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*Hypoxic gene regulation and
oncogenic pathways in neuroblastoma*

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LUND UNIVERSITY
Faculty of Medicine

Academic dissertation

By due permission of the Faculty of Medicine, Lund University Sweden to be defended at the main lecture hall, Pathology building, University Hospital MAS, Malmö, on Friday 28th of March, 2008, at 09.15 a.m. for the degree of Doctor of Philosophy, Faculty of Medicine

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<p>Abstract</p> <p>Neuroblastoma is a childhood tumor stemming from neural crest derivatives of the sympathoadrenal lineage. Neuroblastoma patients show remarkable clinical heterogeneity, with courses ranging from spontaneous regression to fatal tumor progression despite intense multi-modal treatment.</p> <p>Previous studies have shown that hypoxia pushes neuroblastoma cells towards a more immature phenotype, which correlates to aggressive disease. Here we define a role for the hypoxia inducible factor-2α in neuroblastoma tumor progression. While HIF-1α was transiently stabilized at hypoxia (1% oxygen), HIF-2α was induced and regulated HIF-specific target genes, such as VEGF, at later time points. Furthermore, HIF-2α was stabilized and transcriptionally active in cells grown at physiological oxygen levels (5% O₂). Subsequent microarray analysis showed that HIF-2α induced genes, previously identified as hypoxia regulated, at this physiological oxygen level. Several of these genes have been implicated in tumorigenic processes and correlated to adverse patient outcome in various tumor forms. Indeed, siRNA mediated knock-down of HIF-2α in neuroblastoma cells significantly reduced xenograft tumor growth, as compared to siHIF1-α treated or wild-type cells. Moreover, immunohistochemical analyses of a large neuroblastoma tumor material arranged in a tissue microarray showed that HIF-2α expression correlated to VEGF, and that high HIF-2α levels was predictive of poor patient prognosis.</p> <p>Prognostic markers of neuroblastoma patient adverse prognosis include amplification of the MYCN oncogene and an undifferentiated morphology. While these features discriminate high- from low-risk patients with precision, identification of poor outcome low- and intermediate-risk patients is more challenging. We analyze two large neuroblastoma microarray data sets by using a priori-defined gene expression signatures. The results show that differential overexpression of Myc transcriptional targets and low expression of genes involved in sympathetic neuronal differentiation predict relapse and death from disease. This was evident not only for high-risk patients, but also was robust in identifying groups of poor prognosis patients otherwise judged to be at low- or intermediate-risk for adverse outcome. These data suggest that pathway-specific gene expression profiling might be useful in the clinic to adjust treatment strategies for children with neuroblastoma.</p>		
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*”Om det inte är gjort i neuroblastom,
så är det inte gjort”*
Sven Påhlman

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List of papers

This thesis is based on the following papers, referred to as Papers I-IV:

- I** Recruitment of HIF-1 α and HIF-2 α to common target genes is differentially regulated in neuroblastoma: HIF-2 α promotes an aggressive phenotype
Linda Holmquist-Mengelbier*, Erik Fredlund*, Tobias Löfstedt, Rosa Noguera, Samuel Navarro, Helén Nilsson, Alexander Pietras, Johan Vallon-Christersson, Åke Borg, Katarina Gradin, Lorenz Poellinger and Sven Pålman
Cancer Cell 10: 413-23, 2006
*These authors contributed equally to this work
- II** Transcriptional adaptation of neuroblastoma cells to hypoxia
Erik Fredlund, Marie Ovenberger, Åke Borg and Sven Pålman
Biochem Biophys Res Comm 366:1054-60, 2008
- III** HIF-1 α induces MXI1 by alternate promotor usage with limited effects on the Myc network but strong enhancement of specific hypoxic target gene induction
Tobias Löfstedt, Erik Fredlund, Rosa Noguera, Samuel Navarro, Linda Holmquist-Mengelbier, Siv Beckman, Sven Pålman and Håkan Axelson.
Manuscript
- IV** Myc-pathway activation and stage of neuronal differentiation identify more malignant neuroblastomas
Erik Fredlund, Markus Ringnér, John M. Maris and Sven Pålman
Manuscript

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Abbreviations

Numbers in square brackets refer to the Entrez Gene database (ncbi.nlm.nih.gov)

AKT	v-akt murine thymoma viral oncogene homolog 1 [207]
ARNT	Aryl hydrocarbon receptor nuclear translocator [405]
bFGF	Fibroblast growth factor 2 (basic) [2247]
bHLH	Basic helix-loop-helix domain
BMP	Bone morphogenetic protein
CAIX	Carbonic anhydrase 9 [768]
CASH-1	Achaete-scute complex homolog 1 (Drosophila), chicken homolog [386573]
CBP	CREB binding protein [1387]
CHD5	Chromodomain helicase DNA binding protein 5 [26038]
COG	Children's oncology group
DBH	Dopamine beta-hydroxylase [1621]
dHAND	Heart and neural crest derivatives expressed 2 [9464]
E	Embryonal day
E2F	E2F transcription factor
EGF	Epidermal growth factor receptor [1956]
EPAS1	see HIF-2alpha
ERK	Extracellular signal-regulated kinase
FIH	Factor inhibiting HIF [55662]
FISH	Fluorescent in situ hybridization
GATA2	GATA binding protein 2 [2624]
h	Hours
HASH-1	Achaete-scute complex homolog 1 (Drosophila) [429]
HER2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) [2064]
Hes-1	Hairy and enhancer of split 1, (Drosophila) [3280]
HEY1	Hairy/enhancer-of-split related with YRPW motif 1 [23462]
HIF-1alpha	Hypoxia-inducible factor 1, alpha subunit [3091]
HIF-1beta	see ARNT [
HIF-2alpha	Endothelial PAS domain protein 1 [2034]
HIF-3alpha	Hypoxia-inducible factor 3, alpha subunit [64344]
HRE	Hypoxia responsive element
IGF	Insulin like growth factor
IGF-1	Insulin-like growth factor 1 [3479]
INSS	International neuroblastoma staging system
KIF1Bbeta	Kinesin family member 1B, beta isoform [23095]
LZ	Leucine zipper domain
Mad1	MAX dimerization protein 1 [4084]
Mad3	MAX dimerization protein 3 [83463]
Mad4	MAX dimerization protein 4 [10608]

MASH-1	Achaete-scute complex homolog 1 (Drosophila), mouse homolog [17172]
Max	MYC associated factor X [4149]
Mga	MAX gene associated [23269]
mmHg	Millimeters of mercury
Mnt	MAX binding protein [4335]
mTOR	Mammalian target of rapamycin [2475]
Mxi1	MAX interactor 1 [4601]
MYC/Myc	v-myc myelocytomatosis viral oncogene homolog (avian) [4609]
MYCL/Mycl	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian) [4610]
MYCN/MycN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian) [4613]
NF	Neurofilament (medium polypeptide, 150 kDa) [4741]
NGF	Nerve growth factor [4803]
Notch1	Notch homolog 1, translocation-associated (Drosophila) [4851]
NOTCH3	Notch homolog 3 (Drosophila) [4854]
NTF3	Neurotrophin 3 [4908]
ODD	Oxygen dependent degradation domain
p19ARF	Cyclin-dependent kinase inhibitor 2A (p16, inhibits CDK4) [1029]
p21Cip1	cyclin-dependent kinase inhibitor 1A (p21, Cip1) [1026]
p27Kip1	Cyclin-dependent kinase inhibitor 1B (p27, Kip1) [1027]
p300	E1A binding protein p300 [2033]
p53	Tumor protein 53 [7157]
PAS	Per-Arnt-Sim domain
PHD1	egl nine homolog 2 (C. elegans) [112398]
PHD2	egl nine homolog 1 (C. elegans) [54583]
PHD3	egl nine homolog 3 (C. elegans) [112399]
Phox2a	Paired-like homeobox 2a [401]
Phox2b	Paired-like homeobox 2b [8929]
PI3K	Phosphoinositide-3-kinase
pO2	Oxygen partial pressure
RCC	Clear cell renal cell carcinoma
SAGE	Serial analysis of gene expression
Scg10	Superior cervical ganglion 10 [11075]
SHH	Sonic hedgehog [6469]
SIF	Small intensely fluorescent (cell)
Skp2	S-phase kinase-associated protein 2 (p45) [601436]
TH	Tyrosine hydroxylase [7054]
TrkA	Neurotrophic tyrosine kinase, receptor, type 1 [4914]
TrkC	Neurotrophic tyrosine kinase, receptor, type 3 [4916]
TRRAP	Transformation/transcription domain-associated protein [8295]
VEGF	Vascular endothelial growth factor [7422]
VHL	von Hippel-Lindau tumor suppressor [7428]
v-myc	avian myelocytomatosis viral oncogene

Introduction

The first case of neuroblastoma was mentioned by the German pathologist Virchow in a 1864 in study on hyperplasia of the pineal and the adrenal glands. Virchow regarded his finding as a form of glioma, but in 1891 Marchand histologically linked the disease to the sympathetic nervous system. This notion was further supported by Wright who in 1910 coined the term neuroblastoma. Grade of tumor differentiation was early recognized as a prognostic factor and in 1927 Cushing and Wolbach described the spontaneous differentiation of a malignant neuroblastoma into a benign ganglioneuroblastoma. Interestingly, differentiation therapy was one of the first treatments other than surgery, radiation and chemotherapy tried on neuroblastoma patients. In the 1954 British report on “progress in cancer research” (a whole three pages) it was reported on the partly successful use of vitamin B₁₂ as a differentiation inducing therapy. Neuroblastoma research was brought into the molecular era in the early 1980’s, when the *MYCN* proto-oncogene was found to be amplified and to contribute to poor patient outcome. This genetic alteration is still one of the most powerful prognostic markers in neuroblastoma. Tumor hypoxia is a physiological state that occurs due to excessive tumor growth accompanied with a lack of vascular supply of oxygen. Hypoxia has been correlated to resistance to treatment, a diminished response to radiation therapy and to tumor progression. Specifically in neuroblastoma, hypoxia pushes cells towards a more undifferentiated state.

In this thesis, I have investigated the role of hypoxia and the hypoxia inducible transcription factors in neuroblastoma. Furthermore, I examine signaling downstream of the Myc-family transcription factors and stage of neuronal differentiation in relation to neuroblastoma patient prognosis. With the results presented here I emphasize the importance of these pathways in the understanding of neuroblastoma progression and aggressiveness.

Background

The sympathetic nervous system

Overview

The efferent branch of the autonomous nervous system is divided into two counteracting parts involved in the regulation of organism homeostasis. As a general difference to the somatic part of the peripheral nervous system the autonomous nervous system has pre- and postganglionic neurons connected from the spinal cord and medulla oblongata in extra-spinal ganglia to effector organs. In addition, the synapses of the autonomous nervous system are less effective in transmitter substance re-uptake, leading to a local as well as a systemic effect, as elegantly demonstrated by Otto Loewi in 1921 (Loewi,

1921). The parasympathetic nervous system emanates from preganglionic neurons in the brain stem and spinal cord. Axons from these neurons exit the spinal cord at the cranial and sacral regions and connect to parasympathetic ganglia, which generally are located in proximity to or incorporated in their affected organs. Stimulation from this sector of the autonomous nervous system results in the body having a resting state, e.g. lowering heart rate and blood pressure and also enabling food intake through stimulation of the digestive system.

On the contrary, the sympathetic nervous system regulates bodily functions into a “*fight or flight*” response. Blood flow and

pressure are raised by increased heart rate and vasoconstriction, pupils become dilated and glucose is released from the liver into the blood. This systemic response is due to the release of the catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline) by sympathetic postganglionic neurons to their affected organs, as well as into the blood stream, thus generating a true systemic response. Preganglionic sympathetic neurons originate from the thoracic and lumbar regions of the spinal cord and their axons link to postganglionic neurons in ganglia which, in contrast to the parasympathetic nervous system, are distant from their peripheral target organs. These ganglia are the interconnected sympathetic chain ganglia located in the paravertebral sympathetic trunks, and the cervical and mesenteric ganglia. Neurons from the sympathetic ganglia connect the sympathetic nervous system to its peripheral targets. Contributing to the systemic response of sympathetic nervous system activation is the medulla of the adrenal gland. This juxtarenal organ consists of chromaffin cells that resemble sympathetic neurons, but are phenotypically distinct. The adrenal gland is in essence a sympathetic ganglion able to release catecholamines directly into the circulatory system, thus giving a prolonged and enhanced nonspecific response to sympathetic stimuli. The enteric nervous system of the digestive tract is often regarded as a third and independent part of the autonomous nervous system. Even though stimuli from both the sympathetic and parasympathetic nervous systems converge and adjust this system, it can also function self-sufficiently.

Development of the sympathetic nervous system

All parts of the nervous system are derived from the neural plate, defined as a thicken-

ing along the midline of the dorsal part of the embryonal ectoderm (Fig. 1). This structure is formed early in embryogenesis. At approximately week 4 of human embryonal development it folds inwardly to form the neural tube, which eventually will develop into the central nervous system. The neural crest is a transient structure present on the dorsalmost part of the neural tube or on the fold itself, i.e. the border between the developing neural tube and the ectoderm. The cells of the neural crest are migratory, explaining the transient existence of the structure, and are best known for their multipotent potential. Dependent on anteroposterior origin the neural crest cells give rise to such diverse structures as the peripheral nervous system, glial cells, facial bones and cartilage, melanocytes of the skin and hair, and the outflow tract of the heart (Gilbert, 2000). The neural crest cells migrate non-randomly along specified pathways in the embryo and these paths of migration determine cell fate. Cues from the surrounding extracellular matrix provide both migratory guidance and affect cell transcriptional programs, thus leading to lineage commitment (Gammill and Bronner-Fraser, 2003).

The sympathetic nervous system is derived from neural crest cells. In the trunk region, these cells migrate ventrally through the anterior part of the somites and aggregate adjacent to the dorsal aorta. At this location signals from surrounding embryonal structures lead to commitment to the sympathoadrenal lineage. From here cells migrate further ventrally to develop secondary structures such as the sympathetic ganglia and the cells of the adrenal medulla (Påhlman and Hedborg, 2000). During and after this second line of migration the sympathoadrenal precursor cells encounter environmental cues signaling for further migration, proliferation and eventually differentiation (Harris and Erickson, 2007; Pålman and Hoehner, 1996).

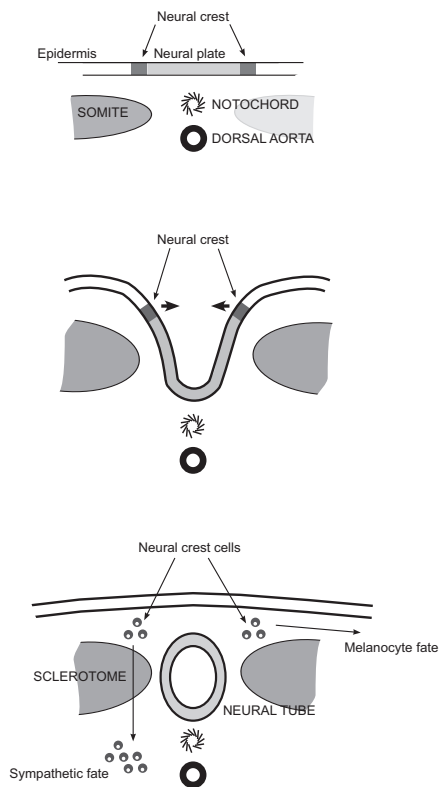


Figure 1. Formation of the neural crest during embryonal development. The neural crest is a transient structure on the border between the epidermis and the developing neural tube. Patterning of the neural plate is induced by signals emanating from the notochord. Subsequent to these signals, the neural plate invaginates and folds to form the neural tube. During this process the neural crest cell population is induced. By migration through specified paths in the embryo, they adapt diverse developmental fates.

At their final locations the precursor cells acquire one of the three postulated specialized fates of cells of the sympathoadrenal lineage: the sympathetic neurons, the chromaffin cells, and the small intensely fluorescent (SIF) cells (Anderson, 1993). The sympathetic neuronal cells initially pattern the paravertebral trunk and subsequent cell

division and differentiation give rise to the sympathetic ganglia. Also developing in the sympathetic ganglia are scattered SIF cells. Morphologically SIF cells have both neuronal and secretory chromaffin features, suggestive of an intermediate sympathetic phenotype, and this cell type has been proposed to function as ganglionic interneurons (Huber, 2006). The secretory chromaffin cells aggregate adjacent to the developing adrenal glands and into distinct extra-adrenal bodies, the paraganglia, the largest being the Organ of Zuckerkandl. During embryonal development the chromaffin cells enter and expand into the adrenal gland, forming a rudimentary adrenal medulla. This structure remains comparatively small until birth and also contains undifferentiated sympathetic neuroblasts (Hoehner et al., 1996). In the absence of a fully functional adrenal medulla the main embryonic source of catecholamines are the extra-adrenal chromaffin tissues.

After birth the sympathetic nervous system encounters extensive remodeling. The adrenal chromaffin cells expand, creating a proper adrenal medulla at the expense of the adrenal cortex. Simultaneously the undifferentiated neuronal cells of the adrenal medulla differentiate and within the mature medulla present as nests of sympathetic neurons (Holgert et al., 1996). In addition, the SIF cells of the sympathetic ganglia are diminished in number and are sparsely present in the adult (Påhlman and Hedborg, 2000). The gradual process of adrenal medulla maturation occurs in conjunction with regression of the paraganglia, evidencing a shift in catecholamine supply from extra-adrenal to adrenal. This process is thought to be finished at app. 2-3 years of age, however the increase in size of the adrenal medulla continues during childhood and adolescence.

Cues and transcription factors in sympathoadrenal development

Sympathetic lineage determination and diversification are thought to be regulated under the influence of growth factors and hormones secreted by embryonal structures in the path of migrating neural crest cells (e.g. Groves et al., 1995; Stern et al., 1991). One theory proposes the existence of a sympathoadrenal precursor cell common to the above mentioned cell fates; this notion is based on the early expression of marker genes relating to both neuronal and chromaffin precursors (Anderson et al., 1991; Carnahan and Patterson, 1991). Early after trunk neural tube closure, ventrally migrating neural crest cells in avians and mouse arrive at the dorsal aorta where they are exposed to members of the bone morphogenetic protein (BMP) family secreted from this embryonal structure. BMP stimulation leads to neural crest cell upregulation of the primary sympathoadrenal lineage marker gene HASH-1 (CASH-1 and MASH-1 in chicken and mouse respectively), corresponding to sympathetic adrenal lineage determination (Reissmann et al., 1996; Shah et al., 1996). HASH-1 is a member of the basic-helix-loop-helix transcription factors and in mouse and chicken models BMP mediated CASH-1/MASH-1 induction has been shown to be pivotal in sympathoadrenal lineage commitment (Guillemot et al., 1993; Schneider et al., 1999). Independently of HASH-1, the paired homeodomain transcription factor Phox2b is induced by BMP stimulation and this protein too has been shown to be essential for sympathetic structures (Pattyn et al., 1999). Acting under, and regulated by HASH-1 and Phox2b are the transcription factors dHAND, Phox2a and GATA2 (Hirsch et al., 1998; Howard et al., 2000; Pattyn et al., 1997). Together these five factors create an interconnected transcriptional network that concert early sympathetic dif-

ferentiation (Goridis and Rohrer, 2002; Huber, 2006). This is marked by upregulation of the key enzymes tyrosine hydroxylase (TH) and dopamine β -hydroxylase (DBH) of the catecholamine synthesis, as well as pan-neuronal markers such as superior cervical ganglion 10 (Scg10) and neurofilament (medium polypeptide 150kDa; NF) (e.g. Groves et al., 1995; Stanke et al., 1999).

Sympathetic neuronal differentiation is dependent on upregulation of specific neurotrophin receptors (Påhlman and Hoehner, 1996). During development of the sympathetic ganglia proper neuronal precursor cells are exposed to growth factors such as basic fibroblast growth factor (bFGF) and insulin like growth factors (IGF). Initially this leads to upregulation of the neurotrophin receptor TrkC and signaling by binding of the ligand neurotrophin 3 (NT3) results in growth inhibition and upregulation of a second neurotrophin receptor, TrkA. TrkA is the cognate receptor to nerve growth factor (NGF) and its presence corresponds to terminally differentiated sympathetic neurons that are dependent on NGF for survival (Anderson, 1993; Anderson and Axel, 1986). Early *in vitro* experiments showed that exposure of sympathoadrenal precursors to glucocorticoids promoted downregulation of neuronal markers and subsequent differentiation into the SIF and adrenal chromaffin phenotypes (Anderson and Axel, 1986; Carnahan and Patterson, 1991). Glucocorticoids are produced and secreted by cells of the adrenal cortex and it was consequently believed that arrival of precursor cells at this location was pivotal to their terminal chromaffin differentiation. This theory has since been questioned by several lines of evidence showing partial adrenal chromaffin differentiation independently of glucocorticoid stimulation or indeed the adrenal cortex (Finotto et al., 1999; Gut et al., 2005). Thus, the instructive cues leading to this line of differ-

entiation are largely unknown. However, the systemic catecholamine secretory function of chromaffin cells requires access to the vascular system, and it has been suggested that signals from blood vessels contribute to chromaffin differentiation (Hall and Landis, 1991). Furthermore, the theory of sympathoadrenal specification, outlined above, has recently encountered several caveats (reviewed in Harris and Erickson, 2007; Huber, 2006), suggesting the existence of alternate migration routes and perhaps additional sympathetic progenitor lineages (Ernsberger et al., 2005).

Neuroblastoma

Overview

Neuroblastoma is a childhood malignancy of the sympathetic nervous system, more specifically stemming from immature neuroblasts of the sympatho-neuronal lineage (De Preter et al., 2006; Hoehner et al., 1996; Hoehner et al., 1998). Tumors locate to structures where these cells are found during development, the major sites being the sympathetic ganglia and the adrenal gland. Neuroblastoma accounts for 7-8 % of all diagnosed childhood cancers, making it the most common solid extracranial malignancy in this patient group (American Cancer Society, 2005). Furthermore, it is by far the most commonly

diagnosed tumor of infancy, and 90 % of all neuroblastoma patients are diagnosed before the age of five (Brodeur and Maris, 2002). Clinically, neuroblastoma is characterized by considerable heterogeneity, with patient outcome ranging from spontaneous tumor regression (Cushing and Wolbach, 1927) to malignant disease progression despite intense treatment. Approximately 40-50% of all patients present with metastatic spread at diagnosis (Brodeur, 2003; Maris et al., 2007), and thus neuroblastoma is estimated to cause approximately 15 % of all childhood tumor related deaths (American Cancer Society, 2005).

Table 1. The International neuroblastoma staging system. From Brodeur and Maris (2002).

Stage 1	Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (i.e., nodes attached to and removed with the primary tumor may be positive).
Stage 2A	Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically.
Stage 2B	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically.
Stage 3	Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement. The midline is defined as the vertebral column. Tumors originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column.
Stage 4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/or other organs, except as defined for stage 4S.
Stage 4S	Localized primary tumor, as defined for stage 1, 2A, or 2B, with dissemination limited to skin, liver, and/or bone marrow (limited to infants younger than 1 year). Marrow involvement should be minimal (i.e., <10% of total nucleated cells identified as malignant by bone biopsy or by bone marrow aspirate). More extensive bone marrow involvement would be considered stage 4 disease.

Clinical factors

The two most prominent clinical factors relating to patient prognosis are patient age and tumor spread at diagnosis. Regardless of other clinical parameters patients diagnosed at an age less than 12 months (Shimada et al., 1984), or recently suggested 18 months (London et al., 2005a; London et al., 2005b) have a better prognosis than older patients. While this has been known for some time (Breslow and McCann, 1971) no biological explanation has been offered. The international neuroblastoma staging system (INSS) uses a four grade scale to describe neuroblastoma clinical presentation (Table 1; reviewed in Brodeur and Maris, 2003). In general, patients with localized disease (INSS stages 1 to 3) have favorable prognosis, whereas patients diagnosed with disseminated disease (INSS stage 4) have comparatively poor prognosis. Children younger than 1 year with metastatic spread limited

to liver, skin and minimal bone marrow involvement are generally considered to have favorable prognosis; this disease pattern has been termed INSS stage 4S. To date very few genetic and molecular markers have outperformed this system as a prognostic tool for patient outcome.

Genetic markers

Several genetic markers correlating to poor patient outcome have been described, the most prominent being amplification of chromosomal region 2p24, harboring the *MYCN* oncogene (Brodeur et al., 1984) (as described further on). Also of clinical importance is deletion of the 1p36 region of the short arm of chromosome 1, a region frequently deleted in several cancer forms (Knuutila et al., 1999). This deletion has been reported to be present in as much as 20 to 30 % of all neuroblastomas and correlates strongly to poor

patient outcome and *MYCN* amplification (Attiyeh et al., 2005; Caron et al., 1996). The high frequency of deletions of the 1p36 region in neuroblastoma suggests the existence of a tumor suppressor located to this region. Recently the p19^{ARF}/p53 modulator *chromodomain helicase DNA binding domain 5* gene (*CHD5*) was proposed as such a tumor suppressor, due to its location in this region (Bagchi et al., 2007). Chromatin remodeling is developmentally regulated (Kiefer, 2007) and *CHD5* is expressed in neuronal tissues including the adrenal gland. Low expression in neuroblastoma has been correlated to poor prognostic (Thompson et al., 2003). Another gene most recently suggested to be a 1p36 tumor suppressor is *kinesin family member 1B* (*KIF1B β*). This gene, originally described as a microtubule related transporter (Zhao et al., 2001), has been shown to negatively affect NGF withdrawal-induced apoptosis and to have reduced expression in 1p36 deleted neuroblastomas (Kaelin, 2008). Also associated with an adverse patient prognosis are deletions at chromosomal region 11q (Attiyeh et al., 2005; Maris et al., 2001) and partial gain of 17q (Bown et al., 1999; Caron et al., 1996). No putative neuroblastoma tumor suppressors or oncogenes have been reported at these regions.

Total tumor cell DNA content (DNA index) has also been shown to have prognostic value, especially for patients diagnosed before their second year (Bagatell et al., 2005). Less aggressive tumors in this patient group tend to be hyperdiploid (or near-triploid), whereas more malignant tumors have a near diploid karyotype. Based on DNA-index and the specific chromosome region gains and losses mentioned above, a genetic model of neuroblastoma development has been suggested (Brodeur, 2003).

The clinical impact of tumor cell differentiation

The concept of tumor cell differentiation has proven very useful in predicting neuroblastoma patient outcome. On the morphological level a differentiated histopathology has for some 80 years been known to correlate to favorable prognosis (Cushing and Wolbach, 1927). Histologically neuroblastoma is, along with e.g. the childhood neoplasms retinoblastoma, Wilm's tumor, Ewing's sarcoma and rhabdomyosarcoma, described as one of the small round blue cell tumors (Maris and Denny, 2002). These tumors are characterized by an undifferentiated morphology and small cell size with scant cytoplasm and enlarged DNA-rich nuclei (staining blue with hematoxylin-eosin). Neuroblastomas are subcategorized into three histological groups ranging from poorly differentiated tumors (neuroblastoma proper), via semi-differentiated ganglioneuroblastoma, containing both undifferentiated and neuron-like cells, to highly differentiated non-dividing tumor cells of ganglioneuroma. An undifferentiated phenotype correlates to poor patient prognosis, whereas the differentiated ganglioneuromas are considered benign (Shimada et al., 1984). This morphological evaluation of tumor cell differentiation is together with DNA index and the mitotic-karyorrhectic index, relating to number of mitotic and necrotic/apoptotic cells, incorporated into the Shimada histopathological tumor classification system (Shimada et al., 1999; Shimada et al., 1984). Unfavorable biology according to this system is highly correlated to poor patient prognosis (Shimada et al., 1999).

The clinical implications of stage of tumor cell differentiation have also been inferred from expression marker studies on the molecular level. One prominent example of this is the correlation between high expression of the neurotrophin receptors TrkA and

TrkC and favorable patient prognosis (Kogner et al., 1993; Nakagawara et al., 1993; Suzuki et al., 1993; Yamashiro et al., 1996). These proteins have been related to a more differentiated tumor phenotype (Hoehner et al., 1995; Suzuki et al., 1993), but while they have a role in the development of the sympathetic nervous system (Påhlman and Hoehner, 1996) the functional role of e.g. TrkC in mature sympathetic neurons is unclear. The concept is corroborated by studies showing low expression of markers for mature sympathetic neuronal differentiation in malignant neuroblastoma tumor samples (Hedborg et al., 1995). Perhaps contrary to the expected, an undifferentiated morphology does however not seem to correlate to the molecular characteristics of immature cells committed to the sympatho-adrenal lineage. Expression of earlier sympathetic neuronal markers such as dHAND, HASH-1 and Phox2B seems ubiquitous in neuroblas-

toma and does not relate to neither tumor stage of differentiation nor other prognostic factors (Gestblom et al., 1999; Raabe et al., 2008). While this suggests that neuroblastoma cells retain early sympathetic neuronal characteristics (Hoehner et al., 1998), poor differentiation assessed in this way does not seem to relate to an increased tumor aggressiveness. The role of early neural crest expressed genes such as Notch is more enigmatic. While neuroblastoma cell lines and tumors have been shown to express Notch receptors and ligands (De Preter et al., 2006; van Limpt et al., 2000; Van Limpt et al., 2003), the functional data remains sparse. In a recent study high levels of *NOTCH3* and *HEY1* in ganglioneuromas as compared to neuroblastomas suggesting that Notch3 and Hey1 support or maintain neuronal differentiation (Revet et al., 2008). Possibly in contrast to these findings, expression of Notch1 and the down-stream target Hes-1 have been

Table 2. Neuroblastoma risk classification according to the Children's Oncology Group. From National Cancer Institute (www.cancer.gov).

INSS Stage	Age	MYCN Status	Shimada Classification	DNA Ploidy	Risk Group
1	0-21y	Any	Any	Any	Low
2A/2B	<365d	Any	Any	Any	Low
	≥365d-21y	NonAmplified	Any	-	Low
	≥365d-21y	Amplified	Favorable	-	Low
	≥365d-21y	Amplified	Unfavorable	-	High
3	<365d	NonAmplified	Any	Any	Intermediate
	<365d	Amplified	Any	Any	High
	≥365d-21y	NonAmplified	Favorable	-	Intermediate
	≥365d-21y	NonAmplified	Unfavorable	-	High
	≥365d-21y	Amplified	Any	-	High
4	<548d	NonAmplified	Any	Any	Intermediate
	<548d	Amplified	Any	Any	High
	≥548d-21y	Any	Any	-	High
4S	<365d	NonAmplified	Favorable	>1	Low
	<365d	NonAmplified	Any	1	Intermediate
	<365d	NonAmplified	Unfavorable	Any	Intermediate
	<365d	Amplified	Any	Any	High

shown in undifferentiated, neural crest-like perivascular neuroblastoma tumor cell populations (Pietras et al., 2008) and active Notch1 has also been shown to be important for maintaining a pool of neuronal precursor cells in developing sympathetic ganglia (Tsarovina et al., 2008).

vival for this group of patients is estimated to some 40% (Matthay et al., 1999).

Risk assessment and outcome

The overall survival for neuroblastoma patients has been reported to be some 60-70% (Gatta et al., 2005; Spix et al., 2006), but specific groups of patients have substantially better or worse prognosis. The clinico-pathological staging systems of the INSS and Shimada, together with age at diagnosis, DNA index, and amplification status of the *MYCN* oncogene, have been grouped to a risk classification system maintained by the Children's oncology group (COG) (Maris, 2005). The COG risk system (Table 2) can quite accurately predict patient prognosis and is therefore used for treatment guidance. Low and intermediate risk patients have estimated overall survival rates of above 90% (Matthay et al., 1998; Perez et al., 2000). Current recommended treatment interventions for these patients are limited to surgical removal of the primary tumor, in some instances combined with radiation and low to intermediate levels of chemotherapy (Matthay et al., 1998; Perez et al., 2000). High risk patients present with several of the unfavorable prognostic markers as outlined above. These patients receive very intense treatment, including induction chemotherapy, myeloablative chemotherapy followed by autologous bone marrow transplantation, and treatment for reduction of minimal residual disease (Brodeur and Maris, 2002). Despite initial treatment response, a large proportion of high risk patients suffer from relapse or refractory disease (Brodeur and Maris, 2002). As a consequence overall sur-

Tumor hypoxia

Overview

In the 2000 review “The hallmarks of cancer” Hanahan and Weinberg (Hanahan and Weinberg, 2000) in a reductionistic view of cancer stipulate six capabilities that cells must acquire in order to become a malignant cancer. Though referring to diverse cellular processes, the six capabilities all have in common that they in effect subdue extra- and intracellular constraints on limitless cellular growth and tumor expansion. This is particularly true with regard to tumor hypoxia and onset of angiogenesis, since without adequate supply of oxygen and nutrients the tumor cells lose the majority of

their growth advantage. Tumor hypoxia has been estimated to be a rather early event of solid tumor expansion. The diffusion limit for oxygen is 100 to 150 μm and studies have shown necrotic tumor cells located less than 200 μm from blood vessels (Thomlinson, 1977). Reduction of available oxygen will cause cells to suffer from decreased levels of available ATP, disturbing all energy consuming intracellular processes; the cells are experiencing metabolic hypoxia (Höckel and Vaupel, 2001). The oxygen level breakpoint for this energy depletion/hypoxia to occur has been shown in various *in vivo* and *in vitro* studies, including neuroblastoma, to be approximately 8-10 mmHg (1%

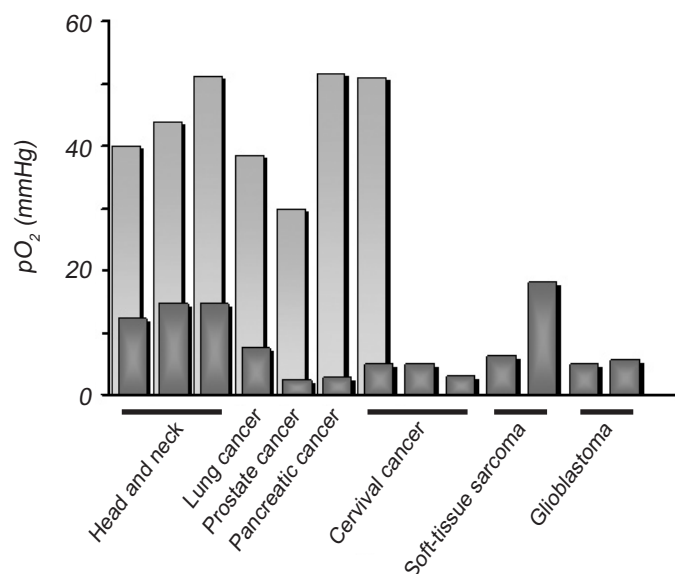


Figure 2. Solid tumors are generally hypoxic, as compared to normal tissue. The tumor oxygenation status (dark gray; shown in front), against surrounding normal tissue (light gray; shown in back). Adapted from Brown and Wilson (2004).

O₂) (Höckel and Vaupel, 2001; Robiolio et al., 1989). Even after onset of angiogenesis the inherent unconstrained tumor growth, as well as the uncoordinated growth and malformed structure of tumor vasculature, causes oxygen shortage to be the regular tissue state (Fig. 2) (Brown and Wilson, 2004; Carmeliet and Jain, 2000). This, in combination with frequently occurring vessel occlusions and caotic blood flow leads to fluctuations in intratumoral oxygen pressure that varies both on a long and a short term basis (Kimura et al., 1996; Lanzen et al., 2006; Torres Filho et al., 1994). In order to maintain intracellular homeostasis and a continued growth advantage tumor cells adjust to these strenuous environmental conditions by manipulating both their intracellular and extracellular space. Two examples of this are metabolic adaptation to a hypoxic environment, leading to an upregulation of glycolysis (Gatenby and Gillies, 2004), and

increased proangiogenic extracellular signaling via e.g. vascular endothelial growth factor (VEGF) (Carmeliet and Jain, 2000).

The hypoxia inducible transcription factors

On the molecular level adaptation to hypoxia is mediated primarily via the hypoxia inducible factors HIF-1 (Semenza and Wang, 1992; Wang et al., 1995) and HIF-2 (Tian et al., 1997; Wiesener et al., 1998). These heterodimeric transcription factors consists of a hypoxia regulated alpha subunit, either HIF-1 α or HIF-2 α , and the constitutively present β dimerization partner aryl hydrocarbon receptor nuclear translocator (ARNT, or HIF-1 β). Structurally the HIF α subunits are bHLH-PAS proteins, where the N-terminal basic domain is responsible for DNA binding and the HLH and PAS domains mediates

ARNT protein interactions. Furthermore, the protein contains one C-terminal and one N-terminal transactivation domain, through which interactions with transcriptional co-regulators occur. The N-terminal transactivation domain also includes an oxygen degradation domain (ODD) responsible for the oxygen sensitive action of the transcription factors (Ivan et al., 2001; Jaakkola et al., 2001).

In the presence of oxygen the HIF α subunits are hydroxylated at conserved proline residues located in the ODD by the prolyl hydroxylase enzymes PHD1, PHD2, and PHD3

(reviewed in Schofield and Ratcliffe, 2004; Wirthner et al., 2007). This leads to recognition by and HIF α interaction with the von Hippel-Lindau (VHL) E3 ligase complex targeting HIFs for ubiquitylation and subsequent proteasomal degradation (Huang et al., 1998; Kallio et al., 1999). At hypoxia the function of the oxygen dependent PHDs is inhibited and HIF α subunits can accumulate and dimerize with ARNT. The fully functional dimers translocate to the nucleus where it binds to specific hypoxia responsive DNA elements and, together with transactivation partners such as CBP/p300, regulate the expression of some one hundred known target

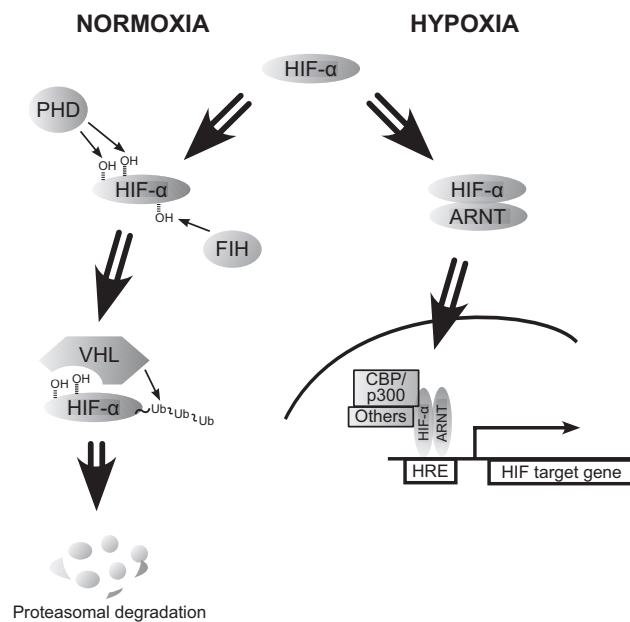


Figure 3. Oxygen mediated regulation of hypoxia inducible factor activity. At normoxia (left panel) the oxygen dependent prolyl hydroxylases (PHD) hydroxylates the HIF α subunits. This leads to recognition by VHL, ubiquitination of the HIF α and subsequent proteasomal degradation. Furthermore, HIF transactivation is inhibited in an oxygen dependent manner by FIH (factor inhibiting HIF)-mediated hydroxylation at a specific asparagine residue. At hypoxia (right panel) the prolyl and asparaginyl hydroxylases are inhibited and consequently HIF α subunits are stabilized. Dimerization with ARNT leads to nuclear translocation and the heterodimers binds to specific DNA hypoxia responsive elements (HRE). Target gene transactivation is performed by recruitment of additional co-factors such as CBP/p300.

genes (Semenza, 2003; Wenger et al., 2005). Further hypoxia-regulated control is mediated via the factor inhibiting HIF (FIH). FIH is an oxygen dependent asparaginyl hydroxylase that suppresses HIF transcriptional activity via hydroxylation of an asparagine residue in the C-terminal transactivation domain, thus abrogating HIF interaction with CBP/p300 (Fig. 3) (Lando et al., 2002). Interestingly FIH seems to have higher affinity for HIF-1 α than for HIF-2 α (Bracken et al., 2006).

The HIF pathway, as with most intracellular signaling pathways, is densely regulated. Negative feedback loops are provided at several levels. The PHD proteins have been shown to be regulated by hypoxia both at the protein and mRNA levels (Aprelikova et al., 2004; Epstein et al., 2001; Holmquist-Mengelbier et al., 2006). This upregulation might reflect a compensatory mechanism leading to some PHD dependent HIF α degradation at lower oxygen levels. It might also be a mechanism for fast shut-down of the hypoxic response at cellular reoxygenation. A third, inhibitory, hypoxia inducible factor alpha subunit HIF-3 α has also been identified (Gu et al., 1998). HIF-3 α contains bHLH, PAS and ODD domains, but lacks the transactivating domains (Makino et al., 2002; Maynard et al., 2005). It has been shown to elicit dominantly negative control on HIF mediated gene regulation (Makino et al., 2001). Besides hypoxia, stabilization of the HIF-1 α and HIF-2 α proteins has been shown to be regulated under non-hypoxic conditions by several oncogenic signaling pathways, including Ras (Blancher et al., 2001), EGFR (Zhong et al., 2000), HER2 (Laughner et al., 2001), IGF-1 (Laughner et al., 2001), VEGF (Calvani et al., 2008) and insulin (Treins et al., 2002). These modulators of HIF protein activity and protein and mRNA levels seem to act via several intracellular signaling pathways including PI3K/AKT, mTOR and

the ERK1/2 MAP kinases (Semenza, 2003). These observations highlights the complexity of the HIF-regulated signaling pathway as also VEGF is positively regulated by ERK and PI3K signaling (Pages and Pouyssegur, 2005).

Another prominent HIF activating mechanism is loss of VHL (reviewed in Kaelin, 2002). Germline mutations in VHL are associated with increased susceptibility to a number of cancers including pheochromocytoma and clear cell renal carcinoma (RCC). VHL-mutation dependent induction of HIF α has been shown to be one of the earliest prelesional events in these tumor forms (Mandriota et al., 2002; Vortmeyer et al., 2006).

Expression of hypoxia-inducible factors

The importance of the hypoxia inducible factors in development has been demonstrated through mouse knock-out models. *Hif1 α ^{-/-}* mice consistently die at approximately embryonal day (E) 11 with neural tube and vascular defects (Iyer et al., 1998; Ryan et al., 1998). Interestingly, wild type embryos showed increased *hif-1 α* expression at E8.5 to E9.5 (Iyer et al., 1998), which in combination with increased levels of hypoxia in *hif1 α ^{-/-}* embryos (Ryan et al., 1998) suggests a role for hypoxia/HIF as a developmental cue in these organ systems. On the contrary, several phenotypes have been reported for *epas1/hif-2 α ^{-/-}* mice (Peng et al., 2000; Scortegagna et al., 2003; Tian et al., 1998), differences that to some extent could be related to mouse strain genetic background (reviewed in Löfstedt et al., 2007). These studies indicate a role for *Epas1* in sympathetic nervous system and vascular development (Peng et al., 2000; Tian et al., 1998), as well as in regulating homeostatic responses during embryonal maturation (Scortegagna et al., 2003). In this context HIF-2 α protein

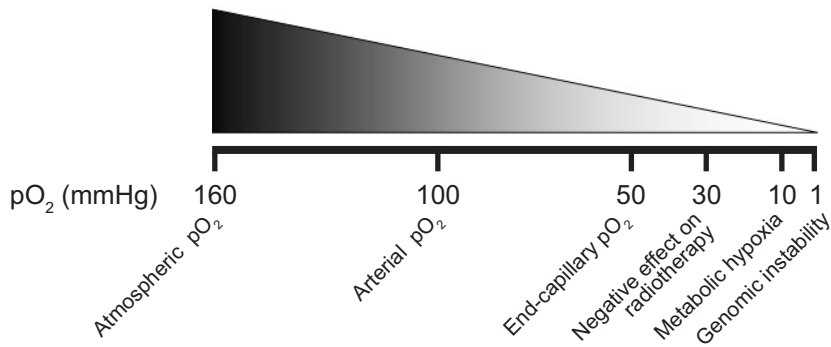


Figure 4. Oxygen pressure at different vascular levels and effects of hypoxia on cellular functions. The blood oxygen partial pressure diminishes substantially from arterial to end-capillary vascular levels. At even lower levels in solid tumors, lack of oxygen impairs radiation treatment, metabolism, and maintenance of genomic stability.

is expressed in the oxygen sensing paranglia of the developing sympathetic nervous system in mouse at E12.5-15.5 (Tian et al., 1998) and in humans during the corresponding developmental period, fetal week 8.5 (Nilsson et al., 2005).

In adult tissues *hif-1α* is ubiquitously expressed (Jain et al., 1998), whereas *epas1* has a more restricted expression pattern, mainly confined to vascular epithelium (Jain et al., 1998) and distinct cell populations of other organs such as smooth muscle cells, and epithelial cells of the lung and kidney (Ema et al., 1997; Flamme et al., 1997; Wiesener et al., 1998). Indeed, HIF-2α has been shown to have a pivotal role in regulation of hematopoiesis (Scortegagna et al., 2005) and in concordance with this a gain of function mutation in *EPAS1* has been associated with erythrocytosis (Percy et al., 2008). At the protein level very few normal human tissues express active HIF, with the exception of HIF-2α in macrophages (Talks et al., 2000).

Hypoxia and HIFs in cancer

Decreased tissue oxygen levels have been

shown to have negative effects on cancer treatment and patient outcome before onset of metabolic hypoxia. End-capillary oxygen pressure is normally about 45-50 mmHg and a drop of about 50% leads to decreased efficiency of radiotherapy (Fig. 4). Onset of metabolic hypoxia is estimated to occur at app. 10 mmHg (Höckel and Vaupel, 2001). Further decreased oxygen partial pressure levels contributes to increased genomic instability by reducing apoptotic potential and inhibiting DNA repair (Huang et al., 2007), but also through inducing gene amplification and DNA breakage (Huang et al., 2007; Russo et al., 1995). Indeed, direct measurements of oxygen partial pressure in primary tumors have demonstrated a correlation between low oxygen levels and poor patient outcome in several cancer forms (Vaupel and Mayer, 2007).

Contrary to what is seen in healthy tissues, the HIF proteins have been shown to be expressed in several cancer forms (Löfstedt et al., 2007; Semenza, 2003), including tumor associated macrophages (Knowles et al., 2004). The hypoxia inducible factors are known to regulate genes involved in diverse cellular processes such as angiogenesis,

glycolysis and apoptosis (Löfstedt et al., 2007; Semenza, 2003). These mechanisms are important in normal processes such as development and tissue homeostasis, but are in tumor cells they in a sense hijacked to contribute to tumor growth, invasion and metastasis. Consequently, expression of the HIF transcription factors correlate to tumor progression and poor patient outcome in several tumor forms (Löfstedt et al., 2007; Semenza, 2003).

Hypoxia induced angiogenesis is primarily mediated through direct HIF regulation of VEGF transcription (Holmquist-Mengelbier et al., 2006; Semenza, 2003). Short VEGF splice forms diffuse from hypoxic areas to induce sprouting from existing vessels. In combination with longer matrix bound isoforms providing more directional cues, VEGF secretion functions both by attracting and by guiding newly formed vessels into hypoxic tumor areas (Carmeliet, 2003). While in theory this would provide the tumor with extensive vascular supply, the lack of coordination between available oxygen and nutrients and tumor cell growth leads to constant evolvement of hypoxic areas in most solid tumors (Brown and Giaccia, 1998).

Hypoxia, through the HIFs, regulates glycolysis via upregulation of the glucose transporters *GLUT1* and *GLUT3* as well as several enzymes involved in (anaerobic) glucose metabolism (Iyer et al., 1998; Semenza et al., 1994). An increased uptake of glucose and induced glycolysis are thought to give a growth advantage and is a common feature of aggressive tumors also in the non-hypoxic setting (Gatenby and Gillies, 2004). An interesting side effect of increased glycolysis is modifications of the tumor microenvironment. Extracellular pH is lowered by two direct HIF regulated processes: extracellular bicarbonate production through carbonic anhydrase 9 (CAIX) (Swietach et al., 2007),

and lactic acid efflux via upregulation of monocarboxylate transporter pumps (Ullah et al., 2006). While induction of these events are prerequisites for maintaining a functional intracellular pH (reviewed in Pouyssegur et al., 2006) the acidification of the surrounding microenvironment furthermore perturbs the extracellular matrix structure and basal membrane integrity, thus abrogating tumor cell invasion into surrounding tissues (Swietach et al., 2007).

Interestingly, efforts are now being made to make use of tumor hypoxia in itself as a therapeutic handle. The strategies behind this uses inventive techniques such as hypoxia activated prodrugs and hypoxia homing drug secreting Clostridia bacteria (Brown and Wilson, 2004).

***MYCN* and the Myc-family transcription factors**

Overview

The *c-myc* gene was originally cloned as the chicken cellular homologue of the viral myelocytomatosis (*v-myc*) gene (Sheiness and Bishop, 1979). The *v-myc* gene was known to cause various neoplasms in chickens and subsequent identification of the human homologue *v-myc avian myelocytomatosis viral oncogene homolog* (gene: *MYC*, protein: Myc) came paired with the observation that this gene was overexpressed in cancer cells as compared to normal fibroblasts (Eva et al., 1982). The *MYC* gene was shortly after found to be altered by amplification or translocation in myeloid leukemia and Burkitt's

lymphoma cells (Collins and Groudine, 1982; Dalla-Favera et al., 1982). Subsequent Southern blot analyses using the newly found *MYC* gene as genomic probe lead to the discovery of *MYCN* (protein: MycN) and *MYCL* (protein: MycL), with sequence homology to *MYC*. Importantly, *MYCN* was found to be amplified in neuroblastoma (Kohl et al., 1983; Schwab et al., 1983), while *MYCL* was identified as amplified in small cell lung carcinoma (Nau et al., 1985). The Myc-family of proto-oncogenes has since been found to be deregulated through amplification, translocation or overexpression in several cancer forms (Nesbit et al., 1999; Vita and Henriksson, 2006).

The Myc-family members are expressed at discrete stages and tissues during development. Generally, the expression is highest during embryonic stages and then downregulated at organ differentiation and maturation (e.g. Zimmerman et al., 1986). During embryonal mouse development, *c-myc* seems to be generally expressed in all proliferating tissues, except neuronal, where *n-myc* prevails (e.g. Kato et al., 1991; Zimmerman et al., 1986). *N-myc* expression is consequently strongest in the central and peripheral nervous system and in neural crest derivatives, however expression is also found in specific tissues of e.g. lung and liver and during limb development (reviewed in Hurlin, 2005; Strieder and Lutz, 2002). *L-myc* expression is mainly confined to the developing kidney and nervous system and to the new born and adult lung, tissues that also show expression of *c-myc* and *N-myc* (Hatton et al., 1996; Zimmerman et al., 1986). This is in sharp contrast to *c-myc* and *N-myc* expression. Mice with germline homozygous deletions of either *c-myc* or *N-myc* show embryonic lethality at E9.5 and E11.5, respectively, and present gross organ abnormalities corresponding to sites of developmental expression (e.g. Davis et al., 1993; Sawai et al., 1993). This is in sharp contrast to the lack of phenotype and apparently normal development of *L-myc*^{-/-} mice (Hatton et al., 1996), suggesting the idea that Myc proteins can substitute for each other. Further confirmation of Myc protein redundancy was provided in a study where mice homozygous for the replacement of *c-myc* coding sequences with those of *N-myc* developed as normal, with only slight differences in e.g. body weight (Malynn et al., 2000). Therefore, differential expression might be the key to the divergent functions of the Myc proteins during development.

The Myc/Max/Mad network

The Myc proteins are transcription factors containing C-terminal basic helix-loop-helix / Leucine zipper (bHLH/LZ) domains, where the basic domain is responsible for DNA binding and HLH/LZ domains function in protein-protein interactions (Grandori et al., 2000; Hurlin and Huang, 2006). The N-terminal parts contain a transactivation domain harboring conserved sequences known as Myc-boxes. The Myc proteins are part of a densely controlled network, the centre of which is the bHLH/LZ protein Max. When Max heterodimerizes with any of the Myc homologs an active transcription factor competent to bind specific DNA E-box sequences (CACGTG) is formed. Through the Myc-boxes in the N-terminal transactivation domain the Myc-Max heterodimers interact with transcriptional cofactors thus leading to interaction with the general RNA polymerase II transcriptional machinery. The Myc-Max complex also engages histone acetyl transferases such as TRRAP and CBP/p300, which leads to chromatin remodeling with subsequent alleviation of transcriptional activation. Virtually all mitogenic signaling pathways promotes Myc, however Myc-Max complexes not only stimulates proliferation but also apoptosis. Therefore, to avoid induced cell death activation of Myc must be balanced by pro-survival signaling (Grandori et al., 2000; Pelengaris et al., 2002). Interestingly, global studies of Myc-Max DNA binding have shown binding to several non-E-box sequences (e.g. Fernandez et al., 2003; Zeller et al., 2006) evidencing a more generalized role for Myc in e.g. chromatin remodeling. Myc induced transcription is counteracted by Mnt, Mga and proteins of the Mad-family (Mad1, Mxi1, Mad2 and Mad3). These proteins are also bHLH/LZ proteins that can form E-box binding heterodimers with Max, but in doing so they repress transcription by

recruiting histone deacetyl transferases via the Sin3A/B transcriptional co-repressors. While Mnt is suggested to serve as a general competitive antagonist of Myc transcriptional activity, Max heterodimerization with Mad proteins has been shown to promote differentiation and cellular quiescence (Grandori et al., 2000; Hurlin and Huang, 2006; Wahlstrom and Henriksson, 2007). Thus, the Myc family proteins are part of a densely controlled network that through transactivation of target genes control diverse cellular processes (Grandori et al., 2000). Both Myc and MycN have also been shown to regulate transcription of ribosomal RNAs and tRNAs, thus upregulating the total translational machinery (Boon et al., 2001; White, 2005). Therefore enhanced Myc activity not only leads to upregulation of specific target genes, but also through a generally increased capacity in protein biosynthesis, to cellular growth (accumulation of mass) (Iritani and Eisenman, 1999; Kim et al., 2000; Ruggero and Pandolfi, 2003).

MYCN and differentiation

One of the first observations regarding *MYC* transcription was a downregulation in response to induced differentiation accompanied with growth control (Westin et al., 1982). Thus, with few exceptions, *MYC* expression is: high in proliferating and low in differentiated tissues/cells (Grandori et al., 2000). The role of *MYCN* is more complex. *In vitro* studies in neuroblastoma cells have shown that *MYCN* expression decreases during induced differentiation (Hammerling et al., 1987; Smith et al., 2004) and a recent study showed that *MYCN* knock-down lead to spontaneous morphological differentiation and upregulation of TrkA and TrkC (Nara et al., 2007). Furthermore, overexpression of *MYCN* enhances proliferation and confers resistance to induced differentiation (Lutz et

al., 1996; Peverali et al., 1996). *In vivo*, the *N-myc* levels in developing mice are higher in undifferentiated as compared to more mature organs (Kato et al., 1991; Zimmerman et al., 1986). However, there does not seem to be a direct contradiction between *MYCN* expression and differentiation. In chicken and quail embryos MycN expression was shown to be present in distinct cells of the sympathetic and dorsal root ganglia at the same time as these cells are differentiating (Wakamatsu et al., 1997). The same study showed that transient MycN overexpression induced neuronal differentiation in neural crest cells *in vitro* and increased migration and differentiation *in vivo* (Wakamatsu et al., 1997). In human tissues *MYCN* mRNA has been shown to be expressed in human sympathetic ganglia at embryonal week 8.5 (Edsjö et al., 2004) at the same time as these structures express markers for neuronal differentiation and the cells are non-migratory (Gestblom et al., 1999; Gestblom et al., 1997; Hoehner et al., 1996). Furthermore, in human week 16-19 fetuses *MYCN*-expression was found in the neural crest derived striated ocular musculature (Hirvonen et al., 1989). While *MYCN* levels clearly are low or not expressed in mature tissues these data suggests that there is an instructive, rather than inhibitory, role for MycN in differentiation of neural crest derived tissues.

MYCN in neuroblastoma

As previously mentioned, *MYCN* was cloned from neuroblastoma tumors and shortly after amplification of the *MYCN* locus was found to be a characteristic of high stage aggressive tumors (Brodeur et al., 1984; Schwab et al., 1984). Approximately 20-25% of neuroblastoma patients present with *MYCN* amplification at diagnosis and the majority of these are clinically considered as high risk irrespective of other clinical features

Table 3. MYCN mRNA and protein expression in relation to neuroblastoma patient outcome.
MYCN mRNA or protein correlation to prognosis before and after correction for MYCN amplification is presented.

Study name [#]	Year	No patients	Method	MYCN/MycN prognosis	Correction for MYCN amplification
Nisen	1988	33	NB	No	Not prognostic
Hashimoto	1989	18	IHC	Yes	-
Nakagawara	1990	35	NB	Yes	-
Slavc	1990	45	NB	No	-
Hiyama	1991	41	IHC	Yes	-
Nakada	1993	36	IHC	Yes	-
Nakagawara	1993	77	NB	Yes	Not prognostic
Chan	1997	57	IHC	Yes	Poor prognosis
Bordow	1998	60	RT-PCR	Yes	Poor prognosis*
Cohn	2000	69	qPCR, WB	No**	-
Matsunaga	2000	70	RT-PCR	Yes	Not prognosis
Tanaka	2004	66	qPCR	No	Not prognosis
Alaminos	2005	99	qPCR, WB	Yes	Poor prognosis
Tang	2006	91	qPCR	No	Good prognosis***

(-) Correction for MYCN amplification was not performed.

(#) Study named after first author on publication.

(*) Children younger than 12 months showed comparatively high levels of MYCN expression, but MYCN expression was only prognostic in patients diagnosed after 12 months of age.

(**) This study only investigated high stage MYCN non-amplified cases.

(***) MYCN expression levels only correlated to poor prognosis in MYCN amplified cases.

Abbreviations: NB: Northern blot; IHC: immunohistochemistry; RT-PCR: reverse transcriptase polymerase chain reaction; qPCR: quantitative real time polymerase chain reaction; WB: Western blot.

(Table 2). Exceptions from this rule are infants diagnosed with localized disease and INSS stage 1 patients showing favorable tumor histology (e.g. Schneiderman et al., 2008). Consequently, MYCN-amplification correlates strongly to poor outcome and tumors with this amplification are often refractory to treatment (Brodeur and Maris, 2002; Cohn and Tweddle, 2004; Schneiderman et al., 2008).

Surprisingly, the clinical significance of MYCN amplification does not transform to MYCN mRNA or MycN protein levels. Several investigations regarding prognostic significance of MYCN/MycN expression have been performed and the results are at best inconclusive (Table 3) (Alaminos et al.,

2005; Chan et al., 1997; Cohn et al., 2000; Hashimoto et al., 1989; Hiyama et al., 1991; Marchand and Roux, 1998; Matsunaga et al., 2000; Nakada et al., 1993; Nakagawara et al., 1993; Nakagawara et al., 1990; Nisen et al., 1988; Slavc et al., 1990; Tanaka et al., 2004; Tang et al., 2006). Two concordant observations are made in the studies referenced in Table 2: MYCN-amplified, as compared to non-amplified tumors, generally also express high levels of mRNA and protein, and both MYCN mRNA and protein levels can be expressed at high levels also in tumors defined as MYCN single-copy. Several of the reports show correlations between MYCN/MycN levels and patient prognosis, however only eight of the fourteen studies corrected the results for MYCN amplification. After

correction three studies showed a correlation between high *MYCN*/MycN expression and poor prognosis, four showed no correlation to prognosis, and interestingly, one study showed a positive correlation between high *MYCN* expression and favorable outcome (Table 3). A fairly recent study by Cohn et al. (Cohn et al., 2000) used a carefully selected cohort containing *MYCN* non-amplified high-stage cases only. These results did not show any correlation between *MYCN* level and patient outcome.

The results presented in Table 3 are further complicated by the fact that most of these studies are performed using rather small cohorts and with varying techniques, both for measuring transcript and protein levels and for detecting *MYCN* amplification. Errors at these stages are then further propagated when correcting *MYCN*/MycN prognostic significance for *MYCN* amplification. Of special interest with regard to this issue is one study where a combination of Southern blot, FISH and gene dosage was used to determine *MYCN* amplification status, as no specific correlation between *MYCN* expression and other markers for poor prognosis were found (Tanaka et al., 2004). A possible explanation for the discordant results shown in Table 2 is provided in the study by Tang et al. (Tang et al., 2006), where it is suggested that high *MYCN* expression in *MYCN* amplified tumors leads to aggressive disease whereas high *MYCN* expression in non-amplified tumors correlates with high *NTRK1* (TrkA) expression, consequentially leading to a less malignant phenotype. Indeed, overexpression of *MYCN* mRNA in *MYCN* non-amplified neuroblastoma cell lines has been shown to induce apoptosis, indicating a connection high *MYCN* expression and a benign tumor phenotype (Tang et al., 2006; van Noesel et al., 2003). However, *MYCN* expression when induced in developing tissues of the sympathetic nervous system in a murine model leads to neuroblastoma like

tumors (Weiss et al., 1997), arguing for the importance of MycN in neuroblastoma etiology.

The present investigation

The specific aims of the work presented in this thesis are centered around the roles of tumor hypoxia, sympathetic neuronal differentiation and Myc/MycN dependent gene regulation, in relation to neuroblastoma aggressiveness.

Paper I: Recruitment of HIF-1 α and HIF-2 α to common target genes is differentially regulated in neuroblastoma: HIF-2 α promotes an aggressive phenotype

Summary

In this paper we investigate the different roles of HIF-1 α and HIF-2 α in the response of neuroblastoma cells to hypoxia. Four major conclusions were drawn:

- 1) HIF-1 α and HIF-2 α function in different time-frames. HIF-1 α is rapidly stabilized, binds to promoter elements in target genes such as *VEGF* and regulates their transcription. HIF-2 α on the other hand is stabilized at later time points than HIF-1 α and this late increase in HIF-2 α levels is followed by diminishing levels of HIF-1 α , whereas HIF-2 α remains expressed.
- 2) HIF-2 α is stabilized and active at near physiological oxygen levels (5% O₂). Importantly, HIF-1 α is not stabilized at this non-hypoxic oxygen pressure.
- 3) HIF-1 α and HIF-2 α generally regulate expression from the same target genes.
- 4) HIF-2 α expression is present in apparently well vascularized regions of neuroblastoma specimens. Furthermore, high HIF-2 α protein levels correlates to poor outcome in neuroblastoma patients.

Discussion

Since their discovery, the function of the hypoxia inducible factors in tissue homeostasis and pathology have been extensively investigated and the HIFs have been shown to be expressed and to correlate to adverse outcome in several cancer forms (Löfstedt et al., 2007; Semenza, 2003). We now show a connection between high HIF-2 α levels and neuroblastoma tumor progression. HIF-2 α has been shown to strongly correlate to tu-

mor angiogenesis and high microvascular density (e.g. Giatromanolaki et al., 2006) and we demonstrate a pivotal role for HIF-2 α in regulation of *VEGF* at both hypoxia and at physiological oxygen levels. In neuroblastoma, angiogenesis has been correlated to tumor aggressiveness. Low stage tumors are rich in infiltrating Schwannian stroma and poorly vascularized. A causal link between these two features has been suggested as Schwannian derived stroma cells secrete anti-angiogenic factors and exhibit *in vivo* inhibition of angiogenesis (Chlenski et al., 2002; Huang et al., 2000; Liu et al., 2005). On the other hand, aggressive neuroblastomas are generally highly vascularized and express high levels of pro-angiogenic factors (Eggert et al., 2000; Meitar et al., 1996; Peddinti et al., 2007). Consequently, a pro-angiogenic tumor cell phenotype correlates to tumor progression and HIF-2 α could contribute to this. Recently, overexpression of HIF-2 α in a neuroblastoma xenograft model was shown to exhibit increased tumor vascularization (Favier et al., 2007).

Stability of both HIF α subunits is known to be regulated by oxygen dependent PHD hydroxylation, although substrate specificity for the different PHDs apply. PDH2 has been suggested to be the major regulator of HIF-1 α levels (Berra et al., 2003) and recently HIF-2 α was shown to be stabilized in *PHD1*, but not *PDH2* and *PHD3* knock-out mice (Aragones et al., 2008). Therefore the rapid increase in *PHD2* and *PHD3* levels seen in hypoxic SK-N-BE(2)c cells could explain the time-dependent decrease in HIF-1 α protein. Interestingly, both PHD2 and PHD3 are induced by HIF-1 α suggesting a negative feed-back regulation on HIF-1 α levels (Metzen et al., 2005; Pescador et al., 2005). The reasons why HIF-2 α levels are gradually increased at 1% O₂ and why HIF-2 α is stabilized at 5% O₂ in neuroblastoma cell lines needs further investigation. Clear-

ly *EPAS1* (HIF-2 α) mRNA is increased both at 1 and 5% O₂, the regulatory events behind this are however unknown. As HIF-2 α is expressed in the developing human sympathetic nervous system (Nilsson et al., 2005) one mechanism might be hypoxia induced dedifferentiation (Jögi et al., 2002).

A number of genes have been suggested to be regulated specifically by one of the hypoxia inducible factors and not by the other, for example HIF-1 α has been suggested to exclusively regulate glycolytic enzymes (Covello et al., 2006; Hu et al., 2003; Wang et al., 2005). We clearly show that in SK-N-BE(2)c cells both HIF-1 α and HIF-2 α contribute to hypoxia mediated induction of the *PGK1* gene, encoding a key glycolytic enzyme. The discrepancy in these results could be cell line specific, but also due to interactions with other molecular pathways. For instance, HIF-2 has been shown to augment Myc transcriptional activity (Gordan et al., 2007) and Myc is a known regulator of glycolytic enzymes (Osthus et al., 2000).

In conclusion, we show that HIF-2 α correlates to neuroblastoma tumor aggressiveness and we provide molecular support as to why HIF-2 α might contribute to tumor progression. It is of interest to note that in the tumor specimens, specific intensity of HIF-2 α staining in positive cells correlated more to poor patient outcome than fraction of HIF-2 α positive cells. That is, and as recently suggested (Pietras et al., 2008), there seems to be a specific subpopulation of HIF-2 α positive cells that contribute to neuroblastoma tumor progression and unfavorable patient outcome.

Paper II: Transcriptional adaptation of neuroblastoma cells to hypoxia

Summary

One of the main findings in this study is that neuroblastoma cell lines grown at hypoxia (1% O₂) elicit heterogeneous gene regulatory responses. This was seen both with regard to specific target genes and to what levels known hypoxia regulated genes were induced. However, a core set of known HIF target genes were concomitantly regulated and gene sets, relating to a general hypoxic response, were significantly induced.

Discussion

As mentioned above, the cellular response to hypoxia is primarily regulated by the potent HIF transcription factors. These in turn regulate the expression of other factors such as *VEGF* and *BHLHB2* (shown here), thus leading to activation of secondary signal transduction and transcriptional networks (Das et al., 2005; Ivanov et al., 2007). Therefore the striking heterogeneity seen in the transcriptional response among the seven neuroblastoma cell lines is perhaps not surprising. An important technical aspect is that we only performed one measurement per cell line. The number of errors can be expected to be fairly large, and it is likely that the extensive gene regulatory effects observed would diminish if additional replicates had been included.

However, when looking at a set of known HIF-regulated genes (Semenza, 2003; Wenger et al., 2005) we do see a similar transcriptional response among the seven cell lines. Furthermore, when using a microarray analysis strategy that takes generalized transcriptional responses into account, several significantly altered transcriptional

themes were observed. From the gene set enrichment analysis it was clear that the hypoxic response of the seven neuroblastoma cell lines did correspond to a universal hypoxia regulated pattern as determined by analyses in many different cellular systems. This conclusion is also supported by the fact that all seven cell lines stabilize/become enriched for HIF-1 α and HIF-2 α , presumably leading to activation of HIF-regulated transcription. One important part of this response was changes in metabolic patterns. There was also a massive response relating to downregulation of proliferation, both with regard to gene sets and to enrichment of E2F-type transcription factor motifs. The presence of the p21^{Cip1}/p53 gene sets in the top enriched downregulated gene sets is intriguing. However, promoter analyses of the genes in these gene sets showed presence of DNA sequences relating to the E2F1, E2F2 and E2F4 transcription factors (Wu et al., 2002). Indeed, the majority of the gene sets shown as downregulated in response to hypoxia overlap with each other and with the E2F transcription factor DNA binding motif sets (unpublished observations). With the experiments and analyses presented in this paper we considerably extend our previous data on hypoxic gene regulation in neuroblastoma cells (Holmquist-Mengelbier et al., 2006; Jögi et al., 2002; Jögi et al., 2004). Moreover, we obtain valuable knowledge on general gene expression in cultured neuroblastoma cells.

Paper III: HIF-1 α induces MXI1 by alternate promoter usage with limited effects on the Myc network but strong enhancement of specific hypoxic target gene induction.

Summary

In this paper we show hypoxia-mediated upregulation of *MXI1* (Max interactor 1) in neuroblastoma and breast carcinoma cells. We define a specific hypoxia responsive element in the *MXI1* gene and show HIF dependent transcriptional activation. However, hypoxia mediated *Mxi1* upregulation did not affect known Myc/MycN target genes and at a general level only weak effects were seen on the Myc/MycN regulated pathway. We did however, see *Mxi1* dependent effects on HIF-1 α induced transcription, both when looking at target genes and when employing a HIF-specific reporter assay.

Discussion

Like the other members of the Mad protein family, *Mxi1* is a bHLH/LZ protein and is therefore able to dimerize with Max. Indeed, several studies have shown repression of Myc transcriptional activation by competitive *Mxi1*:Max heterodimerization and E-box binding (e.g. Lahoz et al., 1994; Zervos et al., 1993). In the presented study, specific Myc/MycN target genes, such as *ODC*, were affected by the general hypoxia-mediated downregulation of MycN levels (Jögi et al., 2002), however, no additional effects were seen when knocking down *MXI1* using siRNA.

As mentioned in the article, two *MXI1* splice variants (*MXI1A* and *MXI1B*) with differential effects on Myc dependent transactivation (Dugast-Darzacq et al., 2007) are transcribed in SK-N-BE(2)c cells. We show a later in-

duction of the Myc-inhibitory *MXIIA*, as compared to the *MXIIB* isoform. This could to some extent explain the weak effects of *MXII* knock down seen with both microarray and reporter construct assays. However, at 24 h of hypoxia, when these assays were performed, both *MXIIA* and *MXIIB* are expressed at more or less equal levels. Moreover, there were only minor differences in *siMXII* effect on Myc/MycN dependent transcription between the *MYCN* amplified SK-N-BE(2)c and the non-amplified SK-N-SH cell line. This could be due to the fact that while SK-N-SH cells show relatively low levels of *MYCN* mRNA, it does express high levels of both *MYC* mRNA and protein (Vandesompele et al., 2003). Recently published data evidences that hypoxia-induced *Mxi1* indeed does affect Myc dependent transcription in RCC cell lines (Zhang et al., 2007), but we clearly do not see such strong effects in the neuroblastoma cell lines assayed here.

However, our results suggest that *Mxi1* can act as a cooperative transcription factor to HIF-1 α . Knock-down of *MXII* affected several previously identified hypoxia target genes (Paper I; Paper II) and furthermore a general decrease in hypoxia responsiveness was found in neuroblastoma cells by microarray analyses. Detailed analyses, including a HIF reporter assay, showed that both *MXII* and HIF-1 α were pivotal in the transactivation of these genes.

This adds a totally new function to *MXII*, implicating it in a direct role during adaptation of cells to an hypoxic environment. Moreover, *MXII*:HIF-1 α dependent regulation of *JAG2* transcription suggests an additional mechanism for hypoxia/HIFs to maintain neuroblastoma cells in an undifferentiated state (Jögi et al., 2002; Löfstedt et al., 2004; Pietras et al., 2008). However, it should be mentioned that results concerning HIF and Notch cooperative effects on differ-

entiation are at present contradictory (Gustafsson et al., 2005; Yun et al., 2005).

Our results add to the emerging data regarding an intricate HIF α and Myc cooperative network. Indeed, besides HIF-1 α functionally counteracting Myc by induction of *MXII* and promotion of Myc degradation (Zhang et al., 2007), HIF-1 α and Myc/MycN have been shown to regulate expression of the same metabolic genes (e.g. Boon et al., 2001; Osthus et al., 2000; Wenger et al., 2005). Specifically HIF-1 α and Myc have been shown to cooperate in shunting cells towards glycolytic metabolism via induction of the *HK2* and *PDK1* genes (Kim et al., 2007). It has been proposed that this shift in cellular metabolism is a key feature in tumorigenesis and tumor aggressiveness (Gillies and Gatenby, 2007; Thompson and Thompson, 2004).

Paper IV: Myc-pathway activation and stage of neuronal differentiation identify more malignant neuroblastomas

Summary

By using transcriptional signatures relating to sympathetic neuronal differentiation and Myc/MycN/MycL dependent gene regulation we show that low stage of differentiation and high expression of Myc/MycN/MycL target genes correlate to advanced stage and unfavorable outcome in neuroblastoma. These measurements outperformed clinically used prognostic markers such as INSS stage, COG risk, and amplification of *MYCN*.

Discussion

Amplification of the *MYCN* oncogene has for long been known to correspond to unfavorable neuroblastoma patient prognosis, however this genetic alteration is present in only approximately 40% of tumors in patients classified as high risk (Attiey et al., 2005). Since the outcome for patients with high-risk tumors is less than 40% (De Bernardi et al., 2003; Matthay et al., 1999), investigations leading to objective markers of disease progression are warranted. Furthermore, a subgroup of the patients initially diagnosed as low- to intermediate risk eventually progress to aggressive disease. Early identification of patients with elevated risk for disease progression could lead to more adjusted therapeutic intervention.

The basis for the Myc dependent gene expression signature employed here is that Myc- and MycN-regulated transcription is largely redundant. Reports comparing Myc and MycN dependent transcription using expression microarrays or SAGE showed a 40-50 % overlap in target genes (Boon et al., 2001; Raetz et al., 2003). Importantly,

in these studies *MYC* and *MYCN* transcriptional effects were compared between cells stemming from different malignancies (neuroblastoma, medulloblastoma and melanoma) and, as argued in this thesis, different cell lines elicit differential gene regulatory responses, even to potent transcription factors (*Paper II*). Myc and MycN functional redundancy has been shown in assays where *c-myc* and *N-myc* were replaced with each other in an E μ mouse plasmacytoma model or during mouse development (Malynn et al., 2000; Wang et al., 1992). Moreover, *MYCN* has repeatedly been identified as the only proliferation rescuing factor in *MYC* depleted fibroblasts (Berns et al., 2000; Nikiforov et al., 2000). Chromatin immunoprecipitation analyses have also provided data that Myc and MycN recognize the same promoter elements and regulates expression from the same subset of target genes (Perini et al., 2005). Of notable interest, SK-N-SH and its subclone SH-SY-5Y are neuroblastoma cell lines that express very little *MYCN*, but instead high levels of *MYC* (Vandesompele et al., 2003 and unpublished observations).

As discussed previously, the data regarding *MYCN* mRNA and protein expression in correlation to neuroblastoma patient outcome are ambiguous. Since *MYCN* amplified tumor specimens also express high levels of *MYCN/MycN* it is likely that this is the factor leading to elevated activity of the Myc pathway in these tumors. As for the *MYCN* non-amplified tumor specimens presenting high signaling through the Myc transcriptional network, several explanations are possible. These fall into two general categories: upstream regulators of *MYC/MYCN* transcript and protein levels, and modulators of transactivation. It is known that neuroblastoma tumors can show high levels of MycN in the absence of *MYCN* amplification, and indeed high *MYCN* expression does correlate with an aggressive tumor phenotype *in*

vitro (reviewed in Cohn and Ikegaki, 2000). There are several signaling pathways acting as positive regulators of *MYCN* expression in neuroblastoma cells, including E2F and IGF-1 (Misawa et al., 2000; Strieder and Lutz, 2002; Strieder and Lutz, 2003). Differentiation induced by e.g. NGF/TrkA has been shown to downregulate *MYCN* (Matsushima and Bogenmann, 1993). Therefore, another possible explanation to elevated *MYCN* levels is failure to differentiate, perhaps associated with low *NTRK1* (TrkA) levels as seen in aggressive neuroblastomas (Nakagawara et al., 1993). In medulloblastoma, sonic hedgehog (SHH) induces *MYCN* expression and in mouse models of this disease MycN contributes to SHH dependent tumor induction (Hatton et al., 2006; Oliver et al., 2003). However, a putative role for SHH in neuroblastoma has not been investigated. In vitro studies have also shown a role for enhancers of *MYCN* mRNA stability and protein half-life in *MYCN* non-amplified cell lines (Cohn et al., 1990; Manohar et al., 2002). Since high levels of *MYCN* mRNA or protein generally does not correlate to adverse patient outcome, this can only be a possible explanation for elevated signaling through the Myc pathway for a subset of cases. Recently, another deregulated pathway with implications for Myc dependent signaling was observed in neuroblastoma (Liu et al., 2007). Wnt/ β -catenin is a developmentally regulated pathway involved in neuronal development and regulation of *MYC* (Bellmeyer et al., 2003; Raible and Ragland, 2005). Elevated Wnt/ β -catenin signaling in combination with high *MYC* levels were found in high-risk *MYCN* non-amplified neuroblastoma tumors. The finding that also some neuroblastoma cell lines expressed low *MYCN* and high *MYC* in combination with nuclear β -catenin makes this a very interesting hypothesis (Liu et al., 2007).

Skp2 is a member of the SCF^{Skp2} E3 ubiqui-

tin ligase complex that is involved in degradation of e.g. p27^{Kip1} and p21^{Cip1} (Bornstein et al., 2003; Carrano et al., 1999; Sutterlüty et al., 1999). Skp2 has also been shown to ubiquitylate Myc and in doing so it not only targets Myc for degradation, but also functions as a coactivator of Myc-dependent transcription (Kim et al., 2003; von der Lehr et al., 2003). Whether Skp2 is also involved in MycN dependent transactivation is not known, however a recent publication showed that high Skp2 levels were associated with an adverse prognosis in neuroblastoma (Westermann et al., 2007).

A mature stage of differentiation as defined by morphology has for long been known to correlate to favorable neuroblastoma prognosis (Cushing and Wolbach, 1927; Shimada et al., 1999; Shimada et al., 1984). The differentiation gene expression signatures used for the analyses in the presented paper are based on observations concerning human development, as well as inferred from studies on mouse and avian systems (e.g. Gammill and Bronner-Fraser, 2003; Gestblom et al., 1997; Hedborg et al., 1995; Hoehner et al., 1995; Hoehner et al., 1996; Hoehner et al., 1998). Our model is based on two sets of genes: those expressed in migratory and pre-migratory neural crest cells, and those that are expressed in mature sympathetic neuronal tissues. The rationale behind this is a gradual shift between these two signatures as cells progress from precursors towards a terminally differentiated state (Hoehner et al., 1998). While this might not be entirely true on the single gene level (especially in a tumor setting) it is however reasonable to assume that a cell expressing high levels of the sympathetic neuronal markers is indeed more differentiated than a cell expressing low levels. Observations regarding higher expression of neuronal markers in differentiated and more benign neuroblastomas have previously been reported. Furthermore, a

prognostic implications of gene expression patterns with reference to a differentiated neuronal phenotype have been inferred from clinical neuroblastoma microarray studies (Asgharzadeh et al., 2006; Krasnoselsky et al., 2005; Ohira et al., 2005). However, the data presented in Paper IV is the first comprehensive study where stage of neuronal differentiation measured at the molecular level is related to patient outcome.

Populärvetenskaplig sammanfattning.

Cancer är ett samlingsnamn på en grupp sjukdomar som har gemensamt att de yttrar sig i att celler som normalt inte ska växa och dela sig börjar göra det okontrollerat. Dessa celler bildar en tumör och man skiljer på benigna och maligna tumörer. En benign tumör är godartad och sprider sig inte, men kan ändå orsaka skada hos patienter genom att exempelvis trycka på blodkärl. Maligna tumörer är elakartade. De växer invasivt, det vill säga de går in i intilliggande vävnad. Ett ytterligare kännetecken är att de sprider sig till andra delar av kroppen, de metastaserar. Både invasiv växt och metastaserande sjukdom bidrar kraftigt till att försämra patientens chanser att överleva. Varför vi får cancer vet man i vissa fall, i andra inte. Generellt sett ökar risken för cancer med stigande ålder, två tredjedelar av alla fall inträffar efter 65 års ålder. Detta kan bland annat bero på att man under längre tid utsatt sig för riskfaktorer, till exempel asbest, radioaktiva ämnen, rökning och vissa typer av kost. Med tanke på detta kan det vara speciellt svårt att förstå varför barn får cancer. Eftersom barn vanligtvis inte utsätts för riskfaktorer på samma sätt som vuxna tror man att vissa barntumörer kan bero på att något gått fel under fosterutvecklingen.

Min forskning handlar om en barntumör som kallas neuroblastom. Det är en cancer i nervsystemet, utanför skallen. En väldigt stor del av tumörerna hittas i binjuren, som är ett mycket litet organ lokaliserat ovanpå njuren. Binjurens normala funktion är att släppa ut adrenalin i blodomloppet. Neuroblastom är en relativt ovanlig sjukdom, 10 till 15 fall diagnostiseras i Sverige varje år. De flesta patienterna är väldigt unga, bara 10% av de drabbade diagnostiseras när de är över fem år. Att få neuroblastom är inte nödvändigtvis livshotande för ett barn. Hälften av alla patienter har en godartad sjukdom och nästan

alla av dessa botas. Den andra halvan får en mycket aggressiv variant med både invasiv och metastaserande växt. Av dessa patienter är det bara cirka 40 % som överlever.

I de arbeten som presenteras i den här avhandlingen har jag försökt förstå och påvisa varför vissa neuroblastom är aggressiva, medan andra är godartade.

Ett centralt tema för min forskning är tumörhypoxi, det vill säga syrebrist i tumören. Detta uppkommer till följd av att tumören växer snabbare än vad det bildas blodkärl till den. Det uppkommer då ett scenario som till viss del liknar vad som sker när hårt ansträngda muskler förbrukar mer syre än vad cirkulationssystemet kan leverera. Bland annat bildas mycket mjölksyra. I den första artikeln i avhandlingen (Paper I), visar vi att det cellulära systemet för att känna av och anpassa sig till omgivande syrenivåer ofta är felreglerat i neuroblastom. Våra resultat pekar tydligt på att detta bidrar till att öka tumörens aggressivitet.

I den fjärde artikeln (Paper IV) försöker vi skilja ut aggressiva från icke-aggressiva tumörer med hjälp av beräkningar på teman som vi sedan tidigare vet bidrar till sjukdomens beteende. Vi visar att man på detta sätt med relativt god säkerhet redan i samband med diagnos skulle kunna förutsäga patientens sjukdomsförlopp. Förhoppningen är att man genom att använda sig av den här typen av metodik kommer kunna anpassa cancerbehandling mer efter individen.

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Det finns väldigt många jag skulle kunna tacka nu när jag har chansen, men jag tror jag gör det i en annan bok, en annan gång. Följande är kategorier av människor som jag känner förtjänar extra uppmärksamhet. Ni är alla med.

- Kollektivister
- Italienare
- Fiskare
- Varbergare
- Icke-arbetskamrater
- Onsdagsvänner
- Romanistas
- Kanadaresenärer
- Bussresenärer
- Barhängare
- och inte sist och inte minst Olis
- Greker
- Polacker
- Skidåkare
- Arbetskamrater
- Allmänna vänner
- Snookerentusiaster
- Institutet
- Berlinresenärer
- Dansare
- Scooterägare

Slutligen vill jag tacka min familj. Tack så mycket.

References

- Alaminos, M., Gerald, W. L., and Cheung, N. K. (2005). Prognostic value of MYCN and ID2 overexpression in neuroblastoma. *Pediatric blood & cancer* *45*, 909-915.
- American Cancer Society (2005). *Cancer facts & figures*. In (Atlanta, GA: American Cancer Society).
- Anderson, D. J. (1993). Molecular control of cell fate in the neural crest: the sympathoadrenal lineage. *Annual review of neuroscience* *16*, 129-158.
- Anderson, D. J., and Axel, R. (1986). A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. *Cell* *47*, 1079-1090.
- Anderson, D. J., Carnahan, J. F., Michelsohn, A., and Patterson, P. H. (1991). Antibody markers identify a common progenitor to sympathetic neurons and chromaffin cells in vivo and reveal the timing of commitment to neuronal differentiation in the sympathoadrenal lineage. *J Neurosci* *11*, 3507-3519.
- Aprelikova, O., Chandramouli, G. V., Wood, M., Vasselli, J. R., Riss, J., Maranchie, J. K., Linehan, W. M., and Barrett, J. C. (2004). Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. *Journal of cellular biochemistry* *92*, 491-501.
- Aragones, J., Schneider, M., Van Geyte, K., Fraisl, P., Dresselaers, T., Mazzone, M., Dirx, R., Zacchigna, S., Lemieux, H., Jeoung, N. H., *et al.* (2008). Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. *Nature genetics* *40*, 170-180.
- Asgharzadeh, S., Pique-Regi, R., Sposto, R., Wang, H., Yang, Y., Shimada, H., Matthay, K., Buckley, J., Ortega, A., and Seeger, R. C. (2006). Prognostic significance of gene expression profiles of metastatic neuroblastomas lacking MYCN gene amplification. *Journal of the National Cancer Institute* *98*, 1193-1203.
- Attiyeh, E. F., London, W. B., Mosse, Y. P., Wang, Q., Winter, C., Khazi, D., McGrady, P. W., Seeger, R. C., Look, A. T., Shimada, H., *et al.* (2005). Chromosome 1p and 11q deletions and outcome in neuroblastoma. *The New England journal of medicine* *353*, 2243-2253.
- Bagatell, R., Rumcheva, P., London, W. B., Cohn, S. L., Look, A. T., Brodeur, G. M., Frantz, C., Joshi, V., Thorner, P., Rao, P. V., *et al.* (2005). Outcomes of children with intermediate-risk neuroblastoma after treatment stratified by MYCN status and tumor cell ploidy. *J Clin Oncol* *23*, 8819-8827.
- Bagchi, A., Papazoglu, C., Wu, Y., Capurso, D., Brodt, M., Francis, D., Bredel, M., Vogel, H., and Mills, A. A. (2007). CHD5 is a tumor suppressor at human 1p36. *Cell* *128*, 459-475.
- Bellmeyer, A., Krase, J., Lindgren, J., and LaBonne, C. (2003). The protooncogene c-myc is an essential regulator of neural crest formation in xenopus. *Developmental cell* *4*, 827-839.
- Berns, K., Hijmans, E. M., Koh, E., Daley, G. Q., and Bernards, R. (2000). A genetic screen to identify genes that rescue the slow growth phenotype of c-myc null fibroblasts. *Oncogene* *19*, 3330-3334.
- Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., and Pouyssegur, J. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. *The EMBO journal* *22*, 4082-4090.
- Blancher, C., Moore, J. W., Robertson, N., and Harris, A. L. (2001). Effects of ras and von Hippel-Lindau (VHL) gene mutations on hypoxia-inducible factor (HIF)-1alpha, HIF-2alpha, and vascular endothelial growth factor expression and their regulation by the phosphatidylinositol 3'-kinase/Akt signaling pathway. *Cancer research* *61*, 7349-7355.
- Boon, K., Caron, H. N., van Asperen, R., Valentijn, L., Hermus, M. C., van Sluis, P., Roobeek, I., Weis, I., Voute, P. A., Schwab, M., and Versteeg, R. (2001). N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. *The EMBO journal* *20*, 1383-1393.
- Bornstein, G., Bloom, J., Sitry-Shevah, D., Nakayama, K., Pagano, M., and Hershko, A. (2003). Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. *The Journal of biological chemistry* *278*, 25752-25757.
- Bown, N., Cotterill, S., Lastowska, M., O'Neill, S., Pearson, A. D., Plantaz, D., Meddeb, M., Danglot, G., Brinkschmidt, C., Christiansen, H., *et al.* (1999). Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *The New England journal of medicine* *340*, 1954-1961.
- Bracken, C. P., Fedele, A. O., Linke, S., Balrak, W., Lisy, K., Whitelaw, M. L., and Peet, D. J. (2006). Cell-specific regulation of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha stabilization and transactivation in a graded oxygen environment. *The Journal of biological chemistry* *281*, 22575-22585.
- Breslow, N., and McCann, B. (1971). Statistical estimation of prognosis for children with neuroblastoma. *Cancer research* *31*, 2098-2103.
- Brodeur, G., and Maris, J. M. (2002). Neuroblastoma. In *Principles and Practice of Pediatric Oncology*, P.A. Pizzo, and D.G. Poplack, eds. (Philadelphia, PA: Lippincott Williams & Wilkins).
- Brodeur, G. M. (2003). Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* *3*, 203-216.
- Brodeur, G. M., Seeger, R. C., Schwab, M., Varmus, H. E., and Bishop, J. M. (1984). Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science (New York, NY)* *224*, 1121-1124.
- Brown, J. M., and Giaccia, A. J. (1998). The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer research* *58*, 1408-1416.
- Brown, J. M., and Wilson, W. R. (2004). Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer* *4*, 437-447.
- Calvani, M., Trisciuoglio, D., Bergamaschi, C., Shoemaker, R. H., and Melillo, G. (2008). Differential involvement of vascular endothelial growth factor in the survival of hypoxic colon cancer cells. *Cancer research* *68*, 285-291.
- Carmeliet, P. (2003). Blood vessels and nerves: common signals, pathways and diseases. *Nat Rev Genet* *4*, 710-720.

- Carmeliet, P., and Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature* 407, 249-257.
- Carnahan, J. F., and Patterson, P. H. (1991). Isolation of the progenitor cells of the sympathoadrenal lineage from embryonic sympathetic ganglia with the SA monoclonal antibodies. *J Neurosci* 11, 3520-3530.
- Caron, H., van Sluis, P., de Kraker, J., Bokkerink, J., Egeler, M., Laureys, G., Slater, R., Westerveld, A., Voute, P. A., and Versteeg, R. (1996). Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *The New England journal of medicine* 334, 225-230.
- Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nature cell biology* 1, 193-199.
- Chan, H. S., Gallie, B. L., DeBoer, G., Haddad, G., Ikegaki, N., Dimitroulakos, J., Yeager, H., and Ling, V. (1997). MYCN protein expression as a predictor of neuroblastoma prognosis. *Clin Cancer Res* 3, 1699-1706.
- Chlenski, A., Liu, S., Crawford, S. E., Volpert, O. V., DeVries, G. H., Evangelista, A., Yang, Q., Salwen, H. R., Farrer, R., Bray, J., and Cohn, S. L. (2002). SPARC is a key Schwannian-derived inhibitor controlling neuroblastoma tumor angiogenesis. *Cancer research* 62, 7357-7363.
- Cohn, S. L., and Ikegaki, N. (2000). Expression of MYCN mRNA and protein. In *Neuroblastoma*, G. Brodeur, Y. Tsuchida, and T. Sawada, eds. (Amsterdam: Elsevier).
- Cohn, S. L., London, W. B., Huang, D., Katzenstein, H. M., Salwen, H. R., Reinhart, T., Madafoglio, J., Marshall, G. M., Norris, M. D., and Haber, M. (2000). MYCN expression is not prognostic of adverse outcome in advanced-stage neuroblastoma with nonamplified MYCN. *J Clin Oncol* 18, 3604-3613.
- Cohn, S. L., Salwen, H., Quasney, M. W., Ikegaki, N., Cowan, J. M., Herst, C. V., Kennett, R. H., Rosen, S. T., DiGiuseppe, J. A., and Brodeur, G. M. (1990). Prolonged N-myc protein half-life in a neuroblastoma cell line lacking N-myc amplification. *Oncogene* 5, 1821-1827.
- Cohn, S. L., and Tweddle, D. A. (2004). MYCN amplification remains prognostically strong 20 years after its "clinical debut". *Eur J Cancer* 40, 2639-2642.
- Collins, S., and Groudine, M. (1982). Amplification of endogenous myc-related DNA sequences in a human myeloid leukaemia cell line. *Nature* 298, 679-681.
- Covello, K. L., Kehler, J., Yu, H., Gordan, J. D., Arsham, A. M., Hu, C. J., Labosky, P. A., Simon, M. C., and Keith, B. (2006). HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes & development* 20, 557-570.
- Cushing, H., and Wolbach, S. (1927). The transformation of a malignant paravertebral sympathicoblastoma into a benign ganglioneuroma. *The American journal of pathology* 3, 203-216.
- Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R. C., and Croce, C. M. (1982). Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proceedings of the National Academy of Sciences of the United States of America* 79, 7824-7827.
- Das, B., Yeager, H., Tsuchida, R., Torkin, R., Gee, M. F., Thorner, P. S., Shibuya, M., Malkin, D., and Baruchel, S. (2005). A hypoxia-driven vascular endothelial growth factor/Fit1 autocrine loop interacts with hypoxia-inducible factor-1alpha through mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 pathway in neuroblastoma. *Cancer research* 65, 7267-7275.
- Davis, A. C., Wims, M., Spotts, G. D., Hann, S. R., and Bradley, A. (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes & development* 7, 671-682.
- De Bernardi, B., Nicolas, B., Boni, L., Indolfi, P., Carli, M., Cordero Di Montezemolo, L., Donfrancesco, A., Pession, A., Provenzi, M., di Cataldo, A., et al. (2003). Disseminated neuroblastoma in children older than one year at diagnosis: comparable results with three consecutive high-dose protocols adopted by the Italian Co-Operative Group for Neuroblastoma. *J Clin Oncol* 21, 1592-1601.
- De Preter, K., Vandesompele, J., Heimann, P., Yigit, N., Beckman, S., Schramm, A., Eggert, A., Stallings, R. L., Benoit, Y., Renard, M., et al. (2006). Human fetal neuroblast and neuroblastoma transcriptome analysis confirms neuroblast origin and highlights neuroblastoma candidate genes. *Genome biology* 7, R84.
- Dugast-Darzacq, C., Grange, T., and Schreiber-Agus, N. B. (2007). Differential effects of Mxi1-SRalpha and Mxi1-SRbeta in Myc antagonism. *The FEBS journal* 274, 4643-4653.
- Edsjö, A., Nilsson, H., Vandesompele, J., Karlsson, J., Pattyn, F., Culp, L. A., Speleman, F., and Pählman, S. (2004). Neuroblastoma cells with overexpressed MYCN retain their capacity to undergo neuronal differentiation. *Laboratory investigation; a journal of technical methods and pathology* 84, 406-417.
- Eggert, A., Ikegaki, N., Kwiatkowski, J., Zhao, H., Brodeur, G. M., and Himelstein, B. P. (2000). High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas. *Clin Cancer Res* 6, 1900-1908.
- Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., and Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proceedings of the National Academy of Sciences of the United States of America* 94, 4273-4278.
- Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., et al. (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107, 43-54.
- Ermsberger, U., Esposito, L., Partimo, S., Huber, K., Franke, A., Bixby, J. L., Kalcheim, C., and Unsicker, K. (2005). Expression of neuronal markers suggests heterogeneity of chick sympathoadrenal cells prior to invasion of the adrenal anlagen. *Cell and tissue research* 319, 1-13.
- Eva, A., Robbins, K. C., Andersen, P. R., Srinivasan, A., Tronick, S. R., Reddy, E. P., Ellmore, N. W., Galen, A. T., Lautenberger, J. A., Papas, T. S., et al. (1982). Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells. *Nature* 295, 116-119.

- Favier, J., Lapointe, S., Maliba, R., and Sirois, M. G. (2007). HIF2 alpha reduces growth rate but promotes angiogenesis in a mouse model of neuroblastoma. *BMC cancer* 7, 139.
- Fernandez, P. C., Frank, S. R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003). Genomic targets of the human c-Myc protein. *Genes & development* 17, 1115-1129.
- Finotto, S., Kriegelstein, K., Schober, A., Deimling, F., Lindner, K., Bruhl, B., Beier, K., Metz, J., Garcia-Ararras, J. E., Roig-Lopez, J. L., et al. (1999). Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells. *Development (Cambridge, England)* 126, 2935-2944.
- Flamme, I., Frohlich, T., von Reutern, M., Kappel, A., Damert, A., and Risau, W. (1997). HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. *Mechanisms of development* 63, 51-60.
- Gammill, L. S., and Bronner-Fraser, M. (2003). Neural crest specification: migrating into genomics. *Nature reviews* 4, 795-805.
- Gatenby, R. A., and Gillies, R. J. (2004). Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 4, 891-899.
- Gatta, G., Capocaccia, R., Stiller, C., Kaatsch, P., Berrino, F., and Terenziani, M. (2005). Childhood cancer survival trends in Europe: a EURO-CARE Working Group study. *J Clin Oncol* 23, 3742-3751.
- Gestblom, C., Grynfeld, A., Ora, I., Ortoft, E., Larsson, C., Axelson, H., Sandstedt, B., Cserjesi, P., Olson, E. N., and Pahlman, S. (1999). The basic helix-loop-helix transcription factor dHAND, a marker gene for the developing human sympathetic nervous system, is expressed in both high- and low-stage neuroblastomas. *Laboratory investigation; a journal of technical methods and pathology* 79, 67-79.
- Gestblom, C., Hoehner, J. C., Hedborg, F., Sandstedt, B., and Pahlman, S. (1997). In vivo spontaneous neuronal to neuroendocrine lineage conversion in a subset of neuroblastomas. *The American journal of pathology* 150, 107-117.
- Giatromanolaki, A., Sivridis, E., Fiska, A., and Koukourakis, M. I. (2006). Hypoxia-inducible factor-2 alpha (HIF-2 alpha) induces angiogenesis in breast carcinomas. *Appl Immunohistochem Mol Morphol* 14, 78-82.
- Gilbert, S. F. (2000). *Developmental Biology*, 6th edn (Sunderland, MA: Sunderland).
- Gillies, R. J., and Gatenby, R. A. (2007). Adaptive landscapes and emergent phenotypes: why do cancers have high glycolysis? *Journal of bioenergetics and biomembranes* 39, 251-257.
- Gordan, J. D., Bertout, J. A., Hu, C. J., Diehl, J. A., and Simon, M. C. (2007). HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. *Cancer cell* 11, 335-347.
- Goridis, C., and Rohrer, H. (2002). Specification of catecholaminergic and serotonergic neurons. *Nature reviews* 3, 531-541.
- Grandori, C., Cowley, S. M., James, L. P., and Eisenman, R. N. (2000). The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annual review of cell and developmental biology* 16, 653-699.
- Groves, A. K., George, K. M., Tissier-Seta, J. P., Engel, J. D., Brunet, J. F., and Anderson, D. J. (1995). Differential regulation of transcription factor gene expression and phenotypic markers in developing sympathetic neurons. *Development (Cambridge, England)* 121, 887-901.
- Gu, Y. Z., Moran, S. M., Hogenesch, J. B., Wartman, L., and Bradfield, C. A. (1998). Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene expression* 7, 205-213.
- Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* 75, 463-476.
- Gustafsson, M. V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U., and Bondesson, M. (2005). Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Developmental cell* 9, 617-628.
- Gut, P., Huber, K., Lohr, J., Bruhl, B., Oberle, S., Treier, M., Ernsberger, U., Kalcheim, C., and Unsicker, K. (2005). Lack of an adrenal cortex in Sf1 mutant mice is compatible with the generation and differentiation of chromaffin cells. *Development (Cambridge, England)* 132, 4611-4619.
- Hall, A. K., and Landis, S. C. (1991). Principal neurons and small intensely fluorescent (SIF) cells in the rat superior cervical ganglion have distinct developmental histories. *J Neurosci* 11, 472-484.
- Hammerling, U., Bjelfman, C., and Pahlman, S. (1987). Different regulation of N- and c-myc expression during phorbol ester-induced maturation of human SH-SY5Y neuroblastoma cells. *Oncogene* 2, 73-77.
- Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100, 57-70.
- Harris, M. L., and Erickson, C. A. (2007). Lineage specification in neural crest cell pathfinding. *Dev Dyn* 236, 1-19.
- Hashimoto, H., Daimaru, Y., Enjoji, M., and Nakagawara, A. (1989). N-myc gene product expression in neuroblastoma. *J Clin Pathol* 42, 52-55.
- Hatton, B. A., Knoepfler, P. S., Kenney, A. M., Rowitch, D. H., de Alboran, I. M., Olson, J. M., and Eisenman, R. N. (2006). N-myc is an essential downstream effector of Shh signaling during both normal and neoplastic cerebellar growth. *Cancer research* 66, 8655-8661.
- Hatton, K. S., Mahon, K., Chin, L., Chiu, F. C., Lee, H. W., Peng, D., Morgenbesser, S. D., Horner, J., and DePinho, R. A. (1996). Expression and activity of L-Myc in normal mouse development. *Molecular and cellular biology* 16, 1794-1804.
- Hedborg, F., Bjelfman, C., Sparen, P., Sandstedt, B., and Pahlman, S. (1995). Biochemical evidence for a mature phenotype in morphologically poorly differentiated neuroblastomas with a favourable outcome. *Eur J Cancer* 31A, 435-443.

- Hirsch, M. R., Tiveron, M. C., Guillemot, F., Brunet, J. F., and Goridis, C. (1998). Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. *Development (Cambridge, England)* *125*, 599-608.
- Hirvonen, H., Sandberg, M., Kalimo, H., Hukkanen, V., Vuorio, E., Salmi, T. T., and Alitalo, K. (1989). The N-myc proto-oncogene and IGF-II growth factor mRNAs are expressed by distinct cells in human fetal kidney and brain. *The Journal of cell biology* *108*, 1093-1104.
- Hiyama, E., Hiyama, K., Yokoyama, T., and Ishii, T. (1991). Immunohistochemical analysis of N-myc protein expression in neuroblastoma: correlation with prognosis of patients. *Journal of pediatric surgery* *26*, 838-843.
- Höckel, M., and Vaupel, P. (2001). Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *Journal of the National Cancer Institute* *93*, 266-276.
- Hoehner, J. C., Gestblom, C., Hedborg, F., Sandstedt, B., Olsen, L., and Pählman, S. (1996). A developmental model of neuroblastoma: differentiating stroma-poor tumors' progress along an extra-adrenal chromaffin lineage. *Laboratory investigation; a journal of technical methods and pathology* *75*, 659-675.
- Hoehner, J. C., Hedborg, F., Eriksson, L., Sandstedt, B., Grimelius, L., Olsen, L., and Pählman, S. (1998). Developmental gene expression of sympathetic nervous system tumors reflects their histogenesis. *Laboratory investigation; a journal of technical methods and pathology* *78*, 29-45.
- Hoehner, J. C., Olsen, L., Sandstedt, B., Kaplan, D. R., and Pählman, S. (1995). Association of neurotrophin receptor expression and differentiation in human neuroblastoma. *The American journal of pathology* *147*, 102-113.
- Holgert, H., Dagerlind, A., and Hokfelt, T. (1996). Phenotype of intraadrenal ganglion neurons during postnatal development in rat. *The Journal of comparative neurology* *371*, 603-620.
- Holmquist-Mengelbier, L., Fredlund, E., Löfstedt, T., Noguera, R., Navarro, S., Nilsson, H., Pietras, A., Vallon-Christersson, J., Borg, Å., Gradin, K., *et al.* (2006). Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. *Cancer cell* *10*, 413-423.
- Howard, M. J., Stanke, M., Schneider, C., Wu, X., and Rohrer, H. (2000). The transcription factor dHAND is a downstream effector of BMPs in sympathetic neuron specification. *Development (Cambridge, England)* *127*, 4073-4081.
- Hu, C. J., Wang, L. Y., Chodosh, L. A., Keith, B., and Simon, M. C. (2003). Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Molecular and cellular biology* *23*, 9361-9374.
- Huang, D., Rutkowski, J. L., Brodeur, G. M., Chou, P. M., Kwiatkowski, J. L., Babbo, A., and Cohn, S. L. (2000). Schwann cell-conditioned medium inhibits angiogenesis. *Cancer research* *60*, 5966-5971.
- Huang, L. E., Bindra, R. S., Glazer, P. M., and Harris, A. L. (2007). Hypoxia-induced genetic instability—a calculated mechanism underlying tumor progression. *Journal of molecular medicine (Berlin, Germany)* *85*, 139-148.
- Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proceedings of the National Academy of Sciences of the United States of America* *95*, 7987-7992.
- Huber, K. (2006). The sympathoadrenal cell lineage: specification, diversification, and new perspectives. *Developmental biology* *298*, 335-343.
- Hurlin, P. J. (2005). N-Myc functions in transcription and development. *Birth Defects Res C Embryo Today* *75*, 340-352.
- Hurlin, P. J., and Huang, J. (2006). The MAX-interacting transcription factor network. *Seminars in cancer biology* *16*, 265-274.
- Iritani, B. M., and Eisenman, R. N. (1999). c-Myc enhances protein synthesis and cell size during B lymphocyte development. *Proceedings of the National Academy of Sciences of the United States of America* *96*, 13180-13185.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001). HIF1alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science (New York, NY)* *292*, 464-468.
- Ivanov, S. V., Salnikow, K., Ivanova, A. V., Bai, L., and Lerman, M. I. (2007). Hypoxic repression of STAT1 and its downstream genes by a pVHL/HIF-1 target DEC1/STRA13. *Oncogene* *26*, 802-812.
- Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y., and Semenza, G. L. (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes & development* *12*, 149-162.
- Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., *et al.* (2001). Targeting of HIF-1alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science (New York, NY)* *292*, 468-472.
- Jain, S., Maltepe, E., Lu, M. M., Simon, C., and Bradfield, C. A. (1998). Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and Ah receptor mRNAs in the developing mouse. *Mechanisms of development* *73*, 117-123.
- Jögi, A., Ora, I., Nilsson, H., Lindeheim, A., Makino, Y., Poellinger, L., Axelson, H., and Pählman, S. (2002). Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. *Proceedings of the National Academy of Sciences of the United States of America* *99*, 7021-7026.
- Jögi, A., Vallon-Christersson, J., Holmquist, L., Axelson, H., Borg, A., and Pählman, S. (2004). Human neuroblastoma cells exposed to hypoxia: induction of genes associated with growth, survival, and aggressive behavior. *Experimental cell research* *295*, 469-487.
- Kaelin, W. G., Jr. (2002). Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer* *2*, 673-682.
- Kaelin, W. G., Jr. (2008). The VHL gene: oxygen sensing and cancer. In *Molecular, Cellular, Physiological and Pathogenic Responses to Hypoxia*, (Vancouver, Canada: Keystone

Symposia).

Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y., and Poellinger, L. (1999). Regulation of the hypoxia-inducible transcription factor 1 α by the ubiquitin-proteasome pathway. *The Journal of biological chemistry* 274, 6519-6525.

Kato, K., Kanamori, A., Wakamatsu, Y., Sawai, S., and Kondoh, H. (1991). Tissue Distribution of N-myc Expression in the Early Organogenesis Period of the Mouse Embryo. (N-myc/mouse embryo/in situ hybridization/neural/crest/sclerotome). In, pp. 29-39.

Kiefer, J. C. (2007). Epigenetics in development. *Dev Dyn* 236, 1144-1156.

Kim, J. W., Gao, P., Liu, Y. C., Semenza, G. L., and Dang, C. V. (2007). Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Molecular and cellular biology* 27, 7381-7393.

Kim, S., Li, Q., Dang, C. V., and Lee, L. A. (2000). Induction of ribosomal genes and hepatocyte hypertrophy by adenovirus-mediated expression of c-Myc in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 97, 11198-11202.

Kim, S. Y., Herbst, A., Tworowski, K. A., Salghetti, S. E., and Tansey, W. P. (2003). Skp2 regulates Myc protein stability and activity. *Molecular cell* 11, 1177-1188.

Kimura, H., Braun, R. D., Ong, E. T., Hsu, R., Secomb, T. W., Papahadjopoulos, D., Hong, K., and Dewhirst, M. W. (1996). Fluctuations in red cell flux in tumor microvessels can lead to transient hypoxia and reoxygenation in tumor parenchyma. *Cancer research* 56, 5522-5528.

Knowles, H., Leek, R., and Harris, A. L. (2004). Macrophage infiltration and angiogenesis in human malignancy. *Novartis Foundation symposium* 256, 189-200; discussion 200-184, 259-169.

Knuutila, S., Aalto, Y., Autio, K., Björkqvist, A. M., El-Rifai, W., Hemmer, S., Huhta, T., Kettunen, E., Kiuru-Kuhlefelt, S., Larramendy, M. L., et al. (1999). DNA copy number losses in human neoplasms. *The American journal of pathology* 155, 683-694.

Kogner, P., Barbany, G., Dominici, C., Castello, M. A., Raschella, G., and Persson, H. (1993). Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. *Cancer research* 53, 2044-2050.

Kohl, N. E., Kanda, N., Schreck, R. R., Bruns, G., Latt, S. A., Gilbert, F., and Alt, F. W. (1983). Transposition and amplification of oncogene-related sequences in human neuroblastomas. *Cell* 35, 359-367.

Krasnoselsky, A. L., Whiteford, C. C., Wei, J. S., Bilke, S., Westermann, F., Chen, Q. R., and Khan, J. (2005). Altered expression of cell cycle genes distinguishes aggressive neuroblastoma. *Oncogene* 24, 1533-1541.

Lahoz, E. G., Xu, L., Schreiber-Agus, N., and DePinho, R. A. (1994). Suppression of Myc, but not E1a, transformation activity by Max-associated proteins, Mad and Mxi1. *Proceedings of the National Academy of Sciences of the United States of America* 91, 5503-5507.

Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., and Bruick, R. K. (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes & development* 16, 1466-1471.

Lanzen, J., Braun, R. D., Klitzman, B., Brizel, D., Secomb, T. W., and Dewhirst, M. W. (2006). Direct demonstration of instabilities in oxygen concentrations within the extravascular compartment of an experimental tumor. *Cancer research* 66, 2219-2223.

Laughner, E., Taghavi, P., Chiles, K., Mahon, P. C., and Semenza, G. L. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1 α (HIF-1 α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Molecular and cellular biology* 21, 3995-4004.

Liu, S., Tian, Y., Chlenski, A., Yang, Q., Zage, P., Salwen, H. R., Crawford, S. E., and Cohn, S. L. (2005). Cross-talk between Schwann cells and neuroblasts influences the biology of neuroblastoma xenografts. *The American journal of pathology* 166, 891-900.

Liu, X., Mazanek, P., Dam, V., Wang, Q., Zhao, H., Guo, R., Jagannathan, J., Cnaan, A., Maris, J. M., and Hogarty, M. D. (2007). Deregulated Wnt/beta-catenin program in high-risk neuroblastomas without MYCN amplification. *Oncogene*, doi:10.1038/sj.onc.1210769.

Loewi, O. (1921). Über humorale übertragbarkeit der Herznervenwirkung. *Pflügers Archiv European Journal of Physiology* 189, 239-242.

Löfstedt, T., Fredlund, E., Holmquist-Mengelbier, L., Pietras, A., Ovenberger, M., Poellinger, L., and Pahlman, S. (2007). Hypoxia inducible factor-2 α in cancer. *Cell cycle (Georgetown, Tex)* 6, 919-926.

Löfstedt, T., Jögi, A., Sigvardsson, M., Gradin, K., Poellinger, L., Pahlman, S., and Axelson, H. (2004). Induction of ID2 expression by hypoxia-inducible factor-1: a role in dedifferentiation of hypoxic neuroblastoma cells. *The Journal of biological chemistry* 279, 39223-39231.

London, W. B., Boni, L., Simon, T., Berthold, F., Twist, C., Schmidt, M. L., Castleberry, R. P., Matthay, K. K., Cohn, S. L., and De Bernardi, B. (2005a). The role of age in neuroblastoma risk stratification: the German, Italian, and children's oncology group perspectives. *Cancer letters* 228, 257-266.

London, W. B., Castleberry, R. P., Matthay, K. K., Look, A. T., Seeger, R. C., Shimada, H., Thorer, P., Brodeur, G., Maris, J. M., Reynolds, C. P., and Cohn, S. L. (2005b). Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. *J Clin Oncol* 23, 6459-6465.

Lutz, W., Stohr, M., Schurmann, J., Wenzel, A., Lohr, A., and Schwab, M. (1996). Conditional expression of N-myc in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. *Oncogene* 13, 803-812.

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, A., and Poellinger, L. (2001). Inhibitory PAS domain protein is a negative regulator

- of hypoxia-inducible gene expression. *Nature* 414, 550-554.
- Makino, Y., Kanopka, A., Wilson, W. J., Tanaka, H., and Poellinger, L. (2002). Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. *The Journal of biological chemistry* 277, 32405-32408.
- Malynn, B. A., de Alboran, I. M., O'Hagan, R. C., Bronson, R., Davidson, L., DePinho, R. A., and Alt, F. W. (2000). N-myc can functionally replace c-myc in murine development, cellular growth, and differentiation. *Genes & development* 14, 1390-1399.
- Mandriota, S. J., Turner, K. J., Davies, D. R., Murray, P. G., Morgan, N. V., Sower, H. M., Wykoff, C. C., Maher, E. R., Harris, A. L., Ratcliffe, P. J., and Maxwell, P. H. (2002). HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron. *Cancer cell* 1, 459-468.
- Manohar, C. F., Short, M. L., Nguyen, A., Nguyen, N. N., Chagnovich, D., Yang, Q., and Cohn, S. L. (2002). HuD, a neuronal-specific RNA-binding protein, increases the in vivo stability of MYCN RNA. *The Journal of biological chemistry* 277, 1967-1973.
- Marchand, S., and Roux, B. (1998). Molecular dynamics study of calbindin D9k in the apo and singly and doubly calcium-loaded states. *Proteins* 33, 265-284.
- Maris, J. M. (2005). The biologic basis for neuroblastoma heterogeneity and risk stratification. *Current opinion in pediatrics* 17, 7-13.
- Maris, J. M., and Denny, C. T. (2002). Focus on embryonal malignancies. *Cancer cell* 2, 447-450.
- Maris, J. M., Guo, C., White, P. S., Hogarty, M. D., Thompson, P. M., Stram, D. O., Gerbing, R., Matthay, K. K., Seeger, R. C., and Brodeur, G. M. (2001). Allelic deletion at chromosome bands 11q14-23 is common in neuroblastoma. *Medical and pediatric oncology* 36, 24-27.
- Maris, J. M., Hogarty, M. D., Bagatell, R., and Cohn, S. L. (2007). Neuroblastoma. *Lancet* 369, 2106-2120.
- Matsunaga, T., Shirasawa, H., Hishiki, T., Yoshida, H., Kouchi, K., Ohtsuka, Y., Kawamura, K., Etoh, T., and Ohnuma, N. (2000). Enhanced expression of N-myc messenger RNA in neuroblastomas found by mass screening. *Clin Cancer Res* 6, 3199-3204.
- Matsushima, H., and Bogenmann, E. (1993). Expression of trkA cDNA in neuroblastomas mediates differentiation in vitro and in vivo. *Molecular and cellular biology* 13, 7447-7456.
- Matthay, K. K., Perez, C., Seeger, R. C., Brodeur, G. M., Shimada, H., Atkinson, J. B., Black, C. T., Gerbing, R., Haase, G. M., Stram, D. O., *et al.* (1998). Successful treatment of stage III neuroblastoma based on prospective biologic staging: a Children's Cancer Group study. *J Clin Oncol* 16, 1256-1264.
- Matthay, K. K., Villablanca, J. G., Seeger, R. C., Stram, D. O., Harris, R. E., Ramsay, N. K., Swift, P., Shimada, H., Black, C. T., Brodeur, G. M., *et al.* (1999). Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *The New England journal of medicine* 341, 1165-1173.
- Maynard, M. A., Evans, A. J., Hosomi, T., Hara, S., Jewett, M. A., and Ohh, M. (2005). Human HIF-3alpha4 is a dominant-negative regulator of HIF-1 and is down-regulated in renal cell carcinoma. *Faseb J* 19, 1396-1406.
- Meitar, D., Crawford, S. E., Rademaker, A. W., and Cohn, S. L. (1996). Tumor angiogenesis correlates with metastatic disease, N-myc amplification, and poor outcome in human neuroblastoma. *J Clin Oncol* 14, 405-414.
- Metzen, E., Stiehl, D. P., Doege, K., Marxsen, J. H., Hellwig-Burgel, T., and Jelkmann, W. (2005). Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene: identification of a functional hypoxia-responsive element. *The Biochemical journal* 387, 711-717.
- Misawa, A., Hosoi, H., Arimoto, A., Shikata, T., Akioka, S., Matsumura, T., Houghton, P. J., and Sawada, T. (2000). N-Myc induction stimulated by insulin-like growth factor I through mitogen-activated protein kinase signaling pathway in human neuroblastoma cells. *Cancer research* 60, 64-69.
- Nakada, K., Fujioka, T., Kitagawa, H., Takakuwa, T., and Yamate, N. (1993). Expressions of N-myc and ras oncogene products in neuroblastoma and their correlations with prognosis. *Japanese journal of clinical oncology* 23, 149-155.
- Nakagawara, A., Arima-Nakagawara, M., Scavarda, N. J., Azar, C. G., Cantor, A. B., and Brodeur, G. M. (1993). Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *The New England journal of medicine* 328, 847-854.
- Nakagawara, A., Kadomatsu, K., Sato, S., Kohno, K., Takano, H., Akazawa, K., Nose, Y., and Kuwano, M. (1990). Inverse correlation between expression of multidrug resistance gene and N-myc oncogene in human neuroblastomas. *Cancer research* 50, 3043-3047.
- Nara, K., Kusafuka, T., Yoneda, A., Oue, T., Sangkhathat, S., and Fukuzawa, M. (2007). Silencing of MYCN by RNA interference induces growth inhibition, apoptotic activity and cell differentiation in a neuroblastoma cell line with MYCN amplification. *International journal of oncology* 30, 1189-1196.
- Nau, M. M., Brooks, B. J., Battey, J., Sausville, E., Gazdar, A. F., Kirsch, I. R., McBride, O. W., Bertness, V., Hollis, G. F., and Minna, J. D. (1985). L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. *Nature* 318, 69-73.
- Nesbit, C. E., Tersak, J. M., and Prochownik, E. V. (1999). MYC oncogenes and human neoplastic disease. *Oncogene* 18, 3004-3016.
- Nikiforov, M. A., Kotenko, I., Petrenko, O., Beavis, A., Valenick, L., Lemischka, I., and Cole, M. D. (2000). Complementation of Myc-dependent cell proliferation by cDNA expression library screening. *Oncogene* 19, 4828-4831.
- Nilsson, H., Jögi, A., Beckman, S., Harris, A. L., Poellinger, L., and Pahlman, S. (2005). HIF-2alpha expression in human fetal paraganglia and neuroblastoma: relation to sympathetic differentiation, glucose deficiency, and hypoxia. *Experimental cell research* 303, 447-456.

- Nisen, P. D., Waber, P. G., Rich, M. A., Pierce, S., Garvin, J. R., Jr., Gilbert, F., and Lanzkowsky, P. (1988). N-myc oncogene RNA expression in neuroblastoma. *Journal of the National Cancer Institute* 80, 1633-1637.
- Ohira, M., Oba, S., Nakamura, Y., Isogai, E., Kaneko, S., Nakagawa, A., Hirata, T., Kubo, H., Goto, T., Yamada, S., *et al.* (2005). Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate risk neuroblastomas. *Cancer cell* 7, 337-350.
- Oliver, T. G., Grasfeder, L. L., Carroll, A. L., Kaiser, C., Gillingham, C. L., Lin, S. M., Wickramasinghe, R., Scott, M. P., and Wechsler-Reya, R. J. (2003). Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. *Proceedings of the National Academy of Sciences of the United States of America* 100, 7331-7336.
- Osthus, R. C., Shim, H., Kim, S., Li, Q., Reddy, R., Mukherjee, M., Xu, Y., Wonsey, D., Lee, L. A., and Dang, C. V. (2000). Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *The Journal of biological chemistry* 275, 21797-21800.
- Pages, G., and Pouyssegur, J. (2005). Transcriptional regulation of the Vascular Endothelial Growth Factor gene—a concert of activating factors. *Cardiovascular research* 65, 564-573.
- Pählman, S., and Hedborg, F. (2000). Development of the neural crest and sympathetic nervous system. In *Neuroblastoma*, G. Brodeur, Y. Tsuchida, and T. Sawada, eds. (Amsterdam: Elsevier).
- Pählman, S., and Hoehner, J. C. (1996). Neurotrophin receptors, tumor progression and tumor maturation. *Molecular medicine today* 2, 432-438.
- Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J. F. (1997). Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. *Development (Cambridge, England)* 124, 4065-4075.
- Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J. F. (1999). The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. *Nature* 399, 366-370.
- Peddinti, R., Zeine, R., Luca, D., Seshadri, R., Chlenski, A., Cole, K., Pawel, B., Salwen, H. R., Maris, J. M., and Cohn, S. L. (2007). Prominent microvascular proliferation in clinically aggressive neuroblastoma. *Clin Cancer Res* 13, 3499-3506.
- Pelengaris, S., Khan, M., and Evan, G. (2002). c-MYC: more than just a matter of life and death. *Nat Rev Cancer* 2, 764-776.
- Peng, J., Zhang, L., Drysdale, L., and Fong, G. H. (2000). The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. *Proceedings of the National Academy of Sciences of the United States of America* 97, 8386-8391.
- Percy, M. J., Furlow, P. W., Lucas, G. S., Li, X., Lappin, T. R., McMullin, M. F., and Lee, F. S. (2008). A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. *The New England journal of medicine* 358, 162-168.
- Perez, C. A., Matthay, K. K., Atkinson, J. B., Seeger, R. C., Shimada, H., Haase, G. M., Stram, D. O., Gerbing, R. B., and Lukens, J. N. (2000). Biologic variables in the outcome of stages I and II neuroblastoma treated with surgery as primary therapy: a children's cancer group study. *J Clin Oncol* 18, 18-26.
- Perini, G., Diolaiti, D., Porro, A., and Della Valle, G. (2005). In vivo transcriptional regulation of N-Myc target genes is controlled by E-box methylation. *Proceedings of the National Academy of Sciences of the United States of America* 102, 12117-12122.
- Pescador, N., Cuevas, Y., Naranjo, S., Alcaide, M., Villar, D., Landazuri, M. O., and Del Peso, L. (2005). Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egl3/phd3) gene. *The Biochemical journal* 390, 189-197.
- Peverali, F. A., Orioli, D., Tonon, L., Ciana, P., Bunone, G., Negri, M., and Della-Valle, G. (1996). Retinoic acid-induced growth arrest and differentiation of neuroblastoma cells are counteracted by N-myc and enhanced by max overexpressions. *Oncogene* 12, 457-462.
- Pietras, A., Gisselsson, D., Ora, I., Noguera, R., Beckman, S., Navarro, S., and Pählman, S. (2008). High levels of HIF-2alpha highlight an immature neural crest-like neuroblastoma cell cohort located in a perivascular niche. *The Journal of pathology* 214, 482-488.
- Pouyssegur, J., Dayan, F., and Mazure, N. M. (2006). Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 441, 437-443.
- Raabe, E. H., Laudenslager, M., Winter, C., Wasserman, N., Cole, K., LaQuaglia, M., Maris, D. J., Mosse, Y. P., and Maris, J. M. (2008). Prevalence and functional consequence of PHOX2B mutations in neuroblastoma. *Oncogene* 27, 469-476.
- Raetz, E. A., Kim, M. K., Moos, P., Carlson, M., Bruggers, C., Hooper, D. K., Foot, L., Liu, T., Seeger, R., and Carroll, W. L. (2003). Identification of genes that are regulated transcriptionally by Myc in childhood tumors. *Cancer* 98, 841-853.
- Raible, D. W., and Ragland, J. W. (2005). Reiterated Wnt and BMP signals in neural crest development. *Seminars in cell & developmental biology* 16, 673-682.
- Reissmann, E., Ernsberger, U., Francis-West, P. H., Rueger, D., Brickell, P. M., and Rohrer, H. (1996). Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. *Development (Cambridge, England)* 122, 2079-2088.
- Revet, I., Huizenga, G., Chan, A., Koster, J., Volckmann, R., van Sluis, P., Ora, I., Versteeg, R., and Geerts, D. (2008). The MSX1 homeobox transcription factor is a downstream target of PHOX2B and activates the Delta-Notch pathway in neuroblastoma. *Experimental cell research* 314, 707-719.
- Robiolo, M., Rumsey, W. L., and Wilson, D. F. (1989). Oxygen diffusion and mitochondrial respiration in neuroblastoma cells. *The American journal of physiology* 256, C1207-1213.
- Ruggero, D., and Pandolfi, P. P. (2003). Does the ribosome translate cancer? *Nat Rev Cancer* 3, 179-192.
- Russo, C. A., Weber, T. K., Volpe, C. M., Stoler, D. L., Petrelli, N. J., Rodriguez-Bigas, M., Burhans, W. C., and Anderson, G. R. (1995). An anoxia inducible endonuclease and enhanced DNA breakage as contributors to genomic instability in cancer.

Cancer research 55, 1122-1128.

Ryan, H. E., Lo, J., and Johnson, R. S. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *The EMBO journal* 17, 3005-3015.

Sawai, S., Shimono, A., Wakamatsu, Y., Palmes, C., Hanaoka, K., and Kondoh, H. (1993). Defects of embryonic organogenesis resulting from targeted disruption of the N-myc gene in the mouse. *Development (Cambridge, England)* 117, 1445-1455.

Schneider, C., Wicht, H., Enderich, J., Wegner, M., and Rohrer, H. (1999). Bone morphogenetic proteins are required in vivo for the generation of sympathetic neurons. *Neuron* 24, 861-870.

Schneiderman, J., London, W. B., Brodeur, G. M., Castleberry, R. P., Look, A. T., and Cohn, S. L. (2008). Clinical significance of MYCN amplification and ploidy in favorable-stage neuroblastoma: a report from the Children's Oncology Group. *J Clin Oncol* 26, 913-918.

Schofield, C. J., and Ratcliffe, P. J. (2004). Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* 5, 343-354.

Schwab, M., Alitalo, K., Klempnauer, K. H., Varmus, H. E., Bishop, J. M., Gilbert, F., Brodeur, G., Goldstein, M., and Trent, J. (1983). Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* 305, 245-248.

Schwab, M., Ellison, J., Busch, M., Rosenau, W., Varmus, H. E., and Bishop, J. M. (1984). Enhanced expression of the human gene N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. *Proceedings of the National Academy of Sciences of the United States of America* 81, 4940-4944.

Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L. J., Marck, B. T., Matsumoto, A. M., Shelton, J. M., Richardson, J. A., et al. (2003). Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in *Epas1*^{-/-} mice. *Nature genetics* 35, 331-340.

Scortegagna, M., Ding, K., Zhang, Q., Oktay, Y., Bennett, M. J., Bennett, M., Shelton, J. M., Richardson, J. A., Moe, O., and Garcia, J. A. (2005). HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. *Blood* 105, 3133-3140.

Semenza, G. L. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 3, 721-732.

Semenza, G. L., Roth, P. H., Fang, H. M., and Wang, G. L. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *The Journal of biological chemistry* 269, 23757-23763.

Semenza, G. L., and Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and cellular biology* 12, 5447-5454.

Shah, N. M., Groves, A. K., and Anderson, D. J. (1996). Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. *Cell* 85, 331-343.

Sheiness, D., and Bishop, J. M. (1979). DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis

virus. *Journal of virology* 31, 514-521.

Shimada, H., Ambros, I. M., Dehner, L. P., Hata, J., Joshi, V. V., Roald, B., Stram, D. O., Gerbing, R. B., Lukens, J. N., Matthay, K. K., and Castleberry, R. P. (1999). The International Neuroblastoma Pathology Classification (the Shimada system). *Cancer* 86, 364-372.

Shimada, H., Chatten, J., Newton, W. A., Jr., Sachs, N., Hamoudi, A. B., Chiba, T., Marsden, H. B., and Misugi, K. (1984). Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. *Journal of the National Cancer Institute* 73, 405-416.

Slavc, I., Ellenbogen, R., Jung, W. H., Vawter, G. F., Kretschmar, C., Grier, H., and Korf, B. R. (1990). myc gene amplification and expression in primary human neuroblastoma. *Cancer research* 50, 1459-1463.

Smith, A. G., Popov, N., Imreh, M., Axelson, H., and Henriksson, M. (2004). Expression and DNA-binding activity of MYCN/Max and Mnt/Max during induced differentiation of human neuroblastoma cells. *Journal of cellular biochemistry* 92, 1282-1295.

Spix, C., Pastore, G., Sankila, R., Stiller, C. A., and Steliarova-Foucher, E. (2006). Neuroblastoma incidence and survival in European children (1978-1997): report from the Automated Childhood Cancer Information System project. *Eur J Cancer* 42, 2081-2091.

Stanke, M., Junghans, D., Geissen, M., Goridis, C., Ernberger, U., and Rohrer, H. (1999). The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons. *Development (Cambridge, England)* 126, 4087-4094.

Stern, C. D., Artinger, K. B., and Bronner-Fraser, M. (1991). Tissue interactions affecting the migration and differentiation of neural crest cells in the chick embryo. *Development (Cambridge, England)* 113, 207-216.

Strieder, V., and Lutz, W. (2002). Regulation of N-myc expression in development and disease. *Cancer letters* 180, 107-119.

Strieder, V., and Lutz, W. (2003). E2F proteins regulate MYCN expression in neuroblastomas. *The Journal of biological chemistry* 278, 2983-2989.

Sutterlüty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Müller, U., and Krek, W. (1999). p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. *Nature cell biology* 1, 207-214.

Suzuki, T., Bogenmann, E., Shimada, H., Stram, D., and Seeger, R. C. (1993). Lack of high-affinity nerve growth factor receptors in aggressive neuroblastomas. *Journal of the National Cancer Institute* 85, 377-384.

Swietach, P., Vaughan-Jones, R. D., and Harris, A. L. (2007). Regulation of tumor pH and the role of carbonic anhydrase 9. *Cancer metastasis reviews* 26, 299-310.

Talks, K. L., Turley, H., Gatter, K. C., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., and Harris, A. L. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *The American journal of pathology* 157, 411-421.

- Tanaka, S., Tajiri, T., Noguchi, S., Shono, K., Ihara, K., Hara, T., and Suita, S. (2004). Clinical significance of a highly sensitive analysis for gene dosage and the expression level of MYCN in neuroblastoma. *Journal of pediatric surgery* 39, 63-68.
- Tang, X. X., Zhao, H., Kung, B., Kim, D. Y., Hicks, S. L., Cohn, S. L., Cheung, N. K., Seeger, R. C., Evans, A. E., and Ikegaki, N. (2006). The MYCN enigma: significance of MYCN expression in neuroblastoma. *Cancer research* 66, 2826-2833.
- Thomlinson, R. H. (1977). Hypoxia and tumours. *Journal of clinical pathology* 11, 105-113.
- Thompson, J. E., and Thompson, C. B. (2004). Putting the rap on Akt. *J Clin Oncol* 22, 4217-4226.
- Thompson, P. M., Gotoh, T., Kok, M., White, P. S., and Brodeur, G. M. (2003). CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. *Oncogene* 22, 1002-1011.
- Tian, H., Hammer, R. E., Matsumoto, A. M., Russell, D. W., and McKnight, S. L. (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes & development* 12, 3320-3324.
- Tian, H., McKnight, S. L., and Russell, D. W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes & development* 11, 72-82.
- Torres Filho, I. P., Leunig, M., Yuan, F., Intaglietta, M., and Jain, R. K. (1994). Noninvasive measurement of microvascular and interstitial oxygen profiles in a human tumor in SCID mice. *Proceedings of the National Academy of Sciences of the United States of America* 91, 2081-2085.
- Treins, C., Giorgetti-Peraldi, S., Murdaca, J., Semenza, G. L., and Van Obberghen, E. (2002). Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *The Journal of biological chemistry* 277, 27975-27981.
- Tsarovina, K., Schellenberger, J., Schneider, C., and Rohrer, H. (2008). Progenitor cell maintenance and neurogenesis in sympathetic ganglia involves Notch signaling. *Molecular and cellular neurosciences* 37, 20-31.
- Ullah, M. S., Davies, A. J., and Halestrap, A. P. (2006). The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. *The Journal of biological chemistry* 281, 9030-9037.
- Wahlstrom, T., and Henriksson, M. (2007). Mnt takes control as key regulator of the myc/max/mxd network. *Advances in cancer research* 97, 61-80.
- Wakamatsu, Y., Watanabe, Y., Nakamura, H., and Kondoh, H. (1997). Regulation of the neural crest cell fate by N-myc: promotion of ventral migration and neuronal differentiation. *Development (Cambridge, England)* 124, 1953-1962.
- van Limpt, V., Chan, A., Caron, H., Sluis, P. V., Boon, K., Hermus, M. C., and Versteeg, R. (2000). SAGE analysis of neuroblastoma reveals a high expression of the human homologue of the Drosophila Delta gene. *Medical and pediatric oncology* 35, 554-558.
- Van Limpt, V. A., Chan, A. J., Van Sluis, P. G., Caron, H. N., Van Noesel, C. J., and Versteeg, R. (2003). High delta-like 1 expression in a subset of neuroblastoma cell lines corresponds to a differentiated chromaffin cell type. *International journal of cancer* 105, 61-69.
- van Noesel, M. M., Pieters, R., Voute, P. A., and Versteeg, R. (2003). The N-myc paradox: N-myc overexpression in neuroblastomas is associated with sensitivity as well as resistance to apoptosis. *Cancer letters* 197, 165-172.
- Vandesompele, J., Edsjö, A., De Preter, K., Axelson, H., Speleman, F., and Pählman, S. (2003). ID2 expression in neuroblastoma does not correlate to MYCN levels and lacks prognostic value. *Oncogene* 22, 456-460.
- Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proceedings of the National Academy of Sciences of the United States of America* 92, 5510-5514.
- Wang, V., Davis, D. A., Haque, M., Huang, L. E., and Yarchoan, R. (2005). Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells. *Cancer research* 65, 3299-3306.
- Wang, Y., Sugiyama, H., Axelson, H., Panda, C. K., Babonits, M., Ma, A., Steinberg, J. M., Alt, F. W., Klein, G., and Wiener, F. (1992). Functional homology between N-myc and c-myc in murine plasmacytomagenesis: plasmacytoma development in N-myc transgenic mice. *Oncogene* 7, 1241-1247.
- Vaupel, P., and Mayer, A. (2007). Hypoxia in cancer: significance and impact on clinical outcome. *Cancer metastasis reviews* 26, 225-239.
- Weiss, W. A., Aldape, K., Mohapatra, G., Feuerstein, B. G., and Bishop, J. M. (1997). Targeted expression of MYCN causes neuroblastoma in transgenic mice. *The EMBO journal* 16, 2985-2995.
- Wenger, R. H., Stiehl, D. P., and Camenisch, G. (2005). Integration of oxygen signaling at the consensus HRE. *Sci STKE* 2005, re12.
- Westermann, F., Henrich, K. O., Wei, J. S., Lutz, W., Fischer, M., Konig, R., Wiedemeyer, R., Ehemann, V., Brors, B., Ernestus, K., et al. (2007). High Skp2 expression characterizes high-risk neuroblastomas independent of MYCN status. *Clin Cancer Res* 13, 4695-4703.
- Westin, E. H., Wong-Staal, F., Gelmann, E. P., Dalla-Favera, R., Papas, T. S., Lautenberger, J. A., Eva, A., Reddy, E. P., Tronick, S. R., Aaronson, S. A., and Gallo, R. C. (1982). Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. *Proceedings of the National Academy of Sciences of the United States of America* 79, 2490-2494.
- White, R. J. (2005). RNA polymerases I and III, growth control and cancer. *Nat Rev Mol Cell Biol* 6, 69-78.
- Wiesener, M. S., Turley, H., Allen, W. E., Willam, C., Eckardt, K. U., Talks, K. L., Wood, S. M., Gatter, K. C., Harris, A. L., Pugh, C. W., et al. (1998). Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* 92, 2260-2268.

- Wirthner, R., Balamurugan, K., Stiehl, D. P., Barth, S., Spielmann, P., Oehme, F., Flamme, I., Katschinski, D. M., Wenger, R. H., and Camenisch, G. (2007). Determination and modulation of prolyl-4-hydroxylase domain oxygen sensor activity. *Methods in enzymology* 435, 43-60.
- Vita, M., and Henriksson, M. (2006). The Myc oncoprotein as a therapeutic target for human cancer. *Seminars in cancer biology* 16, 318-330.
- von der Lehr, N., Johansson, S., Wu, S., Bahram, F., Castell, A., Cetinkaya, C., Hydrbring, P., Weidung, I., Nakayama, K., Nakayama, K. I., *et al.* (2003). The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. *Molecular cell* 11, 1189-1200.
- Vortmeyer, A. O., Tran, M. G., Zeng, W., Glasker, S., Riley, C., Tsokos, M., Ikejiri, B., Merrill, M. J., Raffeld, M., Zhuang, Z., *et al.* (2006). Evolution of VHL tumorigenesis in nerve root tissue. *The Journal of pathology* 210, 374-382.
- Wu, Q., Kirschmeier, P., Hockenberry, T., Yang, T. Y., Brassard, D. L., Wang, L., McClanahan, T., Black, S., Rizzi, G., Musco, M. L., *et al.* (2002). Transcriptional regulation during p21WAF1/CIP1-induced apoptosis in human ovarian cancer cells. *The Journal of biological chemistry* 277, 36329-36337.
- Yamashiro, D. J., Nakagawara, A., Ikegaki, N., Liu, X. G., and Brodeur, G. M. (1996). Expression of TrkC in favorable human neuroblastomas. *Oncogene* 12, 37-41.
- Yun, Z., Lin, Q., and Giaccia, A. J. (2005). Adaptive myogenesis under hypoxia. *Molecular and cellular biology* 25, 3040-3055.
- Zeller, K. I., Zhao, X., Lee, C. W., Chiu, K. P., Yao, F., Yustein, J. T., Ooi, H. S., Orlov, Y. L., Shahab, A., Yong, H. C., *et al.* (2006). Global mapping of c-Myc binding sites and target gene networks in human B cells. *Proceedings of the National Academy of Sciences of the United States of America* 103, 17834-17839.
- Zervos, A. S., Gyuris, J., and Brent, R. (1993). Mx11, a protein that specifically interacts with Max to bind Myc-Max recognition sites. *Cell* 72, 223-232.
- Zhang, H., Gao, P., Fukuda, R., Kumar, G., Krishnamachary, B., Zeller, K. I., Dang, C. V., and Semenza, G. L. (2007). HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. *Cancer cell* 11, 407-420.
- Zhao, C., Takita, J., Tanaka, Y., Setou, M., Nakagawa, T., Takeda, S., Yang, H. W., Terada, S., Nakata, T., Takei, Y., *et al.* (2001). Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta. *Cell* 105, 587-597.
- Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M. M., Simons, J. W., and Semenza, G. L. (2000). Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer research* 60, 1541-1545.
- Zimmerman, K. A., Yancopoulos, G. D., Collum, R. G., Smith, R. K., Kohl, N. E., Denis, K. A., Nau, M. M., Witte, O. N., Toran-Allerand, D., Gee, C. E., and *et al.* (1986). Differential expression of myc family genes during murine development. *Nature* 319, 780-783.