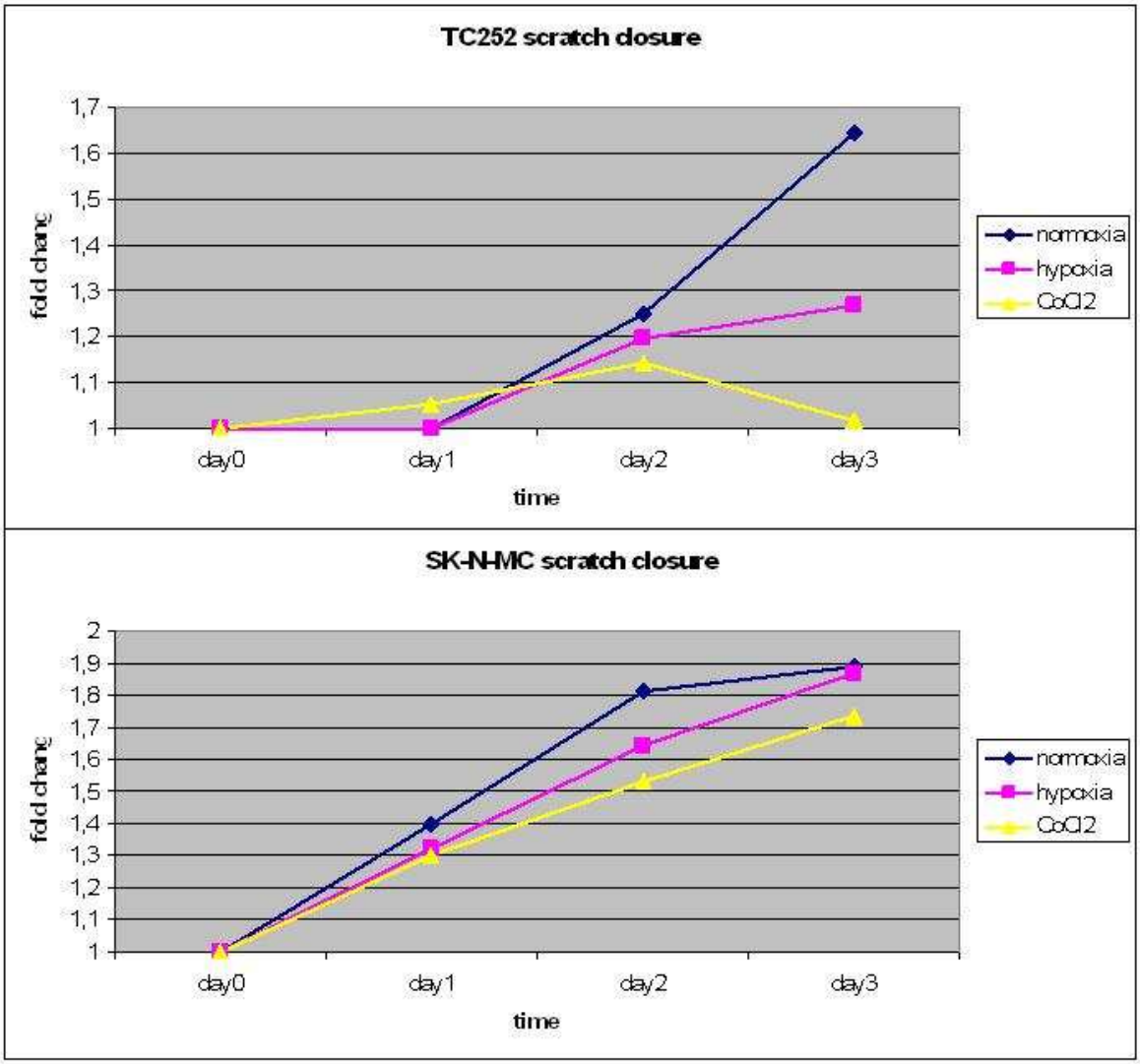

Fig. 26: Schematic representation of ESFT cell lines TC252 and SK-N-MC to close a 400µm scratch over a period of three days.
The experiment was carried out by taking three pictures along the scratch area per day and images were stitched, converted to a uniform dimension and finally analyzed by S.CO LifeScience. The percentage of the cell-covered area at day 0 was utilized as reference-value to calculate fold changes.

This experiment revealed that hypoxia does not induce any increase in the migration ability of Ewing tumor cell lines in vitro.

3.11 Hypoxia affects the invasive capability of ESFT cell lines

Since our previous experiments did not reveal any functional advantage of hypoxia treated ESFT cells on neither proliferation nor migration, we concentrated on the invasive capability of ESFT cell lines that were either cultured under normoxia or hypoxia (Fig.27).

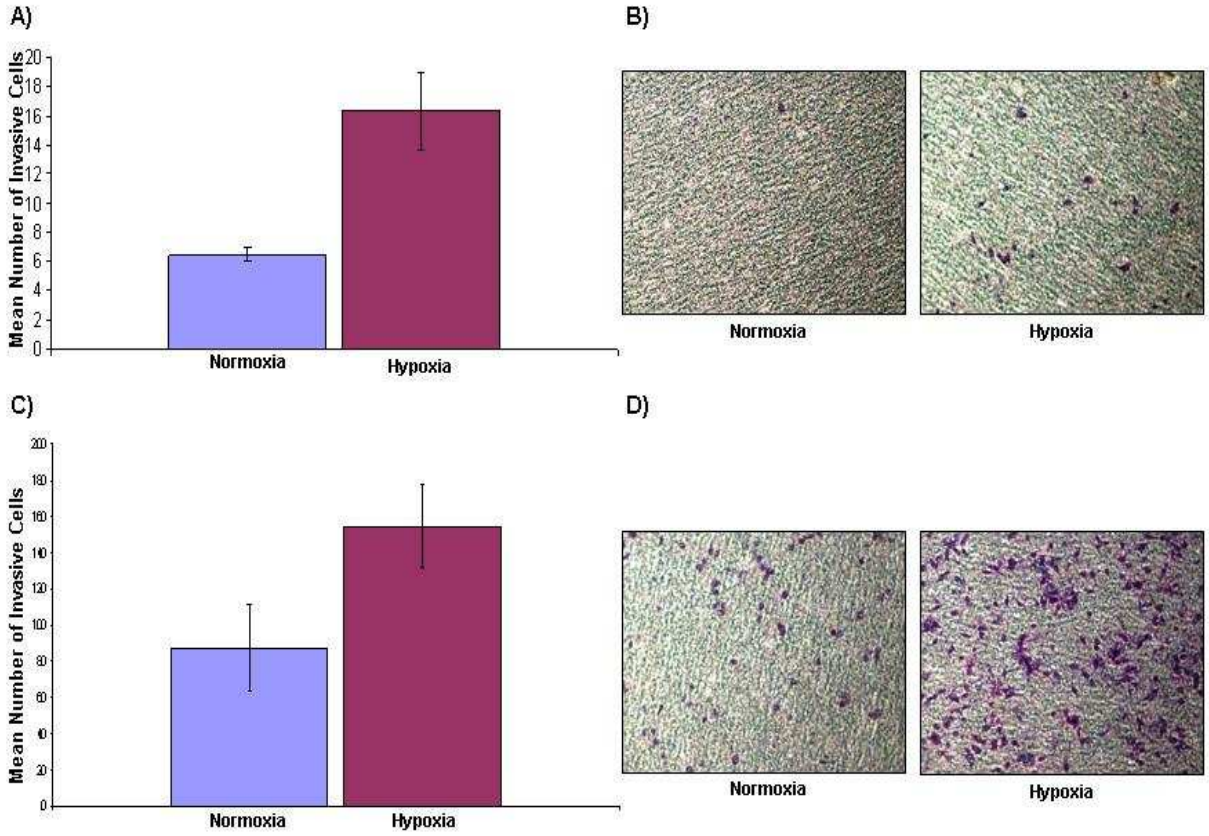

Fig. 27: Invasive capacity of TC252 of ESFT cell lines A) & B) TC252 and C) & D) SK-N-MC. The Experiment was carried out in triplicates for 48 hours. A) and C) Quantification of cells that have invaded into the lower chamber through matrigel. B) and D) Photographs of invading cells on the bottom site of the transwell membrane.

This experiment was carried out by utilizing polycarbonate coated Transwell inserts (Corning Incorporated, Life Sciences, NY, USA) with 8µm pore size and 5 x 10^5 cells were seeded in triplicates. SK-N-MC and TC252 were cultured under normoxic (21% O_2) and hypoxic (1%O_2) conditions for 48 hours, cells were fixed in 4% PFA and stained with 0,2% crystal violet containing 20% methanol. Cells were counted using Image J software and the mean number of invasive cells were monitored.

This experiment clearly showed that both SK-N-MC and TC252 cells that were cultured under hypoxia exhibited an enhanced invasive capability to cross the matrigel barrier compared to the normoxia control.

4 Discussion

The features of many hypoxic tumors have been extensively characterized, such as increased proliferation, invasion and accompanying metastatic potential. These features contribute to the high aggressiveness of hypoxic tumors.

So far, very little is known about the contribution of hypoxia to the aggressiveness of ESFT. Thus our first aim was to investigate whether HIF-1α, which is the main regulator of hypoxia, can be induced during hypoxia treatment of ESFT in vitro.

For that purpose, we chose two representative ESFT cell lines that differed in their p53 status, and treated them with different concentrations of canonical hypoxia mimetics, $CoCl_2$ and DFX. Both compounds have been shown to impede appropriate PHD function, thus stabilizing HIF-1α [152]. The usage of these mimetics was advantageous due to the possibility of culturing the cells under normoxic conditions and an increased probability of stable HIF-1α induction.

We showed that HIF-1α was induced in TC252 and SK-N-MC cell lines, and that the expression magnitude was time and concentration dependent. A similar dose dependent HIF-1α induction by hypoxia mimetics has also been described in a variety of other cancer cell lines [153]. Interestingly, however, our studies revealed that treatment with hypoxia mimetics transiently increased EWS-Fli1 levels with an expression peak at 8 hours of treatment. Since EWS-Fli1 levels are toxic at high concentrations [154] but essential for ESFT cell lines to sustain their cellular functions [155], the fluctuating EWS-Fli1 levels might be explained by an adaptive response mechanism to ensure tolerable EWS-Fli1 levels.

Since both proteins, EWS-Fli1 and HIF-1α, were induced under hypoxia, we tested if these findings were dependent or independent from each other. To assess a potential interplay between these two transcription factors we followed two experimental approaches: First, we over-expressed either a wild-type or a non-degradable HIF-1α protein. Second, we silenced hypoxia induced HIF-1α expression in SK-N-MC and TC252. Ectopic expression of HIF-1α supported our previous findings of enhanced EWS-Fli1 protein levels, whereas EWS-Fli1 was clearly modulated in response to the knockdown of HIF-1α. This indicates that EWS-Fli1 is regulated in an HIF-1α dependent manner. Further investigations are necessary to reveal whether this is due to a direct or indirect mechanism. Collaboration between transcription factors is a well established phenomenon especially in hypoxia

mediated responses [70]. Interestingly, pan-genomic expression profiling studies from our lab revealed that some EWS-Fli1 and HIF-1α target genes are regulated in a synergistic, others in an antagonistic way, indicating that hypoxia influences EWS-Fli1 target regulation (data not shown).

However, EWS-Fli1 mRNA levels were not affected by hypoxia, indicating that the increased protein levels are due to posttranscriptional mechanisms. ASP14 cells represented an exception, showing elevated EWS-Fli1 levels in response to hypoxia, but this finding could not be reproduced in other ESFT cell lines. Further studies on ASP14 revealed that upon EWS-Fli1 knockdown, HIF-2α mRNA was strongly and reproducibly induced, indicating that EWS-Fli1 might repress HIF-2α in ASP14 cells on the transcriptional level.

The question why HIF-2α might be repressed by EWS-Fli1 remains unsolved but it has been shown that HIF-1α and HIF-2α have antagonistic effects [156]. HIF-1α, for example, exhibits both pro- and anti-proliferative properties whereas HIF-2α lacks anti-proliferative properties and is therefore considered to be involved in tumorigenesis even stronger [156].

However, the interplay between HIF-1α and HIF-2α in ESFT, though complex, may be of high relevance to the role of hypoxia for the aggressiveness of Ewing tumors.

We further wanted to know if hypoxia causes any functional consequences to ESFT cell lines in vitro. For that purpose we followed three experimental strategies: a) proliferation assays, to clarify if hypoxia treated ESFT cell lines have any proliferative advantage to normoxic cells, b) 2-D migration assays for testing migration capability under normoxia and hypoxia, and c) invasion assays that should answer the question, if hypoxia can alter the invasive behaviour of Ewing tumor cell lines.

The proliferation assay revealed that there was no hypoxia-driven proliferative advantage for neither SK-N-MC nor TC252 cell lines. More precisely, SK-N-MC cells were almost not affected by hypoxia and proliferation rates were fairly similar to normoxia treated SK-N-MC cells. In contrast, TC252 seemed to be handicapped in their proliferation ability when cultured under hypoxic conditions. Since TC252 cells were wild type for p53 while SK-N-MC cells were mutant, this results may indicate that the p53 status is responsible for an adequate response to hypoxia. It is widely accepted, that hypoxia activates p53 [70] which is one of the major players in mediating stress-sensitivity within a cell [157].

Furthermore, hypoxia mostly induces a G1 arrest in the cell cycle via HIF-1 dependent and independent mechanisms [158], which may explain, why the wildtype p53 cell line TC252 exhibited reduced proliferation ability under hypoxia.

Similar observations were obtained in the wound healing assay. While the capability of SK-N-MC cells to fill the gap was not affected by hypoxia, TC252 cells showed delayed in vitro wound healing. This observation may either be the consequence of impaired migration ability, or reduced proliferation at the margins of the gap.

This result prompted us to analyze whether there was a difference in the cell cycle distribution between the cell lines under hypoxic versus normoxic conditions. Corroborating our results from proliferation assays, hypoxia treated SK-N-MC cells did not exhibit differences to the normoxia treated control cells, but TC252 revealed a strong G1-arrest, explaining the observed disadvantage of TC252 cells to fill the gap in the wound healing assay. Even though proliferation as well as migration are very consistently enhanced under hypoxia in various cell types [159], our experiments did not reveal a similar effect in adherent ESFT cell cultures.

Although there was no functional advantage for hypoxic ESFT cells in either proliferation or migration, we further analyzed the invasive potential of ESFT cell lines under normoxia or hypoxia. Of note, the invasion assay revealed an enhanced invasive capability of both TC252 and SK-N-MC cells, indicating that hypoxia might contribute to the very aggressive phenotype of Ewing tumors. In addition, soft agar assay results from our group revealed that SK-N-MC cells that were cultured under hypoxia tend to form bigger and more colonies than their normoxia counterparts, indicating that hypoxia might increase clonogenicity and induce proliferation under anchorage independent conditions (data not shown).

These results suggest that hypoxia affects proliferation and invasion under anchorage independent conditions, but not in adherent cell cultures. One might speculate that these conditions more closely mirror the in-vivo situation than standard adherent cultures. These in-vitro findings need to be verified in future in-vivo studies.

To address the question which of our findings may be attributed to increased EWS-Fli1 levels and which EWS-Fli1 independent hypoxia-induced effects, it will be necessary to perform invasion and soft agar assays under conditions that mimic increased EWS-Fli1 expression under normoxia, respectively that keep stably low EWS-Fli1 levels under hypoxia.

Taken together, this thesis revealed new insights into the role of hypoxia inducible factors and their putative contribution to the aggressiveness of ESFT cells in vitro, but the question, how these findings correlate to the in vivo situation, should be subject of further investigations in the future.

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