Transcriptional Regulation by Hypoxia-Inducible Factors in Tumor Cells

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TRANSCRIPTIONAL REGULATION BY HYPOXIA-INDUCIBLE FACTORS IN TUMOR CELLS

Akademisk avhandling

Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap i ämnet molekylär medicin kommer att offentligen försvaras i Patologens föreläsningssal, Universitetssjukhuset MAS, Malmö fredagen den 15 juni 2007 kl 08.30

av

Tobias Löfstedt

Fakultetsopponent
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Department of Radiation Oncology
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Stanford, CA 94305, USA
Cancer is a major cause of human morbidity and mortality, and the risk of developing cancer is about one in three lifetime times. Neuroblastoma is the most common extra-cranial solid tumor among children and arises from sympathetic nervous system (SNS) cells arrested in their development. Generally, a low tumor cell differentiation correlates to poor prognosis. Solid tumors, like neuroblastoma, frequently contain regions of oxygen deficiency – hypoxia – caused by a high rate of cellular proliferation and abnormal intratumoral blood supply. In this hypoxic microenvironment cancer cells undergo genetic and molecular changes, allowing continued survival and proliferation. Tumor hypoxia is also associated with increased aggressiveness, resistance to therapy, and poor outcome. Cancer cells become less differentiated in response to hypoxia, which we previously demonstrated in neuroblastomas as well as breast cancer cells, indicating an evolution of a more aggressive phenotype. In the present studies we find evidence of potential mechanisms behind the hypoxia-mediated dedifferentiation of neuroblastoma cells. Hypoxia (1% O2) induced the expression of the negative transcription factor ID3 (Paper I), involving in blocking the function of tissue-specific basic helix-loop-helices (BHLH) proteins, such as the SNS-specifying transcription factors HASS-1 and dHAND. Hypoxic up-regulation of ID3 was dependent on direct or site DNA-binding and activity of hypoxia-inducible factors (HIF), the master transcriptional regulators of oxygen homeostasis. Induction of ID3 expression occurs as an early HIF-mediated hypoxia event, potentially leading to a more immature state.

HIF-1α and HIF-2α, however differently, are both essential for normal development and are highly implicated in tumor progression. In Paper II we show that HIF-1α and HIF-2α share several target genes, but mediate regulation of these under different temporal and oxygen-dependent conditions. Interestingly, HIF-2α, but not HIF-1α, was present in neuroblastoma tumor cells near blood vessels, and thus in apparently better-oxygenized tumor regions. In vitro, HIF-1α protein was transiently stabilized at hypoxia and primarily governed acute hypoxic responses, whereas HIF-2α became more important at prolonged hypoxia. In addition, high HIF-2α activity, including induction of classic hypoxic targets such as VEGF, was detected in cultured neuroblastoma cells already at 5% O2, a physiologically relevant oxygen level, similar to the findings in vivo. In a large clinical neuroblastoma material, significant correlations between high HIF-2α levels and high VEGF content, advanced tumor stage and poor outcome were found. These observations clearly suggest an oncogenic role of HIF-2α, and implicate HIF-2α as an independent prognostic marker in neuroblastoma.

The MX1 (MAX-inhibitor) 1 gene, a reported MYC antagonist, has been detected by us and others as a commonly hypoxia-induced gene. In Paper III we further demonstrated that MX1 proteins, via direct binding to hypoxia-response elements (HREs), up-regulate MX12 mRNA and protein in both hypoxic neuroblastomas and breast cancer cells. Interestingly, reducing MX12 levels had no overall effects on MYC/MYCIN activity in hypoxic neuroblastoma cells. Instead, MX12 appeared to be important in augmenting the hypoxic response, potentially by enhancing specific HIF-1 target gene induction. HIF proteins are primarily stabilized and activated in response to lowered oxygen concentrations. However, growth factor-induced signaling can promote HIF-1α protein synthesis as well as transactivation, even under normoxic conditions. In Paper IV we characterize a novel such a pathway, where stem cell factor (SCF)-evoked c-kit-signaling leads to increased HIF-1α protein, HRE-activation and induction of several HIF-1α targets, such as VEGF and GLUT1, already at normoxia. In addition we find a reciprocal positive feedback loop between c-kit and HIF-1α, where induced HIF-1α mediates reinforcement of c-kit expression.

Overall, this thesis shows the impact of HIF proteins on tumor cell behavior, principally as central hypoxic transcriptional regulators governing the expression of genes with potential importance in several biological processes, such as growth and differentiation, determining cancer cell aggressiveness as well as adaptation to low oxygen conditions.

Key words: Cancer, neuroblastoma, transcription, hypoxia, HIF-1, HIF-2, differentiation, ID2, MX1, MYCN, c-Kit

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Security classification

Signature: Tobias Löfstedt

Date: May 5, 2007
From the Department of Laboratory Medicine, Division of Molecular Medicine, Lund University, Sweden

TRANSCRIPTIONAL REGULATION BY HYPOXIA-INDUCIBLE FACTORS IN TUMOR CELLS

Tobias Löfstedt

Lund University
Faculty of Medicine

Academic Dissertation
Lund 2007
For my Family
CONTENTS

LIST OF PAPERS 8
ABBREVIATIONS 9
INTRODUCTION 11
BACKGROUND 13
   Cancer and tumor progression 13
   Neuroblastoma 16
      Neuroblastoma incidence, prognosis and staging 16
      Genomic abnormalities in neuroblastoma 17
      Aberrant gene expression patterns in neuroblastoma 19
   The sympathetic nervous system (SNS) 20
      Transcription factors in the regulation of SNS development 22
      The MYC-MAX-MAD network 23
      ID proteins 25
   Tumor hypoxia 26
      Responses to hypoxia and malignant progression 28
   Hypoxia-Inducible Factors (HIFs) 30
      Structure of HIF 30
      Oxygen-dependent HIF regulation: stability 32
      Oxygen-dependent HIF regulation: transactivation 34
      Oxygen-independent regulation of HIF proteins 35
      HIF proteins in development 38
Adult tissue expression of HIF 41
Functions and involvement of HIF proteins in tumors 41
HIF-1α versus HIF-2α 44

THE PRESENT INVESTIGATION 47

Aims 47

Results and Discussion 47

The effects of hypoxia on differentiation 47
HIF-1α induces ID2 expression – link to a de-differentiated and aggressive neuroblastoma phenotype (Paper I) 49
Regulation and target gene expression of HIF-1α and HIF-2α in neuroblastoma (Paper II) 52
HIF-2α confers higher neuroblastoma aggressiveness (Paper II) 54
HIF-induced MXI1 does not affect MYC/MYCN signaling, but promotes specific HIF-1α target gene expression in hypoxic neuroblastoma cells (Paper III) 56
Reciprocal regulation between c-Kit signaling and HIF-1α activity (Paper IV) 58

Conclusions 60

POPULARIZED SUMMARY IN SWEDISH 61

ACKNOWLEDGEMENTS 64

REFERENCES 66

APPENDIX

Paper I
Paper II
Paper III
Paper IV
LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals:


III. MXI1 is a direct target of HIF and enhances specific HIF-1 target gene induction under hypoxia. Tobias Löfstedt, Erik Fredlund, Rosa Noguera, Samuel Navarro, Linda Holmquist Mengelbier, Siv Beckman, Sven Påhlman and Håkan Axelson. *Manuscript*.

IV. Positive stem cell factor-induced feedback regulation by Hypoxia-Inducible Factor-1α under normoxia. Malin Pedersen, Tobias Löfstedt, Jianmin Sun, Linda Holmquist Mengelbier, Sven Påhlman and Lars Rönnstrand. *Manuscript*.

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### ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AK3L1</td>
<td>adenylate kinase 3-like 1</td>
</tr>
<tr>
<td>ARD1</td>
<td>arrest defective-1 protein</td>
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<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>ATF3</td>
<td>activating transcription factor 3</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCL</td>
<td>B-cell lymphoma protein</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BNIP3</td>
<td>BCL2/adenovirus E1B 19kD-interacting protein 3</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response element binding protein</td>
</tr>
<tr>
<td>dHAND</td>
<td>deciduum, heart, autonomic nervous system, and neural crest derivatives</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FIH</td>
<td>factor inhibiting HIF</td>
</tr>
<tr>
<td>GAP43</td>
<td>growth-associated protein 43</td>
</tr>
<tr>
<td>GF</td>
<td>growth factor</td>
</tr>
<tr>
<td>HASH-1</td>
<td>human achaete-scute homologue-1</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HES</td>
<td>hairy/enhancer of split</td>
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<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>HK2</td>
<td>hexokinase 2</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia-response element</td>
</tr>
<tr>
<td>ID</td>
<td>inhibitor of differentiation/DNA-binding</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>INSS</td>
<td>International Neuroblastoma Staging System</td>
</tr>
<tr>
<td>IPAS</td>
<td>inhibitory PAS domain protein</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>MAD</td>
<td>MAX dimerization protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAX</td>
<td>MYC-associated factor X</td>
</tr>
<tr>
<td>MCM7</td>
<td>minichromosome maintenance protein 7</td>
</tr>
<tr>
<td>MDM2</td>
<td>mouse double minute 2</td>
</tr>
<tr>
<td>MDR1</td>
<td>multidrug resistance gene 1</td>
</tr>
<tr>
<td>MSH</td>
<td>mutS homologue</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>MXI1</td>
<td>MAX-interactor 1</td>
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<tr>
<td>MYC</td>
<td>myelocytomatosis protein</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NB</td>
<td>neuroblastoma</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NEMO</td>
<td>nuclear factor kappa-B (NF-κB) essential modulator</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>OCT-4</td>
<td>octamer-4 homeodomain transcription factor</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>ODD</td>
<td>oxygen-dependent degradation domain</td>
</tr>
<tr>
<td>PAS</td>
<td>PER/ARNT/SIM</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PHD</td>
<td>prolyl-hydroxylase domain-containing protein</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue, deleted on chromosome 10</td>
</tr>
<tr>
<td>QPCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RB</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>RCC</td>
<td>renal cell carcinoma</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SERPINB9</td>
<td>serine protease inhibitor, member 9</td>
</tr>
<tr>
<td>SHH</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SIF</td>
<td>small intensely fluorescent</td>
</tr>
<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
</tr>
<tr>
<td>SP1</td>
<td>specificity protein 1</td>
</tr>
<tr>
<td>TAD</td>
<td>transactivation domain</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TP53</td>
<td>tumor suppressor protein 53</td>
</tr>
<tr>
<td>TRK</td>
<td>tropomyosin receptor kinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
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INTRODUCTION

This work was undertaken in order to increase our understanding of the biology of the pediatric cancer neuroblastoma, which is the most common extra-cranial solid tumor occurring during childhood. Neuroblastoma arises from the developing sympathetic nervous system (SNS), most likely as a consequence of aberrant or arrested differentiation of sympato-adrenal precursors on their path to a terminal state as functional neurons and neuroendocrine cells. This enigmatic tumor type displays a high degree of heterogeneity, where some lesions are found to differentiate or spontaneously regress into a benign state, but where also severely aggressive high stage tumors with extensive metastases and poor prognosis are exhibited. Large efforts have been made to clarify the molecular mechanisms underlying this malignancy, and an important research field concerns the transcriptional regulation of the approximately 25,000 to 30,000 existing human genes. Knowledge about the transcription factors governing this extensive apparatus is fundamental for deciphering essential biological processes. The basic helix-loop-helix (bHLH) superfamily of transcription factors are key regulators in development, cell fate determination and proliferation of a multitude of cell types. Abnormal function of bHLH proteins is therefore implicated in various diseases, including cancer. Understanding the neuroblastoma gene expression program, which is clearly an aberrant reflection of the corresponding program of the normal tissue from which the tumor originates, for neuroblastoma the early SNS progenitor cells, could aid in the explanation of the vast tumor heterogeneity observed and also in the elucidation of neuroblastoma tumorigenesis.

It is also of importance to consider the impact of tumor microenvironment on cancer cell gene expression and behavior. In most solid tumors, among them neuroblastoma, the high rate of cellular proliferation outpaces the formation of new blood vessels, leading to intra-tumoral regions with high interstitial pressure, insufficient supply of nutrients, acidification, and low availability of oxygen – hypoxia. In this apparently hostile tumor microenvironment cancer cells can ultimately adapt for continued survival and proliferation under hypoxic conditions. Intra-tumoral oxygen measurements have revealed that hypoxic tumors are more aggressive than better-oxygenated ones, and tumor hypoxia also correlates with increased resistance to anti-cancer therapy and worsened patient outcome in several human cancers. Neuroblastoma cells cultured at hypoxia appear to develop a more immature,
stem cell-like phenotype, or de-differentiate, a trait associated with more aggressive tumors in neuroblastoma children.

The most important mammalian proteins controlling the adaptation to low oxygen conditions are the hypoxia-inducible transcription factors (HIF)-1α and HIF-2α. HIF proteins belong to the bHLH/PAS (PER/ARNT/SIM) subgroup and are readily degraded under conditions of sufficient oxygen supply, but rapidly stabilized and activated at hypoxia. By binding to hypoxia-response elements (HREs) within gene regulatory DNA sequences, HIF proteins control the expression of an increasingly growing number of target genes involved in e.g. angiogenesis, glucose transport and metabolism, erythropoiesis, cell growth and apoptosis. Given that HIF proteins are critically involved in development, in postnatal physiology and in cancer patophysiology, the studies behind this thesis therefore focus on the role of hypoxia and HIF proteins in the biology of neuroblastoma and in other tumor cells.
BACKGROUND

Cancer and tumor progression

In the developed countries of the world, more than one person in five will die of cancer. The incidence of cancer is as high as one in three life times, and as the average age of the general population increases so does the rate of cancer diagnoses. More than 100 distinct types of cancer exist with different etiology and epidemiology, and subtypes of tumors can be found within specific organs. Cancer cells, by definition, proliferate in defiance of normal controls, i.e. they are neoplastic, and are able to invade and colonize surrounding or even distant tissues, i.e. they are malignant. By giving rise to secondary tumors, or metastases, cancer cells become hard to eradicate surgically, which also severely reduces patient survival.

Most cancers are thought to originate from a single cell that has undergone a somatic mutation, however, tumorigenesis is a multistep process and the progeny of this cell must undergo further changes before they become cancerous. Several types of human cancers are diagnosed with an age-dependent incidence, implicating four to seven rate-limiting stochastic events required for the development of a full-blown cancerous lesion [1]. Hanahan and Weinberg [2] have set up six acquired capabilities that are shared by most, if not all, types of human cancer: (i) self-sufficiency of growth signals, (ii) insensitivity to growth-inhibitory signals, (iii) evasion of apoptosis, (iv) unstrained replicative potential, (v) maintained angiogenesis and, (vi) tissue invasion and metastasis. The order in which these capabilities are acquired and the underlying mechanisms seem to vary among cancer forms. Since the progression of normal cells into cancer cells largely occurs through a succession of genetic changes, each yielding a certain growth advantage, tumor development is highly analogous to the concept of Darwinian evolution [3].

For initiation of proliferation, normal cells require mitogenic growth signals, transmitted via membrane-bound receptors that bind growth factors (GF), extracellular matrix (ECM) molecules, and cell-to-cell interaction components. In contrast, tumor cells display a greatly reduced dependence on exogenous growth stimulation, and are capable of generating many of their own mitogenic signals. Cancer cells may acquire the ability to synthesize GFs, e.g. PDGF and TGF-α, thereby creating a positive feedback loop known as autocrine stimulation [2]. Over-expression or activation mutations of growth factor-binding receptors, such as EGFR and HER2, may hypersensitize tumor cells to normally non-mitogenic levels of
GFs, or could even elicit ligand-independent signaling [4]. Aberrations of molecules in the
down-stream intracellular signaling pathway can also lead to autonomy of growth stimulation,
one example being RAS which is structurally altered in about 25% of human tumors [5].
Genes such as those mentioned above have normal functions in control of growth and
survival, but are through dysregulated expression or hyperactivation converted into oncogenes, mimicking normal growth signaling in one way or another. In addition, tumors are
heterotypic masses of cells, and successful cancer cells have also acquired the ability to
induce growth-stimulating signals from their normal neighbors, such as fibroblasts, endothelial and immune cells [6].

In normal tissues, mitogenic stimulation is balanced by antiproliferative signals,
mediated by transmembrane receptors coupled to cytoplasmic signaling circuits. Many of
these growth-inhibitory signals ultimately converge onto the RB pathway, regulating the
transition from G1 to S phase of the cell cycle. Human cancers frequently show disruption of
RB function, often via direct RB gene mutation or sequestration by viral oncoproteins (in DNA virus-induced tumors), rendering cells insensitive to antigrowth factors normally
operating along this pathway. RB is the prototype of a classical tumor suppressor gene, the
loss of function of which promotes growth and malignancy. Cancer cells can also avoid
growth-inhibitory terminal differentiation through over-expression of key oncogenic
transcription factors, such as the bHLH MYC (or MYCN as in neuroblastoma) oncoprotein,
favoring the formation of growth-promoting MYC-MAX complexes over differentiation-
inducing MAD-MAX complexes within this transcription factor network [2].

A hallmark of perhaps all types of cancer is the resistance towards programmed
cell death – apoptosis. The apoptotic program is latent in principally all cells throughout the
body, until activated by DNA damage, signaling imbalance, survival factor deficiency or
hypoxia, and is essential for maintaining homeostasis by balancing cell proliferation with cell
elimination. Apoptosis can be avoided by induction of autocrine survival factors, such as
insulin-like growth factors (IGF-1/2), or by directly inhibiting mitochondrial release of
cytochrome C, a potent mediator of apoptosis. Members of the BCL2 protein family, being
either pro-apoptotic (BAX, BAK, and BAD) or anti-apoptotic (BCL2, BCL-XL and BCL-W),
regulate in part mitochondrial death signaling by controlling cytochrome C release [7]. Both
this intrinsic pathway and extrinsic death signals activate downstream caspases, which are
proteases attacking the genome and cellular structures, ultimately leading to a controlled
elimination of the cell. The most common apoptosis-evading strategy used by cancer cells is
the functional inactivation of the TP53 tumor suppressor pathway, which is seen in more than
50% of human tumors. TP53 is central in detection of and cellular response to DNA damage and can elicit the apoptotic cascade, partly by inducing the pro-apoptotic protein BAX.

To ensure expansive tumor growth, in addition to growth signal autonomy, inhibition of antiproliferative signals and apoptotic resistance, cancer cells must also acquire the ability to multiply without limit - a process termed immortalization [8]. All mammalian cells, with the exception of germ cells and other normal cells bearing stem cell-like features, possess an intrinsic, cell-autonomous program that limits their multiplication. After a certain number of doublings normal cells stop dividing, which is known as a state of senescence, but cancer cells can circumvent this process by disruption of e.g. the RB and TP53 tumor suppressor proteins. However, successive cycles of proliferation causes shortening of the chromosomal ends, the telomeres, a process functioning as an internal “cell-clock” and eventually leads to the later stage of senescence, termed crisis, manifested by massive cell death. During embryogenesis and in cells with a high proliferation rate, telomerase protects the ends of the chromosomes, and almost all malignant cells have upregulated the expression of this enzyme and can thereby avoid senescence and crisis [9, 10].

During the continuous proliferation of cancer cells, the need for supply of oxygen and nutrients can at one point not be maintained by the blood vessels pre-existing within the tissue of tumor site. Supply of oxygen is limited to approximately ten cell diameters and induction of neovascularization, or angiogenesis, is required for the progression of a neoplasm to a larger size [11]. This process is carefully regulated by a balance between positive and negative signals in normal tissues, ensuring a functional capillary network. Developing tumors induce a shift, also known as the “angiogenic switch”, in this balance favoring angiogenesis-initiating signals, the most important being vascular endothelial growth factor (VEGF), over prototypical angiogenesis inhibitors such as thrombospondin. VEGF is potently induced by hypoxia, a feature present in most solid tumors (further discussed below). Attraction of blood and lymphatic vessels also greatly facilitates spread of the tumor by invasion and metastasis, two closely related processes involving changes in cell-cell and cell-matrix interactions and activation of extracellular proteases. Metastasizing cancer cells must move out from the primary tumor, invade adjacent tissues, and travel via the circulation to distant tissues for the foundation of new colonies. The dissemination of tumors via metastases is a major cause of human cancer deaths [12].

The progressive genetic alterations leading to the development of a malignant cell are limited by the inefficient process of mutating specific genes in succession. In normal cells, genomic integrity is maintained by several DNA monitoring and repair enzymes,
ensuring that persistent mutations are rare events and that proliferation is blocked upon
detection of genomic damage. Thus, by inducing dysfunction of these DNA caretaker
systems, a premalignant cell can obtain genomic instability, which increases the frequency of
mutations and enables genetic and signaling alterations needed for the six mentioned acquired
capabilities to occur within a human life span.

**Neuroblastoma**

**Neuroblastoma incidence, prognosis and staging**

Neuroblastoma (NB) is the most frequent solid extra-cranial neoplasia affecting children,
accounting for 7-10% of all pediatric malignancies with an annual incidence of 10-12 cases
per million in the population younger than 15 years [13, 14]. An average of 14 children are
diagnosed with neuroblastoma each year in Sweden [15], thereby comprising around 6% of
the pediatric tumors. The median age at diagnosis is 18 months and only 2-4% of the cases
occur after the age of ten [16]. Furthermore, children in whom the diagnosis is made before 1-
2 years often have a better prognosis than cases found in older children. No major etiological
factor causing neuroblastoma has yet been determined, and the importance of environmental
risk factors is uncertain. Although neuroblastoma is a rare disease it is responsible for about
15% of all pediatric cancer deaths [17].

Neuroblastoma originates from abnormal cellular differentiation within the
developing sympathetic nervous system (SNS). Tumors can occur at any SNS location and
primary lesions are most common in the adrenal glands and abdominal sympathetic paraspinal
ganglia, but NB metastases disseminate to other tissues, including bone, liver and lung [18].
Disease symptoms are quite unspecific, but include fatigue, abdominal pain, appetite loss and
diarrhea [19]. Neuroblastoma prognosis is highly dependent on several clinical, biochemical
and genetic features. Tumors are classified according to the International Neuroblastoma
Staging System (INSS), which include parameters such as localization of the primary tumor,
dissemination to lymph nodes and the level of metastasis [20]. This system highlights the vast
degree of heterogeneity among neuroblastomas. Stage 1 and 2 tumors are localized, show
good prognosis and may only require surgery or low-intensity chemotherapy. In contrast,
stage 3 and especially stage 4 comprise extremely aggressive tumors with frequent metastases
and poor prognosis, and are very hard to treat even with advanced radiation or chemotoxic
drugs. An additional stage, 4S, comprises a special subtype of tumors with limited dissemination to liver, skin and/or bone marrow, but with good prognosis and low requirements of treatment. These tumors only occur in children younger than one year of age, and are even known to spontaneously mature/regress, a phenomenon rarely exhibited in tumor biology [20, 21]. Treated neuroblastoma children have an overall survival rate of 50%, but this number vary greatly depending on the classification of the tumor, with approximately 80% survival for stage 4S but only around 20% for aggressive stage 4 neuroblastomas. Tumor classification by INSS and the age of the child at diagnosis are the most important clinically used parameters for predicting neuroblastoma outcome. A set of serum markers has also been proposed, mainly used in monitoring disease activity, including ferritin, lactate dehydrogenase, the disialoganglioside G_{12} and neuron-specific enolase (NSE). Increased serum levels of these markers associates with advanced disease and low neuroblastoma survival [22-24]. In addition, tumors are classified according to a histopathological system [25], utilizing the tumor stromal content, tumor cell mitosis-karyorrhexis index, and the level of cellular differentiation. Several differentiation marker genes, expressed at different neuroblastoma stages, have been identified and compared to corresponding time-point of expression during development of the SNS [26]. Generally, a low level of tumor cell differentiation is associated with more aggressive neuroblastomas and worse prognosis [27, 28].

**Genomic abnormalities in neuroblastoma**

Only a subset of neuroblastoma children display an inherited predisposition to develop the disease. The median age at diagnosis for these cases is substantially lower than that of sporadic neuroblastomas, indicating consistency with Knudson’s two-hit hypothesis for the origin of childhood cancer [29]. Possibly, alterations at a locus on chromosome 16p12-13 could be responsible for neuroblastoma predisposition [30]. However, most neuroblastomas occur spontaneously, and one important developmental factor concerns DNA content of the tumor cells. Somewhat paradoxical, infants with hyperdiploid or near-triploid tumors (but lacking MYCN amplification) show a favorable outcome [31]. The explanation for this finding could be that infants generally have whole chromosome gains without structural alterations, and the prognostic value of ploidy is also lost in neuroblastoma children older than 1-2 years, potentially suffering from several genomic rearrangements [32].
One of the most significant prognostic markers of neuroblastoma is amplification of the \textit{MYCN} oncogene on chromosome 2p24 [33], which has an overall prevalence of 22\% in neuroblastoma tumors [34]. \textit{MYCN} gene amplification correlates with advanced disease stage, rapid tumor progression and poor prognosis, irrespective of patient age [35, 36]. As an exception, this correlation is less evident in children with 4S tumors [37]. The reason why \textit{MYCN} amplification relates to a more aggressive neuroblastoma phenotype is uncertain, but over-expression of the \textit{MYCN} protein causes deregulated proliferation via formation of a transcription-activating complex with its obligate partner MAX. \textit{MYCN} target genes such as \textit{ODC} and \textit{MCM7}, are also involved in progression through the G1 phase of the cell cycle. In addition, other genes located within the amplified region may contribute to the worsened outcome for children with neuroblastoma tumors carrying \textit{MYCN} amplification.

Trisomy of the long arm of chromosome 17 (17q), often as an unbalanced translocation with chromosome 1p, is the most common genetic abnormality in neuroblastoma, found in more than half of the tumors and leads to more aggressive neuroblastomas [38]. The gain of chromosome 17q indicates a dosage effect of gene(s) located in this chromosomal region, providing a selective advantage. One candidate factor could be the anti-apoptotic protein – survivin [39]. Another common abnormality is deletion, or loss of heterozygosity (LOH), of the short arm of chromosome 1 (1p). Loss of this region has an actual prevalence close to 35\% in neuroblastomas, and is associated with advanced disease and \textit{MYCN} amplification [40, 41]. The exact site of deletion is not clarified, but investigations concerning a region at 1p36 have been ongoing for decades to identify one or more potential candidate tumor suppressor genes, the loss of which could be involved in the progression of neuroblastoma [42-44]. Furthermore, other potentially important chromosomal deletions with relatively high frequency have been identified in neuroblastoma tumors, primarily concerning loss of 11q [45] and 14q [46].

Interestingly, the \textit{TP53} tumor suppressor gene, which is the most commonly mutated gene in human cancer, is rarely altered in primary neuroblastomas [47]. Although evidence suggests that \textit{TP53} mutations are more common in tumors from relapse neuroblastoma children [48], the involvement of this gene in neuroblastoma is still controversial. Nevertheless, in the light of its general importance in cancer progression, the \textit{TP53} pathway might still be impeded in this malignancy. A recent report suggests that \textit{TP53} is retained in the cytoplasm by the parkin-like protein, PARC, representing a non-mutational mechanism of \textit{TP53} inactivation [49]. However, exposure of neuroblastoma cells to DNA-damaging agents still causes stabilization and nuclear translocation of \textit{TP53}, despite potent
cytoplasmic sequestration before exposure, and further studies are therefore required to ascertain the precise role of the TP53 pathway in neuroblastoma pathogenesis [49, 50].

**Figure 1. Genetic model of neuroblastoma development.** This model indicates that all neuroblastomas have a common precursor, but commitment is made to progress into one of two main types. Type 1 is characterized by aberrant mitosis causing a hyperdiploid or near-triploid karyotype, but with a low frequency of genomic rearrangements. These tumors often show high TRKA expression, and may undergo differentiation or apoptosis depending on the presence or absence of NGF, respectively. The Type 2 neuroblastomas are generally near-diploid or near-tetraploid, and have several genetic alterations with gain of material on 17q as a common event. Two subsets of this type can be distinguished. One subset more often displays 11q deletion and/or 14q deletion, whereas the second subtype exhibits loss of heterozygosity at 1p, with or without amplification of the MYCN oncogene. The tumors with 1p deletion often have expression of both TRKB and its ligand, BDNF, potentially creating a positive autocrine survival loop. Adapted from [18].

**Aberrant gene expression patterns in neuroblastoma**

One major signaling pathway that could be involved in the malignant transformation of sympathetic neuroblasts to neuroblastoma cells concerns the neurotrophin tyrosine kinase receptors TRKA, TRKB and TRKC, and their respective ligands: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3. Activation of TRKA is linked to survival and differentiation, whereas TRKA inhibition can lead to apoptosis, indicating the important role of NGF on cellular behavior. High expression of TRKA in neuroblastoma has been linked to younger age, low tumor stage, lack of MYCN-amplification, and a favorable outcome [51, 52]. TRKA/NGF signaling is involved in the differentiation of neuroblasts to a more mature stage, and thus potentially in the differentiation or regression of specific neuroblastoma tumors. In contrast, deprivation of NGF in the tumor microenvironment could
result in TRKA-dependent induction of apoptosis, and thereby lead to neuroblastoma regression [51]. Similar to TRKA, the expression of TRKC is predominantly found in lower-stage tumors lacking MYCN-amplification. Expression of the full-length TRKB receptor is, however, strongly associated with MYCN-amplified, unfavorable neuroblastomas [53]. These tumors also frequently express the TRKB ligand, BDNF, possibly creating an autocrine or paracrine loop for increased survival [54].

Other genes, perhaps involved more in general aspects of tumor progression than in neuroblastoma specifically, with high or deregulated expression and of potential clinical significance have also been implicated in this tumor type, such as the multidrug resistance gene 1 (MDR1) [55] and telomerase [56]. A simplified model of neuroblastoma development is depicted in Figure 1.

**The sympathetic nervous system (SNS)**

Neuroblastoma tumors arise from immature cells of the developing sympathetic nervous system (SNS) [57, 58]. Thus, it is of importance to understand the regulation of SNS development, and the differentiation cues affecting the cells involved. The human nervous system is divided into the central nervous system (CNS) and the peripheral nervous system (PNS). The autonomic part of the PNS is subdivided into the sympathetic and the parasympathetic nervous systems, which control inner organ function. Three closely related cell types constitute SNS tissues (Fig. 2), sympathetic neurons, chromaffin cells and small intensely fluorescent (SIF) cells, all derived from sympatho-adrenal progenitors [59]. Sympathetic neurons and, during development, SIF cells make up the chains of sympathetic ganglia, located in parallel on both sides of the spinal cord and in the trunk. Chromaffin cells are the primary cell type of the adrenal medulla and of sympathetic paraganglia. All SNS cells produce catecholamines (adrenaline or noradrenaline) and, thus, the adult SNS controls e.g. respiration and cardiovascular activity, and has a primary function in handling physical and emotional stress responses, preparing the body for emergency situations, the so-called “fight-or-flight” response. In the fetus, catecholamine production is mainly provided by the early SNS structures, the paraganglia (organ of Zuckerkandl), which have an important function in regulating responses to, for instance, hypoxia [60]. The paraganglia regress during early childhood and their role is overtaken by the adrenal glands.
During development, the ectoderm, the endoderm and the mesoderm make up the three germ layers of the human embryo. Between weeks 3–4 of gestation, the ectodermally-derived neural plate invaginates ventrally toward the mesodermal notochord, thereby forming the neural tube which later develops into the central nervous system. From the lateral ridges of the closing neural tube, on the dorsal side, the neural crest cells are formed which later migrate throughout the embryo (Fig. 2). These migrating cells develop into several different cell types, such as melanocytes of the skin, cartilage and bone, smooth muscle cells, glial cells and most cells of the peripheral nervous system, including the sympatho-adrenal progenitors later forming the SNS cells. The ultimate cell fate is dependent on where on the embryonic anterior-posterior axis they are formed, on regional and cell-specific transcription factors [61], in addition to developmental signals derived from organizing structures surrounding the neural crest precursors and their site of destination. Important factors involved in this process are Sonic hedgehog (SHH), fibroblast growth factor (FGF), and the bone morphogenetic proteins (BMP-4 and -7, for example), the latter which are produced by the forming dorsal aorta and can induce tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis [62, 63].
Transcription factors in the regulation of SNS development

Both extrinsic and intrinsic factors, involved in controlling cell fate and differentiation of the migrating neural crest cells and the forming SNS tissues, elicit different gene expression programs that are regulated by transcription factors. Neuroblastoma cells, which are inhibited in their differentiation, show several traits of immature sympathetic precursors, including sustained expression of transcription factors normally found only during certain periods of embryonic development. Many of these transcription factors belong to the basic helix-loop-helix (bHLH) family, such as HASH-1 (human achaete-scute homologue-1) that has been shown to be required for proneuronal cell fate decision within neural crest and SNS development. Most neuroblastoma tumors and cell lines have also been shown to express HASH-1 [64]. This protein forms a transcription-activating complex with ubiquitously expressed bHLH factors named E-proteins, including E2-2, E12 and E47 [65-67]. Similar to the MYC network of transcription factors (see below), HASH-1 together with E-proteins bind E-boxes (consensus: CANNTG) of target genes such as Phox2a, which induces the sympathoadrenal progenitor marker genes choline acetyltransferase, SCG10, dopamine β-hydroxylase and TH, thereby contributing to a sympathetic neuronal phenotype [68]. The related paired homeodomain transcription factor gene, Phox2b, also seems important in autonomic, sympathetic ganglia formation, and is required in mice for maintaining expression of the HASH-1 homologue, Mash-1 [69]. In addition, Mash-1-deficient mice show arrested chromaffin cells and lack of catecholaminergic differentiation, and Phox2b knock-out mice demonstrate a requirement of Phox2b upstream also in this process [70].

Another bHLH factor, dHAND (deciduum, heart, autonomic nervous system, and neural crest derivatives), is expressed during development in early immature chromaffin cells of the adrenal glands, but is reduced upon differentiation [64]. In contrast, dHAND expression appears to persist during embryogenesis in sympathetic neurons and extra-adrenal chromaffin cells. Factors known to promote dHAND expression include Phox2b, GATA proteins and the SHH pathway, the latter leading to dHAND-dependent up-regulation of SHH factors and therefore establishment of a positive feedback loop [71, 72]. The dHAND gene is also expressed in both low- and high-stage neuroblastomas, and could potentially be used in the clinic for distinguishing neuroblastoma cells from other types of pediatric malignant cells [64].

The NOTCH pathway is involved in many cell-fate decisions during development, which has been demonstrated in Drosophila where neuroectodermal cells either
become neurons or epithelial cells, depending on activity and ligand-binding (e.g. by DELTA) to the transmembrane receptor NOTCH. Activation of NOTCH within a cell causes induction of the bHLH HES (Hairy/Enhancer of Split)-factors, which transcriptionally repress genes such as HASH-1 and thus inhibit neuronal differentiation (reviewed in [73]). However, NOTCH can also promote differentiation into glial cells [74]. Down-regulation of pro-neuronal HASH-1, which also is an activator of DELTA expression, leads to reduced amounts of membrane-bound DELTA protein in NOTCH-activated cells. Thus, a cellular determination loop, also known as lateral inhibition, is created between neighboring cells. Neuronal differentiation is repressed in one cell due to low HASH-1 and DELTA levels, whereas an adjacent cell then is less stimulated via the DELTA/NOTCH pathway, and can adopt a neuronal fate [75]. NOTCH-1 and HES-1 have also been implicated in regulating differentiation of neuroblastoma cells [76, 77].

The MYC-MAX-MAD network

As described above, MYCN-amplification is one of the most significant markers determining prognosis of neuroblastoma tumors. Whereas expression of the related factor MYC is frequently present in proliferating cells of several different origins, MYCN expression is primarily found within the CNS and PNS, including neural crest derivatives [78]. These structures usually have low MYC levels, and MYCN is also detected in non-dividing cell that have undergone differentiation. However, MYCN can substitute for the effects of MYC, as shown in a mouse developmental model where MYCN was inserted into the MYC locus [79]. These proteins belong to the bHLHZ (Z = leucine zipper domain) subgroup, binding DNA E-boxes via the basic domain, together with their obligate dimerization partner MAX (MYC-associated factor X). Binding to MAX is, in turn, conferred by the helix-loop-helix and leucine zipper domains. MYC/MYCN-MAX complexes recruit histone acetyltransferases, which make chromatin more accessible, and thereby transcriptionally activate genes (although some transcriptional gene repression also has been reported). These target genes are involved in e.g. cell-growth, differentiation, immortalization and genetic instability, and apoptosis [80] (Fig. 3). MAX, and potentially also MLX (MAX-like protein X), appear in the center of this transcription factor network, and can also interact with the MAD proteins (MAD1, MXI1, MAD3 and MAD4). Heterodimers of MAX and MAD family members interact with the SIN3 co-repressor and recruit histone deacetylases (HDACs), resulting in transcriptional inaccessibility of chromatin and thereby gene repression (Fig. 3). By competing with
MYC/MYCN for MAX binding, and by down-regulating MYC/MYCN target genes, MAD proteins act as antagonists of MYC/MYCN function [81]. Consequently, MYC and MYCN (and their related factor MYCL) are considered to possess oncogenic roles, whereas MAD proteins are potential tumor suppressors. Interestingly, when we manipulate the levels of MXI1, a MAD family member, in neuroblastoma cells we see primarily MYC/MYCN-independent changes in gene expression (paper III).

Figure 3. The MYC-MAX-MAD network of bHLHZ transcription factors. MAX proteins form heterodimers (MAX-MAX homodimers also exist and are usually transcriptionally inert) with proteins from either the MYC or the MAD/MXI1 subgroups, principally leading to activation or repression of target genes, respectively. The general biological responses by these interactions are also given. MYC and MAD/MXI1 proteins compete for binding to MAX, which is central in this transcriptional network, although other proteins such as MLX may appear in the same position. Shown here is also the third identified MYC member, MYCL, which has a more restricted expression pattern than MYC and MYCN.

Mice lacking functional MYC or MYCN display embryonic lethality [78, 82], and MYC and MYCN have been shown to be important inducers of protein synthesis, cell-growth and proliferation. However, cell-growth and cell-death are closely interconnected events, and in the lack of survival signals these proteins are also known to induce apoptosis [80]. During embryogenesis, MYC-expressing cells are associated with a proliferative, non-differentiated state, whereas MYCN can be found in post-mitotic cells of several forming tissues of different origins. Moreover, MYCN knock-out mice show a reduced number of mature neurons in sympathetic ganglia, indicating an important role of MYCN in neural crest and SNS development [78, 83]. MYCN could, by inhibiting premature differentiation, inducing self-renewal, or promoting a neuronal fate be involved in creating a pool of neural crest cells that subsequently can differentiate into sympathetic neurons. In addition, MYCN
might directly regulate embryonic migration of neural crest cells [84], a phenomenon possibly mirrored by the increased motility seen in MYCN-high neuroblastoma cells [85]. The connection to neuroblastoma is further demonstrated in mice with MYCN over-expression targeted to sympathetic progenitors, leading to the development of tumors that in occurrence and genomic changes resemble neuroblastomas [86].

ID proteins

The ID (inhibitor of differentiation/DNA-binding) proteins contain a helix-loop-helix (HLH) domain but lack a basic, DNA-binding region. All ID proteins are highly homologous and principally exert their functions by binding the ubiquitously expressed bHLH E-proteins, forming transcriptionally inactive complexes and/or sequester E-proteins from binding DNA. Because E-proteins themselves are obligate partners for tissue-specific bHLH factors, sequestering E-proteins allows ID to negatively regulate transcriptional responses and inhibit/regulate differentiation in several different cell types, irrespective of which tissue-specific dimerization partner of E-proteins that is expressed. Bone morphogenetic proteins (BMPs), which are members of the TGF-β family, can repress neuronal and myogenic cell fates, and these regulatory activities have been linked to their ability to up-regulate ID proteins [87]. Concerning the nervous system, over- or ectopic expression of ID1 and ID3 has been shown to inhibit neurogenesis, in favor of a glial cell lineage, and combined loss of ID1 and ID3 results in premature neuronal differentiation [88]. Moreover, ID1 ID3 double knock-outs are embryonic lethal, whereas ID1 and ID3 individual knock-out mice are viable, demonstrating the high functional similarity and redundancy between ID proteins. However, loss of ID2 in mice leads to ~ 25% perinatal lethality, and is thereby the most severe phenotype of the single ID knock-outs [89]. Importantly, ID2 has a role in neural crest cell-fate determination, since over-expression of ID2 in the chick neural tube induces conversion of the overlying ectodermal cells to neural crest cells [90].

ID proteins are also highly implicated in cell-cycle regulation, driving proliferation and delaying senescence, partly by reducing expression of cyclin-dependent kinase inhibitors p16^{INK4a} and p21^{Cip1} [91, 92]. Although E-proteins have been implicated in this process [93], the mechanism is more likely to be mediated by ID-dependent inhibition of DNA-binding of ETS transcription factors [91]. ETS proteins can also induce expression of ID2, which has been associated with a role in Ewing sarcoma [94, 95]. The growth-promoting
effect by ID has also been attributed to inhibited function of RB and the related factors p107 and p130, allowing progression through the cell cycle [96]. Conversely, hypophosphorylated RB has been suggested to sequester ID2, thereby counteracting proliferation but promoting differentiation. In addition, the ID proteins have been suggested to contribute to immortalization of several cell types.

It is well-established that ID proteins are over-expressed and implicated in tumorigenesis of several different human cancers (reviewed in [97]). In neuroblastoma, three of the four ID members, ID1, ID2, ID3, but not ID4, are expressed and are down-regulated upon induced differentiation [67]. Furthermore, the ID proteins were found to dimerize with HES-1, and could potentially activate transcription by inhibiting the repressive function of HES-1. Previous studies have suggested a link between MYCN and ID2 in neuroblastoma, with induced ID2 expression in MYCN-transfected cells and high ID2 protein levels only in MYCN-amplified neuroblastoma cells [98]. However, recent investigations performed by our group and others have not confirmed these data [99-101]. Also, MYCN is down-regulated, whereas ID2 is up-regulated in neuroblastoma cells grown under hypoxia ([102] and papers I and III), findings that further disagree with a potential MYCN-ID2 connection in neuroblastoma.

**Tumor hypoxia**

Early work showed that tumors grow in close proximity to blood vessels, and that tumor cells located more than 180 μm away from blood vessels exhibit necrosis [103]. This distance correlates well with the diffusion limit of oxygen (100-150 μm), when it passes from the capillary network and is metabolized within tissues. Similar to normal cells and tissues, cancer cells require supply of oxygen and nutrients for maintained membrane transport, chemical synthesis, growth and overall homeostasis, in addition to elimination of waste products by the blood circulation. When solid tumors expand in size the need for oxygen increases. However, the delivery of O2 to malignant and stromal cells is frequently reduced or even abolished because the induced intratumoral development of new blood vessels (angiogenesis) is insufficient, show structural abnormalities and poor blood flow – ultimately leading to hypoxia [104]. In addition, paraneoplastic symptoms such as anemia can reduce the capacity of oxygen transport and therefore contribute to tumor hypoxia. These processes
generate microregions with very low or even zero (anoxic) oxygen partial pressures, distributed heterogeneously within most solid tumors.

Biochemically, hypoxia is defined as oxygen-limited electron transport, and physiological hypoxia occurs when O₂ availability decreases below a certain threshold that restricts function of organs and cells [105]. Accordingly, tumor hypoxia is present when oxygen levels fall below a critical value, leading to a progressive decrease in ATP production. However, the critical oxygen level leading to hypoxia can vary widely among malignant tumors. Assessments of tumor oxygenation status by both invasive (e.g. via Eppendorf electrodes) and non-invasive methods have been performed (Fig. 4), and results indicate that an oxygen partial pressure (pO₂) of 10 mmHg (corresponding to 1.3% O₂) can be considered hypoxic [106]. This cut-off level was found to distinguish hypoxic cervical cancers with poor prognosis, from less-hypoxic corresponding tumors with significantly better survival. Measuring oxygen distribution within human malignancies, including cervical cancers, soft-tissue sarcomas, squamous cell carcinomas of the head and neck, and breast cancer, has shown values of 10 mmHg or less in about 50% of the tumors [106-109]. Tumors with a pO₂ < 10 mmHg exhibit a substantially increased probability of invasion and metastasis [106, 110]. In contrast to the conditions found in solid tumors, oxygen levels in most normal tissues are in the range of 40-60 mmHg [111], correlating well with end-capillary oxygen levels of 5-6% O₂. Clinical studies reveal that tumor hypoxia is a predictive factor for poor outcome, independent of tumor characteristics such as patient age, tumor size and grade, and extent of necrosis [112].

Furthermore, tumor hypoxia is highly associated with increased resistance to anti-cancer therapy. Due to the decreased levels of available oxygen, which is the substrate for the formation of reactive oxygen species (ROS), causing DNA damage by photon therapy, hypoxia impedes radiation treatment [113]. In addition, hypoxia can confer radiation resistance by increasing the expression of heat-shock proteins, inducing cell growth, or by inhibiting the apoptotic pathway [114]. Furthermore, treatment of cancer patients with chemotherapeutic drugs is also hampered by tumor hypoxia, due to several different mechanisms [111]. The distancing of hypoxic cancer cells from blood vessels causes physical inaccessibility of therapeutic agents, and the abnormal architecture and insufficient circulation of intratumoral blood vessels causes higher interstitial pressure, which also is associated with low penetration of drugs. Furthermore, the therapeutic effect of several substances can be dependent on presence of oxygen. Many drugs are also dependent on a high range of proliferating tumor cells for proper function, and since hypoxia is known to be involved in
decreasing proliferation, a diminished effect of the drugs might be at hand. In addition, hypoxia mediates an intratumoral selection for cells resistant to TP53-mediated apoptosis, thereby desensitizing cancer cells for apoptosis-inducing agents [114]. Finally, hypoxia can induce expression of genes providing drug resistance, as exemplified by the MDR1 gene [115].

Figure 4. Solid tumors are less oxygenated than normal tissues. Measurements of oxygen distribution within normal tissue (top) and head and neck cancer (bottom), using polarographic O2 needle sensors. Tumors show an oxygen tension median of 10 mmHg (~1.3% O2), in contrast to a median of approximately 50 mmHg in normal tissue, with no values lower than 10 mmHg. Adapted from [108].

Responses to hypoxia and malignant progression

In response to hypoxia, normal cells undergo a multitude of biological changes that regulate cell growth, cell death and angiogenesis. Expression of erythropoietin (EPO) is up-regulated by hypoxia in the kidney, leading to raised hemoglobin production and increased blood supply [116]. Another hypoxic response that can mediate a systemic effect (in addition to local actions), is raised TH synthesis [117] which increases catecholamine production, for instance in SNS tissues.

Cancer cells are genetically and phenotypically altered in response to hypoxia, thereby allowing survival and growth of tumors under even very low oxygen tensions. Hypoxic cells switch their glucose metabolism from utilization of the oxygen-dependent tricarboxylic acid (TCA) cycle, to oxygen-independent glycolysis. In hypoxic tumor cells, glycolysis is used as a primary source for ATP production, which occurs even under aerobic conditions (known as the “Warburg effect”) and has also been linked to the process of transformation [118]. Constitutive up-regulation of glycolysis leads to increased acid production through an overload of lactate and CO2, and cancer cells that are selected to resist acid-induced toxicity have a growth advantage within an environment that is toxic to other phenotypes, but harmless to themselves. In addition, environmental acid production facilitates
invasion and metastasis by disrupting cell-cell and cell-matrix interactions. Hypoxic up-regulation of \textit{carbonic anhydrase-9} (CA-9) may as well be involved in lowering pH within tumors and has been associated with poor prognosis [119]. Hypoxia also induces most glycolytic enzymes and the glucose transporters, \textit{GLUT1} and \textit{GLUT3} [118]. Several growth factors involved in promoting proliferation are induced by hypoxia, including IGF-2, TGF-\beta and PDGF, and the hypoxia-activated glycolytic pathway generates metabolites that are essential for cell growth [120].

One of the most studied hypoxia-induced responses is angiogenesis. Hypoxic exposure of cancer cells leads to increased expression and secretion of VEGF, the most important angiogenic factor, and up-regulation of VEGF receptors. Induction of these genes provide both auto- and paracrine survival effects on tumor cells, but primarily increase the vascular blood supply within the tumor by mediating proliferation and recruitment of endothelial cells [120]. Hypoxia also confers immortalization of cancer cells, e.g. by increasing \textit{telomerase} expression [121]. Genetic instability and mutation rates are augmented under hypoxia [122], with hypoxic cells having a diminished capacity for both mismatch and double strand brake DNA repair [123, 124], all processes that would promote cancer progression.

However, the hypoxia-induced changes in tumors, affecting both neoplastic and stromal cells, can promote additional pathways that would appear detrimental for tumor progression, including cellular quiescence, apoptosis and necrosis. Apoptosis is a predominant response to hypoxia in transformed cells, often in a TP53-dependent manner [125], but hypoxia-mediated apoptosis can be avoided via activity of BCL2 family members [126]. The HIF pathway could also be implicated in apoptosis, potentially in co-operation with TP53 (discussed further below). Hypoxia-evoked cell cycle arrest is characterized by increased activity of cyclin-dependent kinase inhibitors such as p21^{Cip1} and p27^{kip1}, and also hypophosphorylation of the RB protein, leading to a block in cell cycle progression [127].

Whether the net phenotypic result of hypoxia-induced intratumoral changes leads to tumor impairment or tumor promotion is influenced by the genomic status of the cancer cells, induced epigenetic changes, and effects by microenvironmental cells and factors, in addition to the degree of hypoxia. Hypoxia-mediated clonal expansion of cancer cells with advantageous alterations, causing reductions in growth-inhibition, less differentiation and lowered sensitivity to apoptosis have been demonstrated [114], and these changes may also favor hypoxia-associated mechanisms that promote tumor growth, angiogenesis, invasion and metastasis (Fig. 5). Furthermore, the increased aggressiveness of hypoxic tumor cells
implicates that anti-angiogenic therapy, which aims at reducing tumor vascular and oxygen supply, could in fact increase hypoxic tumor cell adaptation and selection, and thus promote disease progression. Most, if not all, of these hypoxic responses show involvement by the principal transcription factors controlling adaptation to low oxygen conditions, the hypoxia-inducible factors (HIFs), which are discussed below.

Figure 5. The importance of hypoxia in malignant progression of solid tumors. Dysregulated proliferation of neoplastic cells, and insufficient oxygen supply by the chaotic intratumoral microcirculation leads to the establishment of tumor hypoxia. Sustained or intermittent hypoxia augments genomic instability and heterogeneity, providing a selective pressure for cells that can survive and proliferate under even very low oxygen concentrations. This adaptive response to hypoxia within tumors also results in the emergence of cancer cells with increased potential for angiogenesis, tissue invasion and tumor dissemination, as well as therapeutic resistance. Adapted from [112].

**Hypoxia-Inducible Factors (HIFs)**

**Structure of HIF**

Although several transcription factors have been implicated in the hypoxic response, including NF-κB, CREB, AP-1 and TP53 [128], HIF proteins have convincingly been demonstrated to play the major roles in cellular and systemic O₂ homeostasis, both during development and in postnatal life. HIF proteins are also highly involved in disease processes, including cancer. These proteins are heterodimeric transcription factors consisting of two subunits: an α-subunit, the protein levels of which increase with reduced oxygen tensions, and an oxygen-independent β-subunit. So far, three α-subunits have been discovered. HIF-1α was initially identified by Semenza and Wang as a DNA-binding protein located in the 3´-region of the hypoxia-induced EPO gene [129]. Factors related to HIF-1α in structure and function
were later found, with HIF-2α, also named endothelial PAS domain protein 1 (EPAS1), HIF-like factor (HLF), HIF-related factor (HRF) and member of PAS family 2 (MOP2), having the highest similarity [130-133]. A third member, HIF-3α, has also been cloned, and a splice variant from the HIF-3α locus termed IPAS (inhibitory PAS domain protein) forms transcriptionally inactive heterodimers with HIF-1α that prevents HIF-1α-induced gene expression, e.g. for VEGF, causing maintenance of an avascular phenotype in tissues such as the cornea [134-136]. Although regulation of HIF-3α appears similar to that of HIF-1α and HIF-2α, only a few reports are available for this α-subunit, and thus focus will be directed on HIF-1α and HIF-2α hereafter. The β-subunit, HIF-1β or ARNT (aryl hydrocarbon receptor nuclear translocator), besides functioning as a HIF-α co-factor, is known to bind the dioxin receptor and is involved in responses to environmental pollutants [137]. Lately, ARNT2 and ARNT3 have been identified, the expression of which are more restricted than the ubiquitous factor ARNT. All α-subunits can dimerize with all ARNT proteins, thereby adding complexity to the hypoxic signaling response.

HIF-α and ARNT proteins are relatively large proteins (770-870 amino acids, 90-120 kD) and contain bHLH domains, where the basic region confers DNA-binding and the HLH part mediates protein-protein interactions (Fig. 6). In addition, these proteins have two PAS (an acronym for the PER, ARNT, SIM proteins, in which this motif first was identified) domains, PAS-A and PAS-B, involved in HIF heterodimerization, but also in binding and modifications by other proteins such as the heat-shock protein 90 (HSP90) [138]. Functional HIF-αβ complexes bind a specific DNA sequence (core motif: 5´-CGTG-3´) termed hypoxia-response element (HRE), present in promoters or enhancers of HIF target genes [139]. The HIF-α proteins contain both N-terminal and C-terminal transactivation domains (N-TAD and C-TAD, respectively), the major function of which is to recruit co-activators that are crucial for transcriptional regulation of target genes (no C-TAD is present in HIF-3α, accounting for its inhibitory effect on HIF-1α-mediated transactivation). The N-TAD also comprises an oxygen-dependent degradation domain (ODD), involved in controlling HIF-α protein stability in response to changes in O2 levels (the functions of HIF TADs are further described below). Under hypoxic condition, HIF-α proteins accumulate rapidly (<2 min) in the nucleus due to two nuclear localization signals and bind to ARNT (ARNT is a constitutive nuclear protein and is not necessary for HIF-α nuclear localization), recruit co-activators such as CBP/p300 and regulate target genes [140, 141]. Reoxygenation quickly reduces HIF DNA-binding and HIF-α protein levels, due to the short half-life (<5 min) of these factors [142].
Figure 6. Domain structure of HIF-1α and ARNT, intradomain localization of major post-translational HIF-1α modifications, and interactions of HIF-1α. The oxygen-dependent degradation domain (ODD) of HIF-1α regulates its stability via hydroxylation of proline(P)-402 and -564 (-405 and -531 in HIF-2α) by PHD 1-3 enzymes, and acetylation of lysine(K)-532 by ARD1, leading to binding and ubiquitylation by the VHL E3 ligase complex, and subsequent proteasomal HIF-1α degradation. HIF-1α and HIF-2α have two transactivation domains (TADs), the N-terminal and the C-terminal (HIF-3α lacks the C-TAD), respectively, while ARNT only contains one TAD. Several co-activators, including CBP and p300, interact with the C-TAD and enhance HIF target gene transactivation. HIF-α proteins are also phosphorylated, which may increase transcriptional activity. Oxygen-dependent hydroxylation of asparagine(N)-803 in HIF-1α (-851 in HIF-2α) by the FIH enzyme blocks the interaction with CBP/p300 and thus inhibits HIF-1α-dependent gene transcription. Hypoxia inhibits both PHD and FIH functions, leading to stable and active HIF-α proteins.

Oxygen-dependent HIF regulation: stability

The cellular oxygen-sensing mechanism controlling HIF-α stability (see Figs. 6 and 7) was independently discovered by two groups simultaneously [143, 144], involving a new group of prolyl-4-hydroxylases, now termed prolyl-hydroxylase domain-containing protein (PHD 1, 2 and 3), which in the presence of oxygen hydroxylate two conserved proline residues (Pro-402 and -564 in HIF-1α, and -405 and -531 in HIF-2α) within the respective ODDs, ultimately leading to degradation of the HIF-α subunits [144-146]. PHD2 appears to be the most abundant and primary HIF-α prolyl hydroxylase under normal conditions, although HIF-2α is suggested to be better hydroxylated by PHD3 [147]. PHD1 is found primarily within the nucleus, PHD2 is mostly cytoplasmic, and PHD3 is both cytoplasmic and nuclear with cytoplasmic predominance, but the importance of this distribution is not yet clear [148]. The PHDs require 2-oxoglutarate, derived from the metabolic TCA cycle, and molecular oxygen as substrates, providing a direct link between oxygen availability and HIF regulation. These enzymes also exhibit relatively high $K_m$ values for oxygen (230-250 μM compared to 40 μM for the procollagen prolyl hydroxylase) indicating that they could act as proper sensors for
hypoxia [149]. During catalysis, one oxygen atom is incorporated into the formed hydroxyl group on HIF-α, and the other is coupled to the oxidative decarboxylation of 2-oxoglutarate (2-OG), yielding succinate and CO₂ [150]. These enzymes are also dependent on iron (Fe²⁺) and ascorbate as co-factors, and can therefore be inhibited by hypoxia-mimicking agents such as iron chelators and transition metals, leading to stabilization of HIF-α subunits even at normal oxygen tensions.

Also, nitric oxide (NO) has been shown to inhibit HIF-α hydroxylation and induce HIF activity, primarily by competing with O₂ for binding to the PHDs [151]. However, other reports indicate both NO-induced inhibition of HIF and promotion of PHD function, suggesting a more complex regulation [152]. Both PHD2 and PHD3 (but not PHD1) are transcriptionally induced by hypoxia, potentially in order to mediate a more rapid destruction of HIF-α when cells are reoxygenated [146, 147]. HIF-1α has been shown to activate both PHD2 and PHD3 transcription under hypoxia, and HIF-2α may also be involved in PHD3 up-regulation [153-155]. In contrast, hypoxia, potentially independent of HIF, can induce the accumulation of the Siah2 protein, which is suggested to be involved in polyubiquitylation and degradation of PHDs [156]. Thus, hypoxia causes both negative and positive feedback loops in the reciprocal regulation of PHD and HIF-α proteins. Furthermore, the production of alternative PHD transcripts could influence the rate of HIF hydroxylation [149]. A recent report also demonstrates that the OS-9 protein promotes HIF degradation via interactions with both HIF-1α and PHD2 and PHD3 [157].

Prolyl hydroxylation of HIF-α is of central importance for its degradation pathway (Fig. 7). HIF-α proteins are constitutively expressed, but usually not detectable under normoxic condition [142]. However, the term normoxia is often carelessly used, since most hypoxic experiments on HIF proteins are compared with growth conditions at 21% O₂, which actually corresponds to a hypoxic state, instead of more physiologically relevant oxygen levels as those found in end-capillaries (~5% O₂) [111]. Importantly, we readily find accumulation of nuclear HIF-2α protein, to a higher degree than HIF-1α, at 5% oxygen in neuroblastoma cells ([158, 159] and paper II). Generally though, degradation of HIF-α occurs in the presence of sufficient amounts of oxygen, and starts with binding of the β-domain of the von Hippel-Lindau (VHL) tumor suppressor protein to the hydroxylated prolines within the HIF-α ODD [160]. Mutations in the VHL gene is common both in hereditary (e.g. patients with von Hippel-Lindau disease carrying one defect VHL allele and which develop tumors when the wild-type allele is somatically mutated) and in sporadic cancers such as clear cell
renal cell carcinomas (RCC). In these tumors, HIF-α subunits are not degraded, leading to constitutive up-regulation of HIF targets such as VEGF, and a highly vascular phenotype. VHL belongs to a multiprotein E3 ubiquitin ligase complex, containing elongin B, elongin C, Rbx1 and cullin 2, which polyubiquitylates HIF-α subunits in the presence of oxygen [161-163], leading to proteolysis of HIF-α in the 26S proteasome [144, 164]. Recently, it was found that the E2-EPF ubiquitin carrier protein associates with and targets VHL for proteasomal degradation, thereby stabilizing HIF-1α, which also was implicated in increased tumor growth and metastasis [165].

Another tumor suppressor that is involved in HIF biology is TP53. Recent data suggest that HIF-1α can bind to MDM2, the ubiquitin ligase targeting TP53 for degradation, and thereby indirectly stabilize TP53 [166]. Importantly, TP53 may promote MDM2-mediated ubiquitylation and subsequent proteasomal destruction of HIF-1α, independently of VHL [167]. In addition, acetylation of Lysine-532 of the HIF-1α ODD by the acetyltransferase ARD1 (arrest-defective-1 protein) may increase HIF-VHL interaction, ubiquitylation and degradation of HIF-1α [168], although other reports contradict these findings [169].

Oxygen-dependent HIF regulation: transactivation

Analysis of the HIF-α C-TAD has revealed that hydroxylation of an asparagine residue (Asn-803 in HIF-1α and Asn-851 in HIF-2α), blocks interaction with the CH-1 (cysteine/histidine rich) domain of the transcriptional co-activators p300 and CBP (the binding protein of CREB, c-AMP-response element binding protein), rendering HIF-α transcriptionally inactive under normoxic conditions [170] (Figs. 6 and 7). The CBP/p300 proteins are histone acetyltransferases and when bound to HIF-α they mediate chromatin accessibility, which facilitates transcriptional induction of HIF target genes. The relevant asparaginyl hydroxylase mediating the transcriptional inactivation of HIF-α subunits response was defined as the factor inhibiting HIF (FIH), which belongs to the same group of oxygen-, iron- and 2-oxoglutarate-dependent enzymes as the PHDs and can therefore be inhibited by hypoxia, iron chelators and 2-OG analogues [171, 172]. FIH is mainly localized to the cytoplasm and, unlike the PHDs, the mRNA of FIH is not induced by hypoxia [148]. Also, the FIH K_m for oxygen is approximately 90 μM, which is substantially lower than that for the PHD enzymes, and thus more pronounced hypoxia may be required for full inhibition of FIH-induced
hydroxylation [173]. Nevertheless, asparaginyl hydroxylation provides a second oxygen-dependent mechanism by which HIF-α subunits that escape the degradation pathway can be transcriptionally inhibited (Fig. 7). In addition to p300/CBP, HIF interacts with the co-activator SRC-1 (steroid receptor co-activator-1) and the transcription intermediary factor 2, and these interactions enhance the transactivation potential of HIF-1α in an O2-dependent manner, producing a synergistic effect with CBP [174]. The latter is also potentiated by the redox regulatory protein Ref-1, which interacts with both the N-TAD and the C-TAD, but displays a higher effect on the C-TAD [174].

FIH is also reported to interact with VHL [171]. Although this interaction is not required for FIH activity, normoxic HIF-dependent transcription in cells lacking VHL is essentially complete [161], suggesting that VHL is essential for all oxygen-dependent controls of HIF, and that VHL function extends beyond ubiquitin-mediated HIF-α degradation. These findings also implicate the second HIF-α transactivation domain (N-TAD) in gene regulation. HIF-dependent genes can be insensitive to changes in FIH-mediated C-TAD hydroxylation, for instance observed with some HIF splice variants lacking the C-TAD domain but that still activate certain HIF targets. Recent data also suggest a co-operation between the two TADs in HIF-2α-mediated renal tumorigenesis [175]. Furthermore, it was previously shown that the PHDs can recruit the candidate tumor suppressor ING4 (inhibitor of growth 4), mediating suppression of HIF-1α transcriptional activity even under hypoxic conditions, without affecting HIF-1α stability [176]. Still, the importance of these interactions has not yet been fully clarified.

**Oxygen-independent regulation of HIF proteins**

HIF-α proteins can be stabilized and activated already under normoxic conditions (Fig. 7), and additional post-translational modifications, such as phosphorylation, appear to play major roles. Tumor suppressors (such as PTEN and p14ARF) and oncogenes (e.g. RAS and v-SRC (viral sarcoma oncogene)) are able to affect HIF-α expression, independent on oxygen levels [177]. These pathways are usually not as efficient HIF regulators as hypoxia, and in contrast to hypoxia, they appear to operate in a more cell type-specific manner. Nevertheless, oxygen-independent regulation of HIF is important in several biological processes, and is highly involved in cancer. Binding of growth factors and cytokines, such as EGF, basic FGF, TGF-α, interleukin-1β, IGF-1/2 and insulin to their cognate receptors has been shown to up-
regulate HIF-1α, primarily involving the MAPK (mitogen-activated protein kinase) and the phosphatidylinositol (PI)-3-kinase (PI3K) pathways [178]. In turn, several growth factors (and their receptors) are HIF target genes themselves, possibly creating positive feedback loops [177].

Figure 7. Regulation of HIF-1α. The HIF-1α protein is constitutively expressed within most cells, but constantly degraded in the presence of sufficient oxygen. Under normoxic conditions, newly synthesized cytoplasmic HIF-1α is rapidly hydroxylated by PHD enzymes (primarily PHD2) and acetylated by the ARD1 protein. These modifications lead to binding and ubiquitylation by the VHL E3 ligase complex and subsequent destruction of HIF-1α in the 26S proteasome. Of note, HIF-1α prolyl hydroxylation and degradation may also occur in the nucleus. The FIH protein is also active in the presence of oxygen and hydroxylates HIF-1α proteins that have escaped PHD/VHL-mediated degradation, resulting in blocked interaction with CBP/p300 co-activators and inhibition of HIF target gene activation. Under hypoxic conditions, HIF-1α is not hydroxylated and is therefore stabilized, can translocate to the nucleus where it binds ARNT and co-activators such as CBP/p300, and regulates target genes via hypoxia-response elements (HREs). However, growth-factor (GF)-induced signaling via receptor tyrosine kinases can induce HIF-1α protein accumulation and activity even at normoxia, mainly because the PHDs are not present within cells in a large excess over HIF-1α and therefore have limited function. GF-mediated signaling frequently involves activation of the PI-3-kinase pathway, including major downstream targets such as AKT and mTOR, which stimulates translation- and ribosome-regulatory proteins, thereby increasing the rate of HIF-1α protein synthesis. In addition, signaling via receptor tyrosine kinases can activate the MAPK pathway, leading to phosphorylation of HIF-1α and/or co-activators, which enhances HIF-1α transactivation. See text for additional HIF regulatory mediators and for further details.

Direct phosphorylation of HIF-α appears to occur after stabilization of the protein under normoxic or hypoxic conditions [179, 180]. In one study, activation of the MAPK extracellular signal-regulated kinases (ERK)-1 and ERK-2 (p44 and p42, respectively)
led to HIF-1α phosphorylation, whereas activation of p38 or JNK (c-Jun N-terminal kinase) MAPK kinases did not [180]. However, the C-TAD of HIF-1α has been shown to be phosphorylated by both ERK and p38 MAPKs [179], and ERK-signaling can also increase HIF-2α transactivation [181]. The specific residues phosphorylated by ERK and p38 have yet to be determined, but may involve threonine-796 in HIF-1α and threonine-844 in HIF-2α [182]. Phosphorylation seems not to influence stability or DNA-binding of HIF-α, but instead increases HIF transcriptional activity [180, 183]. One suggested underlying mechanism is that ARNT preferentially binds the phosphorylated form of HIF-α subunits [184]. However, recent studies indicate that direct phosphorylation of the HIF-α C-TAD does not correlate with HIF transcriptional activity, and instead can phosphorylation of the p300 co-activator increase the interaction between HIF-αC-TAD and p300, thereby augmenting HIF transcriptional ability [185].

Several mechanisms involving almost all activation steps within the PI3K pathway have been shown to increase HIF-1α levels [178]. The PI3K downstream kinase AKT, involved in apoptosis, cell growth and translation, activates the mammalian target of rapamycin, mTOR, which in turn is an activator of the p70 ribosomal protein S6 kinase (p70S6K) that can enhance HIF-1α translation. mTOR also phosphorylates and inhibits the translational regulatory protein 4E-binding protein (4EBP), which then prevents 4EBP-mediated inhibition of the eukaryotic translation initiation factor 4E, resulting in an increase in translation rate. Conversely, the PI3K cascade can be inhibited by the tumor suppressor PTEN (phosphatase and tensin homologue), which also inhibits HIF-1α stabilization and activity [186]. The PI3K/AKT pathway is frequently dysregulated in cancer, often through activation by growth factors (see above), leading to up-regulation of HIF-1α. In a study of breast cancer cell lines, inhibition of PI3K caused a reduction in HIF-1α expression and phosphorylation, and lower VEGF levels, without affecting HIF-2α [187]. Cellular transformation by HRAS has been shown to increase HIF-1α protein and target gene expression via PI3K pathway activation, independent on oxygen levels [188, 189]. Activated v-SRC can induce HIF-1α levels under normoxia through a PI3K- or MAPK-dependent pathway, however, likely as a result of enhanced overall protein translation [190]. Also, RAS- and v-SRC-mediated HIF-1α induction under normoxic conditions may be attributed to inhibition of PHDs, although activated AKT stabilized HIF-1α independently of prolyl hydroxylation in the same study [191]. Furthermore, signaling from the epidermal growth factor receptor HER2 can increase HIF-1α protein synthesis in a PI3K-dependent manner,
and in colon cancer cells stimulated with IGF-1 or prostaglandin E2, HIF-1α protein is induced through MAPK and PI3K pathways [192-194].

Finally, several other post-translational modifications, including S-nitrosation may induce HIF transcriptional activity, whereas modification with the small ubiquitin-related modifier (SUMO) may repress activity [195].

**HIF proteins in development**

Functional studies *in vitro* have displayed considerable similarities between HIF-1α and HIF-2α in structure and transactivation properties [196, 197]. However, developmental models in mice show that these proteins are not redundant, probably to a large extent caused by the existing spatial and temporal differences in HIF-α subunit expression patterns during embryogenesis. To date there are no mouse genetic data regarding the function of HIF-3α.

Two highly similar *HIF-1α−/−* mouse phenotypes were initially published [198, 199]. Loss of *HIF-1α* in these mice resulted in morphological abnormalities by embryonic (E) day E8.0-E8.5, developmental arrest by E9.0, and all *HIF-1α−/−* embryos died by E11. The greater severity of embryonic defects in these mice compared with ARNT-deficient mice suggests that HIF-1α can dimerize with for instance ARNT2 or ARNT3 [200]. *HIF-1α-null* mice display a substantial decrease in somites, failure in closing the neural tube and massive defects in vascular structure and organization, especially in the cephalic region. Concomitant with the disruption of vascular development, massive cell death was observed within the cephalic mesenchyme [198]. Cardiac development is also aberrant in *HIF-1α−/−* embryos, primarily with hyperplasia of the presumptive myocardium leading to reduced size of the ventricular cavity and outflow tract. The vascular defects in these mice are similar to those found in *VEGF*-deficient embryos, and hypoxic *VEGF* induction was absent in *HIF-1α−/−* embryonic stem (ES) cells [198, 199].

However, a later study of *HIF-1α* knock-out mice surprisingly demonstrated higher basal *VEGF* expression compared with wild-type embryos, possibly explained by the *HIF-1α−/−* vascular defects resulting in, besides a deficient O₂ supply, glucose deprivation which induces *VEGF* independently of HIF-1α [201]. Interestingly, HIF-2α appears to be more involved in responses to hypoglycaemia than HIF-1α [202]. Rather than being associated with VEGF deficiency, the vascular and morphological abnormalities in *HIF-1α−/−* embryos were temporally and spatially correlated with increased cell death, occurring prior to the onset
of vascular defects [201]. The cell death was especially manifested at the neurosomatic junction, the site at which neural crest cells emigrate from the neural tube to populate the cephalic mesenchyme.

An additional study of HIF-1α−/− embryos, using a different mouse genetic background, confirmed that that loss of HIF-1α results in cardiac defects due to faulty ventricular formation, as well as abnormal aortic outflow tract and cephalic vessels [203]. Furthermore, in concordance with the earlier mouse models, the malformations in these HIF-1α-null embryos were associated with defective neural crest cell migration, involving aberrant expression of neural crest developmental marker genes. Particularly interesting was the reduced expression of ID2 found in E9.5 HIF-1α−/− embryos [203], since this gene normally is required for proper neural crest development [90], and because our group later identified ID2 as a HIF-1 target gene in hypoxic neural crest/SNS-derived neuroblastoma cells ([102] and paper I).

The published HIF-2α−/− mutants show large variations in overall phenotype, to a higher extent than the HIF-1α-null mouse models, likely due to the fact that mice with more pronounced differences in genetic background were used in the HIF-2α−/− studies. The HIF-2α−/− mice created by Tian et al. were embryonic lethal by E16.5, however, displayed normal vascular development and no general morphological defects up to E15.5 [204]. The HIF-2α−/− embryos exhibited pronounced bradycardia, likely caused by severely reduced catecholamine levels. Heterozygous animals also expressed high HIF-2α levels in catecholamine-producing sympathetic ganglia by E11.5-E12.5, and during E12.5-E15.5 in the paraganglia, and these time-points correlated with the onset of death in HIF-2α-deficient embryos. The midgestational lethality was largely rescued by administration of catecholamine precursors to pregnant mice, which led to the conclusion that HIF-2α is involved during development as a sensor of hypoxia, mediating a response of increased levels of circulating catecholamine required for proper cardiac function [204]. Peng et al. generated different HIF-2α−/− mice showing both subtle and severe vascular defects depending on genotype, but embryonic lethality was displayed for all mutants [205]. These embryos could be rescued, however limited, with supplementation of catecholamine precursors, indicating catecholamine-deficiency by loss of HIF-2α similar to the findings by Tian et al. [204]. In a later study, the vascular defects observed in HIF-2α-null mice could be restored by endothelium-specific expression of HIF-2α cDNA, which also allowed embryonal survival [206].
Compernolle et al. showed that HIF-2α-deficiency can cause pre- or perinatally fatal respiratory distress syndrome [207]. Vascular development in these mice was normal until one day before birth when subtle pulmonary vessel defects occurred, temporally coinciding with the induction of HIF-2α and VEGF in alveolar endothelial cells of wild-type mice. VEGF regulates fetal lung maturation and was reduced in embryos lacking HIF-2α. Administration of VEGF improved lung function and survival of HIF-2α-null mice. Catecholamine production was lower also in this mouse model, but not connected with the overall pathology [207]. A postnatal HIF-2α−/− phenotype was obtained by Scortegagna et al, however, these mice demonstrated substantially shorter life spans and multiple organ pathology [208]. Adrenal gland catecholamine levels were reduced, however only slightly in these juvenile HIF-2α−/− mice. Gross metabolic defects were observed, in addition to enhanced production of reactive oxygen species in combination with an impaired response to oxidative stress. Thus, HIF-2α could function as a primary sensor of oxidative stress during development, governing a protective response to maintain ROS homeostasis, which HIF-1α cannot substitute for [208]. In addition, HIF-2α-deficient mice show defects in EPO-dependent hematopoietic development and retinal neovascularization. HIF-2α-null kidneys have severely decreased EPO expression and impaired erythropoietin induction by hypoxia. Treatment of mice lacking HIF-2α with EPO causes a reversal of the hematopoietic and retinal vascularization defects [209-211].

Importantly, several of the HIF-2α knock-out models implicate HIF-2α as a regulator of the sympatho-adrenal lineage, in particular Tian et al. showing strong HIF-2α expression in the developing sympathetic ganglia, paraganglia, and also weaker expression in the adrenal glands, the latter confirmed in a study of HIF tissue distribution during development [212]. Our group has also demonstrated HIF-2α expression in mouse E14.5 paraganglia, and HIF-2α protein in human fetal week 8.5, approximately corresponding to the mouse E16 developmental stage [102, 159]. In addition, we see induction of HIF-2α at near-end capillary oxygen tensions in cultured neuroblastoma cells, as well as high HIF-2α, but not HIF-1α, protein levels in apparently well-oxygenated neuroblastoma tumors ([158, 159] and paper II).
Adult tissue expression of HIF

HIF-1α mRNA is constitutively expressed in most cultured tissue cells, but in vivo, HIF-1α might be transcriptionally regulated in response to hypoxia and ischemia [213, 214]. As described above, HIF-1α protein is constantly expressed in cells but also rapidly degraded, unless stabilized by hypoxia or induced by intracellular signaling pathways. Although these stimuli probably operate in most normal human cells, investigations have shown that HIF-1α protein is usually not detected within normal tissues [215].

Regarding HIF-2α, early findings indicated a more restricted expression pattern than that of HIF-1α, with abundant HIF-2α levels found primarily in vascular endothelial cells of various organs such as brain, lung, kidney, liver and spleen [130-132]. In contrast, HIF-1α expression in endothelial cells is usually much lower than HIF-2α. Investigating adult mouse and human tissues however revealed that substantial HIF-2α expression is also found in other cell types, such as smooth muscle cells, hepatocytes, epithelial cells of the lung, kidney and intestine, glial cells and neurons, including SNS derivatives [131, 132, 196, 212, 216]. Nevertheless, protein levels of HIF-2α are hardly detectable in normal tissues, similar to HIF-1α, with the exception of strongly HIF-2α-expressing bone marrow macrophages [215].

Functions and involvement of HIF proteins in tumors

In contrast to normal tissues, HIF-1α and/or HIF-2α proteins are abundant in most types of human tumors, including breast, lung, skin, colon, ovarian, pancreatic, prostate and renal tumors, however in a heterogeneous pattern [215, 217]. In addition, high HIF protein levels are also common in metastases [217]. Intratumoral HIF expression can be mediated by both physiological induction, primarily hypoxia, and by oncogenic activation/tumor suppressor loss-of-function, and these processes are not mutually exclusive.

HIF-1α is associated with increased mortality in several human cancers, including cervical, breast, glioma, ovarian and endometrial tumors (reviewed in [177]). Data from xenografted tumors in mice are less consistent. Disrupting HIF-1α in embryonic stem (ES) cells causes inhibited xenograft growth in some reports, clearly implicating HIF-1α as an important factor for solid tumor formation [199, 218], whereas other studies show xenograft growth of ES cells to be promoted by loss of HIF-1α [219, 220]. The varying net effects of
HIF-1α-deficiency in these models could be caused by differences in genetic background of the ES cells, but it appears that HIF-1α was inversely correlated with tumor growth only in studies involving ES cells that lack the large complement of genetic aberrations, characteristic of cancer cells. Still, inhibited tumor growth is consistently demonstrated in RCC as a result of normoxic HIF-1α expression caused by VHL-deficiency [221-223].

In contrast, stabilization of HIF-2α under normoxia is required for growth of VHL−/− RCC and hemangioblastoma. Re-introducing VHL in VHL-null RCC causes abrogation of xenograft tumor formation, which is rescued by over-expression of HIF-2α, but not HIF-1α [221, 224, 225]. Nevertheless, despite xenograft data showing HIF-1α to be negative for RCC growth, and the clear dominance of HIF-2α in RCC cell lines, over-expression of HIF-1α is seen in most bona fide primary human RCC tumors [215, 217], and HIF-1α activity appears important in the earliest detectable RCC lesions, whereas HIF-2α seems associated with more advanced disease [226]. HIF-2α protein expression in human tumors has been associated with stromal cells, including tumor macrophages, a feature related to angiogenesis and poor outcome, and HIF-2α also appears to be a negative prognostic marker for an increasing number of sporadic tumors such as non-small-cell lung, breast, bladder and colorectal cancers (reviewed in [227]), in addition to neuroblastoma (paper II).

More than 100 direct HIF target genes have been demonstrated, and it has been estimated that 1-5% of all human genes may be responsive to hypoxia in a HIF-dependent manner [139, 177]. However, for the majority of these genes, hypoxic induction is cell type-specific, and it is the functional interaction between HIF and other transcription factors that determines which HIF-responsive genes that are up-regulated in a particular cell under hypoxic conditions. HIF-1α-dependent induction of glucose transporters (GLUT1, GLUT3) and glycolytic metabolism, via direct up-regulation of most glycolytic enzymes [198, 228], is well appreciated and is directly implicated as an important process for tumor progression, yielding growth advantage and enhancing cancer cell dissemination (see above) [118]. Recently, HIF-1α was also shown to directly inhibit aerobic metabolism, via induction of pyruvate dehydrogenase kinase, potentially directing the limited oxygen supply in tumor cells to other cellular processes and reducing toxic ROS production [229]. In addition, HIF proteins up-regulate the CA-9 enzyme, now considered as one of the best markers for tumor hypoxia, to deal with the increased acidification caused by the shift to glycolytic ATP production [119].
As described above, HIF-1α and HIF-2α, however differently, play essential roles in developmental angiogenesis, and as may be expected HIF proteins are also important in vascularization of tumors. One of the most commonly hypoxia-induced and studied HIF target genes is VEGF, so far identified as the strongest promoter of neoangiogenesis, a process that improves tissue oxygenation through increased capillary density and that is crucial for tumor development [230]. Hypoxia is an important environmental factor directing the “angiogenic switch”, with HIF proteins activating this process through direct up-regulation of VEGF mRNA, tilting the balance in favor of angiogenesis-induction over inhibition. Concomitantly, loss of tumor suppressor genes, in particular TP53, may cause a reduction of inhibitors such as thrombospondin-1 [231], thereby contributing to angiogenesis. VEGF is also an inducer of vascular permeability, causing high interstitial and oncotic pressure within solid tumors, associated with reduced penetration of therapeutic drugs, and inhibition of VEGF in tumors has been shown to improve drug delivery into tumor tissue [178].

In addition to DNA-binding to gene HREs, HIF-1α has been demonstrated to bind and potentiate the activity of the intracellular domain of NOTCH-1, shown in a model where HIF-1α required NOTCH signaling to inhibit muscle and neural cell differentiation under hypoxia [232]. However, in a previous report, inhibition of myogenesis by hypoxia was independent of both NOTCH signaling and HIF-1α [233]. Furthermore, HIF-1α is connected to cell cycle arrest, potentially by directly displacing MYC binding from the p21Cip1 promoter, thereby inhibiting MYC-mediated repression of p21Cip1, leading to a block in cell cycle progression [234]. Similarly, HIF-1α can repress the DNA mismatch repair genes MSH2 and MSH6 via MYC displacement, however only when TP53 is deleted, suggesting a direct role of HIF in hypoxia-induced genetic instability [235].

Invasion and metastasis are defining characteristics of cancer and are promoted by tumor hypoxia. A substantial number of proteins involved in these processes are HIF-induced, including vimentin, fibronectin, keratins 14, 18, 19, matrix metalloproteinase 2, urokinase plasminogen activator receptor, cathepsin D, TGF-α, autocrine motility factor and the proto-oncogene c-MET [177]. Loss of expression of E-cadherin, a hallmark of invasion, is also associated with HIF activity [236]. Key metastatic mediators such as the chemokine receptor CXCR4 and lysyl oxidase (LOX), which is associated with hypoxia and poor outcome in several tumor types, are up-regulated by HIF proteins [237, 238].
Despite all adaptation advantages HIF activity brings, gain-of-function mutations within the HIF genes have not been found in tumors. One explanation could be that HIF-induced processes, such as angiogenesis, are not clonally selected. Another explanation relies on the fact that HIF-1α can induce apoptosis, for example by stabilizing the TP53 protein or via transactivation of the pro-apoptotic BCL2 family member BNIP3 [166, 239]. In such cases, additional mutations that inactivate TP53 or activate anti-apoptotic BCL2 proteins would be required for increased HIF-1α activity to promote, instead of inhibit cancer cell survival. A few reports show enhanced growth of HIF-1α-/- tumors compared to wild-type tumors, and HIF-1α-mediated reduction of proliferation together with increased apoptosis have been ascribed as underlying mechanisms [220]. However, most studies clearly demonstrate that loss of either HIF-1α or ARNT is detrimental for tumor growth, with similar or even increased apoptosis in HIF-1α-/- tumors when comparing with HIF-1α+/- tumors (reviewed in [178]). In addition, increased HIF-1α activity, including up-regulation of p21Cip1 involved in cell cycle arrest, has been shown to confer protection against oxidative stress-induced apoptosis in cortical neurons [240].

**HIF-1α versus HIF-2α**

As mentioned above, the two most important transcription factors mediating adaptive responses to hypoxia, HIF-1α and HIF-2α, appear to play different roles in development and tumor formation. These differences are certainly in part linked to the spatial and temporal variations in HIF-α subunit expression during embryogenesis and in adult tissues, and which are ultimately reflected in cancer cells depending on their origin. Concerning the regulation of HIF-α proteins, differential HIF phosphorylation within the PAS-B domain has been shown to render HIF-2α, but not HIF-1α, unable to repress the expression of the DNA repair gene NBS1 [241]. Interaction between HIF-2α, but not HIF-1α, and the NF-κB essential modulator (NEMO) is also demonstrated, which enhances normoxic HIF-2α transcriptional activity by facilitating CBP/p300 recruitment [242]. In addition, several reports indicate that HIF-2α, compared to HIF-1α, is relatively resistant to inhibition by FIH under normoxic conditions [173, 175, 243], which could have implications in the differences in HIF-α subunit regulation, observed in neuroblastoma cells (paper II).

Differential target gene preferences may also be involved in separating function of the two HIF-α proteins. Investigations, using microarrays and/or HIF-specific small
inhibitory RNAs, aiming at distinguishing between HIF-1α- and HIF-2α-mediated gene induction, indicate that HIF-1α is the predominant HIF transcription factor in several cell types [177, 244]. This HIF-1α dominance is not fully understood and although mechanisms for a potential inhibition of HIF-2α function have been suggested, including cytoplasmic trapping and inactivation by a presumptive HIF-2α-specific co-repressor [245, 246], this inhibitory effect appears to be highly cell type-specific. Furthermore, HIF-2α dominance in HIF gene regulation is commonly found in VHL-deficient RCC [223, 247, 248]. In several VHL-negative RCC cell lines HIF-1α protein is not expressed, possibly due to truncated HIF-1α mRNA transcripts or transcriptional silencing [249]. The differential actions by HIF-proteins seen in RCC cells, may however not be true for other cells with a functional VHL gene. Nevertheless, despite a clear HIF-1α-dominance in gene regulation of breast cancer cells, hypoxia-induced migration of the same cells revealed a requirement of both HIF-1α and HIF-2α [247]. Interestingly, evidence suggest that HIF-2α is not involved in hypoxia-mediated apoptosis, and the pro-apoptotic BNIP3 gene appears to be induced only by HIF-1α, and not HIF-2α [202, 223].

HIF-dependent induction of glycolytic enzymes seems to be preferentially mediated by HIF-1α, but several classic HIF-1α-regulated genes are becoming increasingly appreciated as HIF-2α predominant targets, including EPO [210, 211, 250], TGF-α [223], and VEGF [196, 223, 251]. These finding are not restricted to HIF-2α-only expressing RCC but also other cell types, such as neuroblastoma. Furthermore, transcriptional induction of the angiopoietin receptor, Tie-2, involved in angiogenesis, and the VEGF receptor-2 is mediated in a HIF-2α-dependent manner, which has been associated with the predominant role of HIF-2α in endothelial cells, but for the VEGF receptor-2 also as a result of co-operation between ETS transcription factors and HIF-2α, but not HIF-1α [130, 252]. These findings show, as mentioned before, that additional and potentially cell-type specific transcription factors can contribute to the differences seen in HIF-α subunit function. The HIF-2α-ETS co-operation has been further evaluated in breast cancer cells. In these cells was HIF-1α predominant in hypoxic gene induction, but a number of genes such as lysyl oxidase-like 2, involved in invasion and metastasis, and the HIF-1α negative regulator CITED2 were preferentially up-regulated by HIF-2α in collaboration with ETS family members [253]. Co-operation between HIF-2α and the SP1 transcription factor has also been observed in HIF-2α-mediated induction of the plasminogen activator inhibitor-1 and membrane type-1 matrix
metalloproteinase genes [254], however also HIF-1α has been demonstrated to interact with SP1. Perhaps the best example found so far of a HIF-2α-specific target gene is OCT-4, a transcription factor essential for maintaining stem-cell pluripotency, thus indicating a role of HIF-2α in stem cell function and regulation of differentiation [255].
THE PRESENT INVESTIGATION

Aims

The general objective of the work underlying this thesis was to further explore the role of hypoxia-inducible factors in tumor biology, with focus on HIF-mediated transcriptional responses in the childhood malignancy neuroblastoma.

The specific aims were:

To investigate the effects of hypoxia and HIF-1α on the expression of the HLH transcription factor ID2, a gene involved in early SNS development and thus potentially important in the neuroblastoma phenotype.

To elucidate the differences in oxygen-dependent regulation and function of HIF-1α and HIF-2α in neuroblastoma, and the relevance of HIF-2α in neuroblastoma aggressiveness.

To explore the regulation of the MXI1 gene by hypoxia and HIF proteins in neuroblastoma cells, and the role of MXI1 within the MYC-network and in HIF-signaling.

To investigate the effects of stem cell factor-mediated c-Kit-signaling on HIF-1α activity, and the potential reciprocal regulation between HIF-1α and c-Kit.

Results and Discussion

The effects of hypoxia on differentiation

A small group of neuroblastomas show tumor cells with a spontaneous neuronal-to-neuroendocrine/chromaffin lineage shift, close to zones of tumor necrosis, which led to the suggestion that hypoxia can drive neuroendocrine differentiation [58, 256]. Evidence for this trans-differentiation comes primarily from the observed hypoxic up-regulation of the neuroendocrine markers IGF-2 and TH in neuroblastoma cells. These genes are however foremost directly driven by hypoxia and HIF proteins, and therefore not necessarily associated with induction of differentiation. Also, the established neuroendocrine marker gene Chromogranin A was not induced in hypoxic neuroblastoma cells [256]. Instead,
investigations from our group, based on results from several neuroblastoma cell lines, global
gene expression analysis and xenografted tumor data, clearly show that hypoxic
neuroblastoma cells lose their differentiated characteristics – de-differentiate – and adopt a
more immature, neural crest-like phenotype [102, 159, 257]. Neuroblastoma cells grown at
1% O₂ decrease their expression of neuronal/neuroendocrine markers, including
Chromogranin A and B, NPY, GAP43, dHAND, HASH-1, Phox2b and neurofilament, whereas
genes involved in the determination process of neural crest cells into sympathetic precursors
(c-Kit, NOTCH-1, HES-1 and ID2) are up-regulated. Moreover, in xenografted tumors, the
neuronal markers dHAND and GAP43 were reduced in tumor cells adjacent to
necrotic/hypoxic regions [102]. As part of the hypoxic adaptation, neuroblastoma cells also
induce pro-survival genes, including IGF-2, TGF-β and VEGF [257]. The hypoxia-induced
de-differentiated neuroblastoma phenotype appears to persist for 24 h or more upon re-
oxygenation, which could present a window for tumor cells to spread to secondary sites, in
part as a consequence of their immature and hence possibly more motile characteristics [158].
Other reports further suggest that hypoxia and HIF proteins are involved in inducing an
immature phenotype of neuroblastoma cells. A truncated form of TRKA, which is NGF-
independent and constitutively activated in neural stem cells and in some neuroblastomas, was
found to be up-regulated by cobalt chloride, a hypoxia-mimetic inducing HIF proteins [258].
Conversely, the VHL tumor suppressor can induce neuroblastoma cells into a neuron-like cell
state [259].

Hypoxia-mediated de-differentiation is not restricted to neuroblastoma, since
down-regulation of differentiation markers and induction of some stem cell characteristics by
hypoxia are demonstrated in ductal breast carcinoma in situ [260]. Hypoxia has been shown to
down-regulate differentiation-specific proteins in prostate cancer cells, which was associated
with increased survival [261]. Although hypoxia-mediated differentiation has been suggested,
most reports demonstrate that hypoxia induces block of differentiation and stem cell
characteristics, not only in transformed cells but also in several different non-malignant cell
types [232, 233]. In summary, hypoxia-induced tumor cell de-differentiation may be one
mechanism by which hypoxia contributes to the selection of a malignant phenotype, since low
differentiation is generally correlated with increased aggressiveness and poor outcome in
tumors, including breast cancer [262], and neuroblastoma [27, 28].
**HIF-1α induces ID2 expression – link to a de-differentiated and aggressive neuroblastoma phenotype (Paper I)**

Our observation that hypoxia leads to a less differentiated phenotype of neuroblastoma cells, potentially associated with increased cancer cell aggressiveness, led us to investigate the molecular mechanisms behind these processes. Our focus was first directed at ID2, a gene known to be involved in neural crest development [90], to inhibit pro-neuronal bHLH proteins [67], and that is induced in neuroblastoma cells grown under hypoxic conditions [102, 257]. In paper I we further explore the regulation of ID2, as well as the other two ID factors expressed in neuroblastoma, ID1 and ID3, by hypoxia (1% O2) and the involvement of HIF-1α. By Northern blotting and quantitative real-time PCR (QPCR) we detected a fast induction of ID2, and to some extent ID1 but not ID3, under hypoxic conditions in both neuroblastoma and breast cancer cells. The hypoxia-mediated ID2 up-regulation was dependent on functional transcription machinery and, in terms of kinetics, similar to the induction of the classic HIF target VEGF, suggesting involvement of HIF proteins also in ID2 regulation. Indeed, specific HIF-1α-binding was found in vitro and in vivo, respectively determined by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP), at two hypoxia-response elements within the ID2 promoter. These HREs were also required for HIF-1α-mediated transcriptional activation of ID2, as determined by reporter gene experiments. The finding of two co-operating HIF-binding sites was interesting, since this mode of regulation is present in several previously known HIF targets, such as transferrin, GLUT1, PGK1 and IGF-binding protein-1 [139], however the functional consequences of this HRE distribution is not understood. A potential explanation for the existence of two or more HIF-binding sites regulating a gene, could involve a cellular mechanism that firmly ensures induction of the gene in response to HIF proteins, suggesting that up-regulation of ID2 plays an important role in the adaptation to hypoxic conditions.

Hypoxic neuroblastoma tumors and cells exposed to hypoxia reduce expression of SNS markers, while concomitantly augmenting expression of genes regulating early neural crest development, overall indicating a de-differentiation away from SNS characteristics toward a stem cell-like phenotype with stronger neural crest traits [102, 257]. HIF-dependent ID2 expression could therefore represent an early adaptive and differentiation-reducing response, occurring within hours of hypoxic exposure of neuroblastoma cells. By binding to and sequestering E-proteins, ID2 can indirectly inhibit DNA-binding and transcriptional activity of lineage-specifying transcription factors such as dHAND and HASH-1 (Fig. 8).
Hypoxia also down-regulate these neuronal transcription factors, as well as the E-protein E2-2, the primary dimerization partner for dHAND, HASH-1 and ID2 [67, 102, 257]. This regulatory pattern within the bHLH network acts in concert in the de-differentiation process together with HIF-activated ID2. Mouse developmental models demonstrate that HIF-1α is required for proper neural tube closing and neural crest formation, and that ID2 expression is substantially reduced in HIF-1α−/− embryos [203]. In addition, induced differentiation in neuroblastoma cells leads to down-regulation of the ID proteins [67].

**Figure 8. Potential effects of hypoxia/HIF-activated ID proteins.** Increased levels of ID2 (and ID1) by HIF-1α could potentiate sequestration of E-proteins (e.g. E2-2), thereby reducing interactions between lineage-specifying bHLH transcription factors and E-proteins. This process would lead to decreased expression of e.g. neuronal marker genes, which is also observed in hypoxic neuroblastoma cells, overall suggesting an induction of a less mature phenotype. In addition, hypoxia, through yet unknown mechanisms, down-regulates the tissue-specific transcription factors HASH-1 and dHAND, as well as the E2-2 protein. Hypoxia-induced ID1 could also increase sequestration of ETS transcription factors, leading to reduced p16 expression and less p16-mediated repression of cyclin/cyclin-dependent kinase complexes. These actions would enhance phosphorylation of the RB tumor suppressor protein and thus release the block of RB on E2F-mediated transcription, leading to cell cycle progression. Furthermore, hyperphosphorylated RB may have less potential to bind and inhibit ID2 or, alternatively, HIF-1α-activated ID2 could inhibit hypophosphorylated RB, resulting in increased proliferation.

Hypoxia-induced ID2, as observed in paper I, could play an essential role in several traits associated with increased aggressiveness of tumors, including neuroblastoma (Fig. 8). ID proteins can promote cell proliferation by inhibiting differentiation through E-protein sequestration [263], by antagonizing the ability of the RB tumor suppressor products to repress E2F-dependent transcription and cell cycle progression [96], or by blocking E-protein- and ETS transcription factor-mediated induction of p21Cip1, p15INK4b and p16INK4a.
Furthermore, ID genes are frequently over-expressed in many human tumors, and enforced transgenic expression of ID proteins leads to development of tumors in several tissues (reviewed in [97]). Also, xenografted hypoxic neuroblastoma cells produced slightly larger and faster growing tumors than corresponding normoxic cells [102]. Studies have shown that combined loss of ID1 and ID3 in mice causes defects in developmental angiogenesis in the forebrain, and inhibits growth, metastasis and angiogenesis of transplanted tumors [88]. These findings are attributed to the inability of recruitment of endothelial precursors from the bone marrow in response to VEGF, causing impaired neovascularization of tumors [88, 265]. The ID1/ID3 knock-out angiogenic defects appear confined to the CNS, reasonably as a result of compensation by ID2 in the rest of the body where it is expressed in endothelial cells (ID2 is not present in CNS endothelial cells), implicating ID2 in the angiogenic process as well.

However, these studies deal with aberrant responses due to ID-deficiency in non-transformed endothelial cells. Recent investigations, directly involving cancer cells per se, show that ID1 is a HIF-1α target, and promotes angiogenesis through activation of VEGF [266, 267]. In addition, ID1 can mediate cancer cell invasion and metastasis of tumor types such as breast and endometrial carcinomas, potentially via induction of matrix metalloproteinases [268]. In paper I, we also observed a fast hypoxic induction of the ID1 gene, suggesting a direct HIF target, in both neuroblastoma and T47D breast cancer cells, known to de-differentiate in response to hypoxia [260]. Interestingly, a recent report suggests that hypoxia actually decreases ID1 expression in many cell lines, via repression by the hypoxia-inducible ATF3 transcription factor, mediating a stress response pathway that reduces protein translation [269]. However, neuroblastoma cells appear to have insufficient levels of ATF3, and ID1 is therefore up-regulated by hypoxia and HIF proteins in these cells. Due to the strong homology between ID proteins, it is likely that ID2, if expressed, can perform several of the functions manifested by ID1.

Earlier data implicated that ID2 was required for MYC/MYCN-induced neoplasia, particularly concerning neuroblastoma [98], however subsequent studies from our group and others could not substantiate these findings. ID2 appears not to be critically involved in MYC/MYCN-dependent transformation, and is not a prognostic factor in neuroblastoma [99-101]. Nevertheless, HIF-activated ID2 (and potentially ID1) could still play important roles in hypoxic regions of solid tumors, perhaps in a temporal manner, facilitating the aggressive cancer cell phenotype by counteracting differentiation, promoting proliferation, as well as taking part in angiogenesis and invasion. Thus, future experiments
involving manipulation of ID expression in neuroblastoma cells and tumors could enhance our understanding of the significance regarding HIF-induced ID factors.

Regulation and target gene expression of HIF-1α and HIF-2α in neuroblastoma (Paper II)

Initial work from our group [158, 159], and the findings from paper II demonstrate that neuroblastoma cells grown at hypoxia (1% O₂) induce both HIF-1α and HIF-2α within hours (acute hypoxia). At prolonged hypoxic exposure, however, HIF-1α protein is down-regulated, whereas HIF-2α levels are continuously increased. At 5% oxygen, close to a normoxic pO₂ tension in vivo (similar to end-capillary oxygen levels) [111], HIF-1α is barely detected, in contrast to HIF-2α, which is stabilized and increases over time. Also, low basal levels of HIF-2α can be observed in cultured neuroblastoma cells already at atmospheric oxygen tensions (~21% O₂). The time-dependent differential HIF stabilization seen in hypoxic neuroblastoma cells is consistent with findings from previous reports. Early results from the Semenza group showed a HIF-1α stabilization peak within 4-8 h, and thereafter a decline in protein levels under continuous hypoxia in Hep3B cells [270]. An investigation of several cell types demonstrated similar oxygen-dependent stabilization and activation properties of HIF-1α and HIF-2α [243]. However, of interest regarding to our findings, rat pheochromocytoma PC12 cells were a prominent exception. These cells have neuronal and neuroendocrine SNS traits and exhibited a HIF pattern under prolonged hypoxia corresponding to neuroblastoma cells [243]. Lung epithelial cells also down-regulate HIF-1α under extended hypoxia, possibly due to an increase of a natural antisense against HIF-1α (aHIF) and destabilization of HIF-1α mRNA [271]. The presence of aHIF has not been investigated in neuroblastoma cells, but since HIF-1α mRNA levels are only moderately decreased by hypoxic treatment, translational and post-translational mechanisms are more likely involved in the reduction of HIF-1α protein under prolonged hypoxia. In contrast, both HIF-2α mRNA and protein are up-regulated at hypoxia, also seen in lung epithelial cells [271], and at 5% oxygen in neuroblastoma cells, suggesting multiple levels of oxygen-dependent HIF-2α regulation. Transcriptional induction of HIF-2α expression in response to changes in oxygen concentration is generally uncommon [196] and may be a unique trait of neuroblastoma cells (paper II). More studies are required to determine which transcription factors are involved,
and whether hypoxic HIF-2α mRNA induction is limited to neuroblastoma and a few other cell types.

Activation of HIF-2α protein, but not HIF-1α, at 5% O₂ has also been observed in previous studies. HeLa (cervical cancer) cells were found to induce HIF-2α protein at higher oxygen levels than those required for HIF-1α stabilization, and a number of additional cell lines display basal HIF-2α, but no HIF-1α protein expression under normoxic cell culture conditions [196]. Normoxic HIF-1α protein in mouse tissues in vivo has however been reported [272], but in the same study was HIF-1α up-regulation in e.g. the liver and kidney dependent on severe hypoxia and only transient, whereas in a study of hypoxic rats was HIF-2α protein induced at relatively higher oxygen pressure and appeared sustained in all tested organs [216].

The PHD2 and PHD3 enzymes, highly involved in the HIF-α degradation pathway, are induced by hypoxia and can also possess functional hydroxylation ability even at low oxygen levels [147, 273]. In paper II we detected up-regulation of these enzymes, to a higher degree at 1% than at 5% oxygen, and since the PHDs do not catalyze de-hydroxylation it is likely that at least PHD2, which is the primary HIF-1α-hydroxylating PHD [274], mediates the observed destabilization of HIF-1α over time at reduced oxygen levels in neuroblastoma cells. In contrast, the maintained high HIF-2α protein levels at prolonged hypoxia and at 5% O₂ clearly indicate that HIF-2α is less sensitive to PHD-dependent hydroxylation than HIF-1α. On the other hand, a potential increase in HIF-2α translation at 1% and 5% oxygen, via the MAPK or the PI3K pathway, might also counteract PHD-mediated HIF-2α degradation.

In accordance with the time- and oxygen-dependent HIF protein patterns, increased HIF-2α nuclear accumulation, HRE activation and DNA-binding to target genes were prominent at prolonged hypoxia and at 5% O₂ in neuroblastoma cells. In contrast, HIF-1α appeared to be the predominant HIF protein in acute hypoxic responses. Furthermore, microarray and QPCR analyses of neuroblastoma cells showed that expression of TH mirrored the changes in HIF proteins, with a fast induction at 1% (similar to HIF-1α) and a slower up-regulation at 5% O₂ (similar to HIF-2α). A list of approximately 75 genes with an expression pattern clearly similar to that of TH were identified, and included both previously known and novel oxygen-regulated genes. Selective HIF siRNA analysis showed that HIF-2α is the primary HIF transcription factor under prolonged hypoxia, and that HIF-2α governs the expression of several of the identified genes at 5% O₂. Importantly, HIF-2α appeared as the
main inducer of VEGF at 5% O₂, and the novel target gene SERPINB9, which is associated with metastatic melanoma [275], was severely affected by HIF-2α knock-down. In summary, HIF-1α and HIF-2α seem to regulate several genes in common, however in different temporal and oxygen-dependent manners (Fig. 9). Future experiments will determine if these patterns apply to other tumor cell types, or if they are specific to neuroblastoma. Since the HIF response within a cell also is affected by additional mediators, it is possible that transcription factors such as ETS and SP1, working in co-operation with HIF-2α (and/or HIF-1α) in target gene activation [253, 254], or the NEMO protein, which enhances normoxic HIF-2α but not HIF-1α transcriptional activity [242], may be involved in specifying neuroblastoma HIF function. In addition, stronger inhibition of HIF-1α by FIH, than of HIF-2α could contribute in diversifying HIF-α subunit activity [173, 175, 243].

Figure 9. HIF activity in neuroblastoma cells.
This model summarizes changes in protein levels and transactivation ability of HIF-α subunits in response to decreased oxygen tensions over time, as observed in paper II. HIF-1α is rapidly but transiently increased during hypoxia, mediating early transcriptional responses at 1% O₂. At 5% oxygen, a level close to end-capillary pO₂ tensions, HIF-1α is hardly detectable. In contrast, HIF-2α is continuously accumulated both at 1% and at 5% oxygen, governing prolonged hypoxic gene transcription as well as HIF-dependent responses under “physioxic” conditions in neuroblastoma cells. From paper II.

HIF-2α confers higher neuroblastoma aggressiveness (Paper II)

The in vitro data concerning HIF protein patterns and function were also consistent with findings in vivo from neuroblastoma specimen. Nuclear HIF-2α protein was frequently detected in neuroblastoma cells situated close to blood vessels in apparently well-oxygenized tumor areas. HIF-1α protein was however not present in these vascularized neuroblastoma regions, again indicating that HIF-2α has a more important role than HIF-1α at higher oxygen levels. The HIF-2α-high tumor areas also contained high VEGF protein expression, consistent with the predominant role of HIF-2α over HIF-1α in driving VEGF expression at 5% O₂ in cultured neuroblastoma cells. Furthermore, in a large neuroblastoma tumor material, arranged in a tissue microarray, there was a significant correlation between HIF-2α and VEGF,
suggesting that HIF-2α maintains VEGF expression also in the in vivo situation, and indicates a role of HIF-2α in neuroblastoma angiogenesis and growth. Selective HIF knock-down by siRNA further demonstrated that reduction of HIF-2α, but not HIF-1α, significantly slowed early xenografted neuroblastoma growth, likely due to reduced HIF-2α-dependent induction of genes such as VEGF.

A potentially higher importance of HIF-2α than HIF-1α in tumor growth has repeatedly been implicated in renal cell carcinomas [221, 224, 225], and a preferential role of HIF-2α in up-regulating TGF-α, which activates the growth-stimulatory EGF receptor pathway, in addition to increasing VEGF levels, are suggested underlying mechanisms [223, 276]. Also, teratomas derived from ES cells, where HIF-2α was genetically introduced at the HIF-1α locus, are larger and more proliferative, have higher VEGF and TGF-α expression and show increased vascularization compared to teratomas from wild-type ES cells [277]. In addition, HIF-2α-specific induction of the OCT-4 gene blocks embryonic development and promotes ES cell-derived teratomas [255]. These reports, together with the findings in paper II clearly indicate a substantial role of HIF-2α in tumor growth, and also link HIF-2α activity to the de-differentiation observed in hypoxic tumors. Thus, it would be interesting to investigate possible connections between HIF-2α and TGF-α, OCT-4 or the NOTCH pathway, implicated together with HIF-1α in maintenance of low differentiation [232], as well as a potential role of HIF-2α in ID2 regulation (paper I), under prolonged hypoxia in neuroblastoma cells.

HIF-2α, and in the same study also HIF-1α, have been claimed to operate as tumor suppressors in both glioblastoma and teratoma models [219]. In that report, high HIF-2α was linked to VEGF expression and vascularization, similar to our findings, but the net effect of HIF-2α (and HIF-1α) on xenograft tumor growth was negative, which is in contrast to the situation in neuroblastoma cells. In addition, disagreement with a role of HIF proteins as tumor suppressors comes from several reports, including many showing a positive association between HIF-1α and glioma progression [278]. In the investigated clinical neuroblastoma material, we found a significant correlation between high HIF-2α content and poor survival of neuroblastoma children. Furthermore, HIF-2α levels were also able to separate high-stage tumors, with high HIF-2α predicting poor outcome in advanced neuroblastoma disease. The findings in paper II clearly suggest HIF-2α as a prognostic marker independent of clinical staging, and overall indicate an oncogenic role of HIF-2α. We find that HIF-2α is an important factor mediating neuroblastoma aggressiveness, potentially
by increasing tumor growth and angiogenesis. HIF-2α can also induce genes associated with tumor invasion and metastasis, such as SERPINB9, and has potential to function as a clinical prognostic factor for low neuroblastoma survival.

**HIF-induced MXI1 does not affect MYC/MYCN signaling, but promotes specific HIF-1α target gene expression in hypoxic neuroblastoma cells (Paper III)**

HIF-1α has been demonstrated to inhibit MYC function under hypoxic conditions, mainly by displacing MYC from binding to promoters of target genes, such as cyclin-dependent kinase inhibitors and DNA repair genes, leading to reduced cell cycle progression and increased genetic instability, respectively [234, 235]. We and others have found that the MXI1 (MAX-interactor 1) gene, a reported antagonist of MYC [279], is consistently induced by hypoxia in several different cell types, including neuroblastoma ([257, 280, 281] and paper II), suggesting a general and thus important hypoxic adaptation response. Concerning neuroblastoma, MYCN is amplified in a substantial proportion of the tumors and constitutes a prognostic marker for poor outcome [34-36]. Interestingly, presumably as part of the hypoxia-mediated de-differentiation in neuroblastoma cells, MYCN expression is reduced [102]. Overall, these findings indicate that MYC/MYCN activity, despite a well-documented role of these proteins in proliferation and growth, may be negative for cancer cell progression in hypoxic tumor areas, and must be inhibited to avoid e.g. MYC/MYCN-induced apoptosis [80].

In this study (paper III), we show that MXI1 mRNA and protein is up-regulated by hypoxia in both neuroblastoma and breast cancer cells, and that MXI1 represents a novel direct HIF target gene. HIF siRNA-treatment abrogated hypoxia-induced MXI1, and specific DNA-binding (via ChIP analysis) and reporter gene transactivation by HIF-1α was detected within MXI1 gene regulatory sequences. Although a tissue microarray of clinical neuroblastoma samples indicated that most tumors were negative for MXI1, a significant correlation was found between high MXI1 protein levels and strong HIF-1α staining in this material. As might be expected from the observed differential HIF-α subunit activity in neuroblastoma (paper II), HIF-2α also mediated MXI1 induction at prolonged hypoxia.

By the use of siRNA against MXI1 we aimed at elucidating the function(s) of hypoxia-activated MXI1. Interestingly, despite investigating neuroblastoma cell lines with varying levels of MXI1, MYC and MYCN (amplified and non-amplified), no overall or
consistent effects by MXI1 knock-down on MYC/MYCN function were observed. As expected, MYC/MYCN expression and activity were reduced by hypoxia in some experiments, but MXI1 reduction did not generally alter MYC/MYCN levels or E-box transactivation, and did not largely affect endogenous MYC/MYCN target gene expression. These results were surprising, but a study where the DNA-binding region of MYC was switched with the corresponding domain of MXI1, showed that biological actions of MYC and MXI1 involve not only common genes but also distinct sets of genes [282]. In accordance, specific effects by MXI1 siRNA were found on several genes, previously not reported as direct MYC/MYCN targets. Two alternatively transcribed MXII isoforms, MXIIA and MXIIB, with endogenous protein expression have been identified, both in mouse and human [283, 284], and we show that MXIIB is the primary hypoxia- and HIF-responsive transcript. In human glioblastoma cells, this isoform was found to be a poor repressor of MYC target gene transactivation, which is in agreement with our results [284]. However, the underlying mechanism was suggested to be cytoplasmic retention of MXIIB, but we clearly observe increased nuclear MXII protein, both in neuroblastoma and breast cancer cells upon hypoxic exposure. Therefore our data indicate an augmented role of MXII as a transcription factor at low oxygen, and the need for alternative mechanisms explaining the lack of effects on MYC/MYCN activity by knock-down of MXII. Future studies will hopefully clarify this matter. A recent report suggest that HIF-2α can enhance MYC-induced cell transformation and MYC transcriptional activity, which is opposite to the antagonistic effects by HIF-1α on MYC function [285]. It is possible that this diverging HIF-mediated response also is operational in neuroblastoma cells, given the importance of HIF-2α in neuroblastoma behavior (paper II).

In contrast to the absent effects on MYC/MYCN function, microarray and QPCR experiments identified genes that, in addition to being reduced by MXII knock-down, were induced by hypoxia. A closer examination displayed that reducing MXII levels caused inhibition of specific endogenous HIF-1α target gene induction, and also blocked transactivation of an EPO HRE reporter gene construct, which was primarily regulated by HIF-1α, but not HIF-2α. Of note, the EPO gene is in fact now considered a HIF-2α preferential target, but its activation by HIF-2α requires additional genomic sequences (and likely co-operation with other transcription factors) besides the HRE [250]. Furthermore, the changes in gene expression evoked by MXII siRNA were highly similar in the investigated neuroblastoma cell lines, irrespective of MYCN-amplification status, suggesting that
MYC/MYCN activity does not influence MXI1 function, neither on HIF-1α-dependent nor on -independent gene regulation. Importantly, hypoxia- and HIF-1α-induced expression of the AK3L1 (adenylate kinase 3-like 1) gene, involved in maintaining nucleotide metabolism [286], was dependent on MXI1 activity, implicating an important function of MXI1 in the adaptation to low oxygen conditions. The Jagged-2 gene, encoding a ligand for the NOTCH receptor, was also regulated by both HIF-1α and MXI1, suggesting an additional mechanism in the control of genes with a potential role in keeping neuroblastoma and other cell types at a low-differentiated state under hypoxia ([102, 232] and paper I). In summary, HIF proteins directly up-regulate MXI1 expression, which also appears to be the most pronounced and consistent cellular response within the MYC-MAX-MAD network under hypoxic conditions. Knocking down MXI1 did not affect overall MYC/MYCN activity in neuroblastoma cells, but our results indicate that MXI1 can function as a HIF-1α-specific co-factor, adding to the list of transcription factors and other proteins involved in specifying the HIF response within a cell [139].

Reciprocal regulation between c-Kit signaling and HIF-1α activity (Paper IV)

Signaling via the receptor for stem cell factor (SCF), c-Kit, is critically involved in hematopoiesis, melanogenesis and reproduction. Due to its promotion of cell growth and survival, increased c-Kit activity is implicated in the formation of several human cancers, including gastrointestinal tumors and leukemia [287]. However, in neuroblastoma there are conflicting reports regarding the role of c-Kit in disease progression and prognosis [288, 289]. Nevertheless, c-Kit is involved in determination of neural crest cell fate [290], and c-Kit expression can be upregulated in neuroblastoma cells in response to hypoxia, potentially taking part in the de-differentiation process at low oxygen levels [102].

Here we find that c-Kit activation via SCF leads to stabilization and activation of HIF-1α in both megakaryoblastic leukemia and pro-B hematopoietic cells, overall indicating an induction of a hypoxic response, already under normoxic conditions. SCF-stimulation alone caused HIF-1α accumulation and subsequent up-regulation of HIF-1α target genes, including GLUT1, BNIP3, HK2, and VEGF. When HIF-1α expression was reduced, using siRNA, the transactivation of target genes was decimated. To ensure that the effects of SCF were dependent on c-Kit, we performed HRE reporter gene assays after over-expression of c-Kit in SK-N-BE(2)c neuroblastoma cells, which otherwise lack c-Kit protein
under normoxic conditions. Still, prolonged hypoxia can up-regulate c-Kit mRNA, and thus potentially the cognate protein, suggesting that c-Kit activity is more important in neuroblastoma during hypoxic conditions [102]. SCF-treatment induced HIF-dependent HRE activity only when c-Kit was present. Furthermore, the c-Kit-mediated HIF-1α accumulation occurred at the level of protein translation, and required activity of both the PI3K and the MAPK/ERK pathways. Inhibition of NADPH oxidase also blocked SCF-induced HIF-1α. As expected, c-Kit protein levels were reduced by SCF treatment, presumably via polyubiquitylation and proteasomal degradation [291, 292]. Interestingly, concomitant with the SCF-dependent increase in HIF-1α we observed a re-enhancement of c-Kit mRNA and cell surface protein levels, suggesting a positive feedback loop between c-Kit and HIF-1α. This feedback was also abrogated by inhibition of the signaling pathways leading to HIF-1α accumulation by SCF-stimulation.

Our results are in agreement with a previous report showing a PI3K-dependent induction of HIF-1α-binding to the VEGF promoter, and subsequent VEGF expression and secretion upon SCF treatment in small cell lung cancer cells [293]. These effects were also reduced by the c-Kit inhibitor imatinib. However, the actions of SCF were only markedly evident together with the HIF-1α-stabilizing agent cobalt chloride, whereas we show clear effects with SCF-stimulation alone, and we demonstrate that c-Kit signaling under normoxia leads to a general hypoxic response with induction of several HIF-1α target genes. Results in paper IV also provide evidence showing that the c-Kit tyrosine kinase receptor enforces its own expression via up-regulation of HIF-1α, similar to the autocrine loops formed by HIF-1α and several growth factors, such as IGF-2 [294]. A specific HIF-binding HRE within the c-Kit gene has not yet been identified, but further support to the finding of HIF-regulated c-Kit comes from an evaluation of a large tumor material, showing high c-Kit and SCF activity in perinecrotic glioblastoma tumor areas with high HIF-1α levels [295]. SCF-mediated c-Kit signaling in cells can potentially elicit several biological responses associated with hypoxia and HIF activity, such as angiogenesis via VEGF induction. In addition, the positive c-Kit-HIF feedback loop may be involved in maintaining a stem cell-like phenotype in e.g. hematopoietic precursors, as well as in tumor cells, including neuroblastoma.
Conclusions

Paper I

The ID2 and ID1 genes are induced by hypoxia in neuroblastoma cells. ID2 represents a novel direct target gene of HIF-1α.

Paper II

HIF-1α and HIF-2α are differentially regulated in a temporal and oxygen-dependent manner in neuroblastoma.
HIF-1α mediates acute hypoxic responses in neuroblastoma cells
HIF-2α protein is detected in neuroblastoma tumor cells adjacent to blood vessels, and in cultured neuroblastoma cells HIF-2α protein is present at 5% O₂, equivalent to normoxia in vivo.
HIF-2α regulates gene expression at prolonged hypoxia and at 5% oxygen.
HIF-2α is a positive factor for xenografted neuroblastoma growth, and high HIF-2α protein correlates to overall poor outcome in neuroblastoma children.
High HIF-2α levels correlate to worse prognosis within high stage tumors, and is an independent prognostic marker in neuroblastoma.

Paper III

MXI1 is directly induced by HIF proteins in hypoxic neuroblastoma and breast cancer cells.
Expression and function of MYC and MYCN are unaffected by MXI1 activity under hypoxic conditions in neuroblastoma cells.
MXI1 potentiates specific HIF-1α target gene expression at hypoxia.

Paper IV

SCF/c-Kit signaling mediates accumulation of HIF-1α protein and induction of HIF target gene expression under normoxic conditions.
SCF-induced HIF-1α activity is dependent on multiple regulatory signaling pathways.
A potential positive feedback loop exists between c-Kit and HIF-1α.
**POPULARIZED SUMMARY IN SWEDISH**

Cancer är en av människans vanligaste folksjukdomar och utgör även en av våra vanligaste dödsorsaker. Begreppet "cancer" innefattar egentligen en stor grupp av sjukdomar, men alla uppstår då en cell i kroppen börjar dela sig okontrollerat, vilket ger upphov till en onormal, elakartad massa av celler – en tumör. Cancerceller kan därifrån även sprida sig och skapa dottertumörer, metastaser, i andra delar av kroppen, vilket oftast kraftigt försämrar patientens möjlighet till överlevnad.

Neuroblastom är en cancerform som drabbar barn, med de flesta fall i mycket unga år (0-2 år), och i Sverige diagnostiseras ungefär 14 barn med neuroblastom varje år. Detta är en komplex tumörsjukdom med kraftigt varierande prognos, t ex förekommer fall som uppvisar spontan tillbakabildning av tumören, vilket innebär en god prognos. Dock har många barn med neuroblastom aggressiva tumörer som medför mycket svår sjukdom, och ungefär 50% av de drabbade barnen kan inte räddas till livet. Neuroblastom har sitt ursprung i utvecklingen av det sympatiska nervsystemet, som är en del av det icke-viljestyrda nervsystemet. Tumörerna kan uppkomma vid alla ställen där sympatiska nervsystemet anläggs i kroppen, vanligast är i binjurarna, och utgörs av omogna nervceller vilka inte vidareutvecklas till sin funktionella roll. Genom att undersöka vilka gener, och deras verksamma produkter – proteinerna, som är aktiverade i cancercellerna kan deras mognadsgrad bedömas. Generellt innebär lägre mognad av cancercellerna en aggressivare tumörsjukdom, dålig prognos och sämre överlevnad för barnen.

Neuroblastom är en s.k. solida tumör, som t.ex. lung- och bröstcancer, till skillnad från icke-solida cancerformer som leukemi, vilket drabbar de cirkulerande blodcellerna. Vaxande solida tumörer behöver syre och näring, som tillförs via blodkärlen. Men den expanderande tumören medför att vissa cancerceller distanseras för långt från blodtillförseln, vilket skapar syrebrist i dessa – hypoxi. För att bibehålla inflode av syre sätter tumörerna igång en nybildning av blodkärl. Dock är blodkärl i tumörer inte lika funktionella som motsvarande normala organ, t.ex. anläggs de inte korrekt och de läcker. Således, trots detta iscensättande av ny kärnbildning kommer vissa områden med cancerceller fortfarande vara dåligt syresatta via blodet, vilket skapar kvarstående hypoxi inom tumören. Mätningar i tumörer har visat ett genomsnitt på ca 1% syre, vilket kan jämföras med normala vävnaders syretryck på ca 5-6%. Hypoxi i tumörer har kopplats till en ökad aggressivitet hos flera cancerformer och sämre prognos för patienterna, samt även ett ökat motstånd inom tumörerna mot cancerbehandling. En viktig aspekt som vi och andra påvisat är att hypoxi
orskar lägre mognadsgrad hos cancerceller, inklusive i neuroblastom. Även detta kan vara en viktig del i den förhöjda aggressiviteten som observeras hos hypoxiska tumörer, vid jämförelse med bättre syresatta tumörer.

Alla yttre påverkningar, som t.ex. hypoxi, medför förändringar i aktiviteten av generna i en cell. Om en process i cellen kräver ökad aktivitet av en gen skrivos denna av, eller uttrycks, från arvsmassans DNA (transkription), vilket kontrolleras av proteiner som direkt binder DNA, s.k. transkriptionsfaktorer. De viktigaste transkriptionsfaktorerna som sköter anpassningen av en cell till en syrefattig miljö är de hypoxi-inducerbara faktorerna (HIF-1α och HIF-2α). Dessa proteiner ansamlas i stora mängder inom bara några minuter av syrebrist, medan de lika snabbt bryts ned då tillräckligt syre finns tillgängligt inne i cellen. HIF proteinerarna kontrollerar över 100 gener, vilka arbetar för att säkerställa energibehovet och överlevnaden hos cellen. T.ex. aktiverar HIF viktiga gener som orsakar nybildningen av blodkärl, både i normala vävnader och i tumörer.

I denna avhandling undersöks HIF proteinernas ingående roller i kontrollen av gener, som kan ha stor betydelse för cancercellers beteende vid hypoxi. Dessutom behandlas hur själva regleringen av HIF proteinerna sköts under olika förhållanden, samt vad dessa processer har för betydelse för cancercellers/tumörers aktivitet och aggressivitet, med särskilt fokus på neuroblastom. I delarbete I visas att HIF-1α i hypoxiska (1% syre) tumörceller, framför allt i neuroblastom, kan aktivera genen ID2, genom att direkt binda dess reglerande DNA. ID2, och även ID1 som aktiveras av hypoxi i samma studie, är negativa transkriptionsfaktorer d.v.s. de förhindrar andra transkriptionsfaktorers funktion. ID proteiner kan därmed blockera utmognad av celler, men även öka frekvensen av celldelning samt förmågan till cellspredning. Genom aktivering av ID2 via HIF, kan cancerceller erhålla lägre mognadsgrad och dra nytta av flera fördelsgivande cellulära processer, vilket sammantaget skulle kunna bidra till ökad aggressivitet hos en tumör.

Delarbete II visar att HIF-1α och HIF-2α, vilka liknar varandra strukturnässigt och reglerar flera gemensamma gener, utför sina funktioner i neuroblastomceller beroende på vilken miljö de befinner sig i. Resultaten i studien tyder på att HIF-1α är mest aktivt och fungerande vid akut hypoxi, medan HIF-2α tar över regleringen av HIF målgener vid långvarig hypoxi. Detta kan ha stor betydelse i neuroblastomtumörer, då syre tillförseln kan bli avbruten kortvarigt när ett blodkärl tillfälligt blir blockerat, eller då syrebristen blir mer utdragen. HIF proteinerna återfinns vanligtvis endast i tumörområden med syrebrist, och därför var det särskilt intressant att höga nivåer av HIF-2α kunde identifieras i cancerceller.
belägna intill blodkärl, och således i uppenbarligen mer syresatta områden i neuroblastomtumörer. I neuroblastomceller odlade vid 5% syre, motsvarande en fysiologiskt normal syreövrig i kroppens organ, var dessutom HIF-2α stabilt och kunde aktivera gener som annars bara är förknippade med hypoxi. I motsats upptäcktes inget HIF-1α protein i cancerceller nära blodkärl i neuroblastomtumörer, och i odlade celler var närvaron och aktiviteten av HIF-1α betydligt lägre än HIF-2α vid 5% syre. Dessutom fastställdes en klar koppling mellan höga HIF-2α nivåer och avancerad sjukdom och dålig överlevnad i ett större material med neuroblastomtumörer. Fynden i studien tyder på att HIF-2α medför en ökad aggressivitet i neuroblastom, och att HIF-2α kan användas vid framtida kliniska bedömningar av prognos för barn med neuroblastom.

I delarbete III demonstrieras att HIF proteinerna styr den hypoxiska aktiveringen av genen **MXII**, vars produkt också är en transkriptionsfaktor. MXII tillhör en grupp av transkriptionsfaktorer, vilka reglerar flera funktioner inom cellen såsom cellutbrytning och proteinsyntes, där MXII fungerar som en hämmare medan proteinerna MYC och MYCN agerar som aktiverare. Dock verkar inte den ökade aktiviteten av MXII under hypoxi påverka funktionen av MYC eller MYCN i neuroblastomceller, kanske för att de sistnämnda proteinerna är så centrala i biologin av denna tumörtyp. Istället finns det klara bevis i studien att MXII driver upp nivåerna av andra hypokändsensitive gener, och att MXII kan fungera som ett assistanterande protein till HIF-1α i regleringen av dessa gener.

HIF proteinerna aktiveras främst av sänkta syreövrig. Det finns dock flera exempel på hur olika proteiner via sina receptorer (mottagare) på cellytan signalerar in i cellen och ger ökad ansamling och funktion av HIF. Delarbete IV beskriver en ny sådan HIF-aktiverande signalväg, som omfattar receptorn e-Kit och dess bindande protein SCF (stamcellsfaktor). Signalering via e-Kit ger ökad mängd HIF-1α och även påföljande aktivering av HIF målgener, trots avsaknad av hypoxi i cellerna. Dessutom ger denna signalväg en positiv återkoppling, på sådant sätt att den förhöjda HIF-funktionen av SCF-stimulerar driver upp mängden e-Kit på cellytan.
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