Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

DNA METHYLATION AND GENE EXPRESSION PATTERNS IN ADRENAL MEDULLARY TUMORS

Nimrod G. B. Kiss

Karolinska Institutet

Stockholm 2009

Main supervisor
Catharina Larsson, Professor
Department of Molecular Medicine and Surgery,
Karolinska Institutet, Stockholm, Sweden

Cosupervisor
János Geli, MD, PhD
Department of molecular Medicine and Surgery,
Karolinska Institutet, Stockholm, Sweden

Faculty Opponent
Peter Söderkvist, Professor
Department of Clinical and Experimental Medicine,
Faculty of Health Sciences,
Linköpings Universitet, Linköping, Sweden

Thesis Committee
Stig Linder, Professor
Department of Oncology/Pathology
Karolinska Institutet, Stockholm, Sweden

Eva Severinson, Professor
The Wenner-Gren Institute,
Stockholm University, Stockholm, Sweden

Fredrik Öberg, Associate Professor
Department of Genetics & Pathology
Uppsala University, Uppsala, Sweden

Published by Karolinska Institutet.
Printed by Larserics Digital Print AB, Landsvägen 65, 172 65 Sundbyberg
Cover: *Master of Pipettes*

ISBN: 978-91-7409-750-4

“You are exactly where you want to be.”

-Dr. Alex

ABSTRACT

Pheochromocytomas and abdominal paragangliomas are neuroendocrine tumors of the autonomous nervous system, arising in adrenal medulla and sympathetic ganglia throughout the abdomen. These tumors originate from catecholamine-producing chromaffin cells. Neuroblastomas are common childhood tumors in the adrenal medulla and abdominal ganglia. They derive from an unidentified subset of immature sympathetic nerve cells. Like most other cancers, pheochromocytomas, paragangliomas and neuroblastomas arise as a result of a vast array of genetic and epigenetic events that allow them to circumvent the normal mechanisms governing cell growth and death.

It is the purpose of this thesis and the works contained herein to further elucidate the mechanisms that propel the development of these tumors.

It was the purpose of **Paper I** to assess the involvement of the CDKN2A gene locus and its two resident tumor suppressor genes (TSGs), $p16^{INK4A}$ and $p14^{ARF}$, previously implicated in the pathogenesis of pheochromocytomas and paragangliomas. $p16^{INK4A}$ promoter hypermethylation was prominent in a subset of malignant paragangliomas, and sequence alterations were observed in the gene. Suppressed $p16^{INK4A}$ RNA and protein expression was also evident in these tumors. SDHB gene mutation status was assessed, and overlapped fully with $p16^{INK4A}$ hypermethylation. In contrast, we found little evidence for $p14^{ARF}$ involvement in pheochromocytomas and paragangliomas.

In **Paper II** we assessed global and gene specific methylation levels in pheochromocytomas and paragangliomas in relation to clinical phenotype. CpG islands in the promoter regions of 11 tumor suppressor genes were assessed using a quantitative method. A CpG island methylator phenotype (CIMP; defined as hypermethylation in three or more of the assessed TSGs) was found in the same subset of paragangliomas that displayed $p16^{INK4A}$ hypermethylation in Paper I.

In **Paper III** we strived to verify concerted TSG promoter hypermethylation in a subset of malignant paragangliomas with SDHB mutation in an independent sample series, and to shed light on the temporal occurrence of genetic and epigenetic events in these tumors. Epigenetic changes in the new tumor set mirrored the results from Papers I and II. As in the previous papers, TSG hypermethylation was strongly associated with SDHB mutation, and we show that mutation precedes aberrant TSG methylation and tumor formation.

The purpose of **Paper IV** was to evaluate the involvement of two RAS effector proteins with tumor suppressor functions, RASSF1A and NORE1A, in pheochromocytomas and paragangliomas. Suppressed RASSF1A and NORE1A mRNA were observed in tumors in comparison to normal reference. In agreement with the previous papers we found RASSF1A hypermethylation in malignant paragangliomas. Reconstitution of Nore1a in rat pheochromocytoma cell line suppressed the ability to grow in soft agar and induced apoptosis, supporting a tumor suppressive function for Nore1a in pheochromocytoma.

In **Paper V** we assessed global and gene specific methylation of 14 TSGs in neuroblastomas in relation to clinical phenotype. Frequent TSG hypermethylation was observed with neuroblastomas, but no clear-cut correlations could be made with specific tumor features.

In **Paper VI** we assessed expression and gene methylation of the TSG RIZ in neuroblastomas. We observed suppressed RIZ1 expression in a subset of neuroblastomas with adverse features.

In conclusion we find substantial evidence for epigenetic involvement in TSG inactivation in paragangliomas and neuroblastomas. In light of these findings we propose the evaluation of demethylating agents in the treatment of these tumors.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. Methylation of the p16INK4A promoter is associated with malignant behavior in abdominal extra-adrenal paragangliomas but not pheochromocytomas.
Kiss NB, Geli J, Lundberg F, Avci C, Velazquez-Fernandez D, Hashemi J, Weber G, Höög A, Ekström TJ, Bäckdahl M, Larsson C.
Endocr Relat Cancer. 2008 Jun;15(2):609-21.

II. Global and regional CpG methylation in pheochromocytomas and abdominal paragangliomas: association to malignant behavior.
Geli J, **Kiss N**, Karimi M, Lee JJ, Bäckdahl M, Ekström TJ, Larsson C.
Clin Cancer Res. 2008 May 1;14(9):2551-9.

III. Acquired hypermethylation of the p16INK4A promoter in abdominal paraganglioma: relation to adverse tumor phenotype and predisposing mutation.
Kiss NB, Muth A, Juhlin C, Geli J, Bäckdahl M, Höög A, Wängberg B, Nilsson O, Ahlman H, Larsson C.
Manuscript

IV. The Ras effectors NORE1A and RASSF1A are frequently inactivated in pheochromocytoma and abdominal paraganglioma.
Geli J, **Kiss N**, Lanner F, Foukakis T, Natalishvili N, Larsson O, Kogner P, Höög A, Clark GJ, Ekström TJ, Bäckdahl M, Farnebo F, Larsson C.
Endocr Relat Cancer. 2007 Mar;14(1):125-34.

V. Global and gene-specific promoter methylation in relation to biological properties of neuroblastomas.
Kiss NB, Kogner P, Martinsson T, Larsson C, Geli J.
Manuscript

VI. Suppression of RIZ in biologically unfavourable neuroblastomas.
Geli J, **Kiss N**, Kogner P, Larsson C.
Manuscript

RELATED PUBLICATIONS

I. Array-CGH identifies cyclin D1 and UBCH10 amplicons in anaplastic thyroid carcinoma.
Lee JJ, Au AY, Foukakis T, Barbaro M, **Kiss N**, Clifton-Bligh R, Staaf J, Borg A, Delbridge L, Robinson BG, Wallin G, Höög A, Larsson C.
Endocr Relat Cancer. 2008 Sep;15(3):801-15.

II. Assessment of NORE1A as a putative tumor suppressor in human neuroblastoma.
Geli J, Kogner P, Lanner F, Natalishvili N, Juhlin C, **Kiss N**, Clark GJ, Ekström TJ, Farnebo F, Larsson C.
Int J Cancer. 2008 Jul 15;123(2):389-94.

III. Estrogen receptor β mediates specific DNA methylation changes in the promoter region of the glucose transporter 4: a novel function for ERβ in regulation of epigenetic mechanisms.
Rüegg J, Swedenborg E, Karimi M, **Kiss NB**, Larsson C, Ekström TJ, Pongratz I.
Submitted manuscript.

IV. Frequent promoter hypermethylation of the APC and RASSF1A tumor suppressors and LINE-1 repeats in parathyroid tumors.
Juhlin CC, **Kiss NB**, Villablanca A, Haglund F, Nordenström J, Höög A, Larsson C.
Submitted manuscript

V. Recurrent genomic alterations in sporadic benign and malignant pheochromocytomas and paragangliomas revealed by
whole-genome array-CGH analysis.
Sandgren J , Diaz de Ståhl T, Andersson R, Menzel U, Piotrowski A, Nord H, Bäckdahl M, **Kiss N**, Braukhoff M, Dralle H, Hessman O, Larsson C, Åkerström G, Bruder C, Dumanski JP, Westin G.
Submitted manuscript

VI. Brain region- and gene locus-specific hypomethylation
and expression patterns in the chronic alcoholic.
Johansson S, **Kiss N**, Martinez RM, Thompson RF, Rimondini R, Sheedy D, Garrick T, Harper C, Bakalkin G, Hurd YL, Greally JM, Hatchwell E, Flores-Morales A, Larsson C, Dunn JJ Ekström TJ.
Submitted manuscript

Contents

LIST OF ABBREVIATIONS

ACTB	ß-actin
AFIP	Armed Forces Institute of Pathology
AMP	Adenosine monophosphate
APC	Adenomatous polyposis of the colon
APS	Adenosine 5’ phosphosulphate
ARF	Alternative reading frame
ATP	Adenosine triphosphate
BrdU	Bromodeoxyuridine
B2M	ß-2-microglobulin
CASP8	Caspase 8
CCD	Charge-coupled device
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2 A
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
CIMP	CpG island methylator phenotype
COBRA	Combined bisulfite restriction assay
CpG	Cytosine-phosphate-guanine
CTP	Cytidine triphosphate
DAPK1	Death-associated protein kinase 1
DCR2	Decoy receptor 2
FLICA	Fluorescent inhibition of caspase activity
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HIF1a	Hypoxia inducible factor 1a
HPRT1	Hypoxanthine guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
INSS	International Neuroblastoma Staging System
LINE	Long interspersed nuclear element
LOH	Loss of heterozygosity
LUMA	Luminometric methylation assay
MAPK	Mitogen-activated protein kinase
MEN2	Multiple endocrine neoplasia type 2
MetI	Methylation index
MST	Mammalian Ste20-related kinase
MYCN	Myelocytomatosis viral related oncogene, neuroblastoma
NAP	Normal adrenal pool
NF	Neurofibromatosis
NK-cells	Natural killer cells
NORE1	Novel RAS effector 1
PI3K	Phosphatidylinositol 3-kinase
PPi	Pyrophosphate

PTEN	Phosphatase and tensin homologue
PVDF	Polyvinylidene fluoride
RARB	Retinoic acid receptor beta
RASSF1	Ras association domain family protein 1
RB	Retinoblastoma
RET	Rearranged during transfection protooncogene
RIZ	Retinoblastoma-interacting zinc finger gene
qRT-PCR	Quantitative real-time polymerase chain reaction
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
TNF	Tumor necrosis factor
TP53	Tumor protein p53
TP73	Tumor protein p73
TSG	Tumor suppressor gene
TTP	Thymidine triphosphate
VHL	von Hippel-Lindau disease

1 INTRODUCTION

1.1 CANCER AND ITS GENETIC BACKGROUND

1.1.1 The human cancer spectra

A tumor is an abnormally growing mass of cells derived from the carrier's own tissues[1]. The vast majority of tumors that arise in humans grow locally and do not invade neighboring tissues – and are therefore benign. Tumors that have acquired the ability to invade adjacent tissues or spawn metastases – daughter tumors – in remote tissues pose a considerable threat towards the carrier and are referred to as malignant. Cancer is a term loosely applied to tumorous growths, in particular to the malignant subset. Even though the component cells of a cancer often are grossly aberrant they normally retain some of the characteristics of the tissues from which they derive, a trait that influences the clinical management of the tumor in question. (Weinberg 2007)

1.1.2 Cancer – the result of a multistep process

Cancer is a multistep process. In a normally functioning organism the turnover of cells is carefully controlled by genetic and molecular mechanisms. This control machinery allows for a remarkable plasticity in physiological adaptations, and it admits precise tissue regeneration. In cancer the cells lose control over the mechanisms that regulate their growth, survival and normal function. Over a period of time cells originally displaying normal phenotype accumulate a number of traits that prompt them to replicate uncontrollably, invading other tissues and disrupting normal physiological processes, with detrimental perspectives for the patient (Weinberg 2007). As a tumor develops, its component cells acquire more and more traits that give them a growth advantage, while at the same time losing the "unnecessary", specialized features of fully differentiated normal cells. Thus, highly malignant cancers have lost many of the specific characteristics of their normal precursor cells. (Figure 1) The stages of tumor formation are therefore often possible to distinguish as a full spectrum of tumors with intermediate morphology between normal and overtly malignant tissue. *Hyperplasia* is an early form of tumorous growth characterized by cells with normal morphology that occur in excessive numbers as a result of deregulated proliferation. More abnormal tissues are termed *dysplastic* – here cell appearance is no longer normal. Growths that invade underlying tissues are called *neoplasias* (this term is sometimes applied to all tumorous growth), and tumors reach their progressive apex when they

[1] A notable and virtually unparalleled exception to this is the devil facial-tumor disease, a cancer spread by allografts through bites in fighting Tasmanian devils (*Sarcophilus harrisii*); large, carnivorous marsupials native to the Australian island state of Tasmania (Pearse & Smith, 2006)

acquire the ability to *metastasize*, to spread to remote parts of the affected individual. (Weinberg 2007) A classical example of multistep cancer development was proposed by Vogelstein *et al.* in 1988. According to this model development from normal colon to metastatic disease is paralleled by a series of genetic and epigenetic alterations (Vogelstein *et al.* 1988).

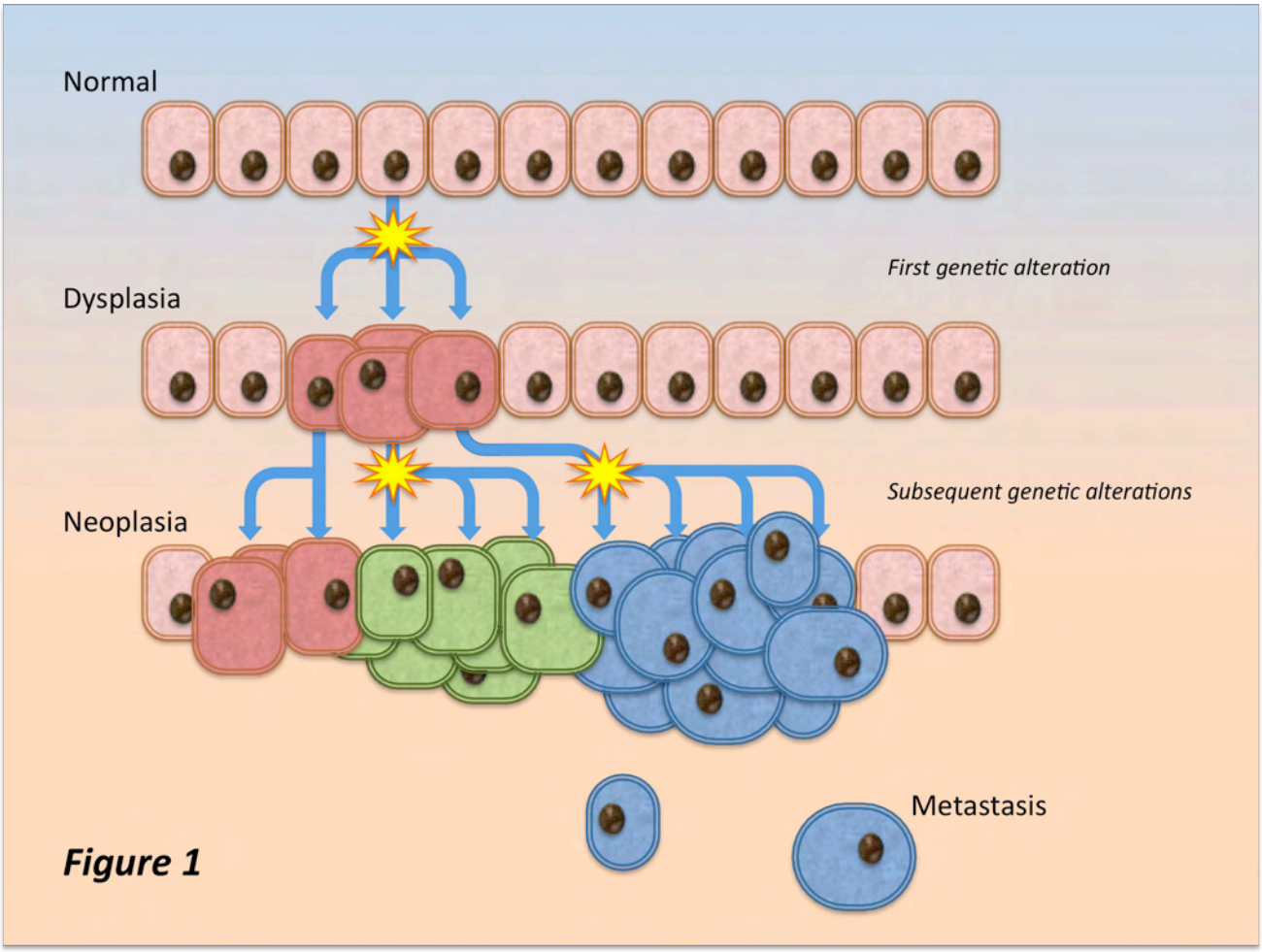

Figure 1. Tumor cell heterogeneity and step-wise accumulation of genetic aberrations leading to development of dysplasia, neoplasia and metastatic disease.

Cancer, like any other tissue composed of cells, proliferate through cell division. Tumors occur because their composite cells have lost the normal constraints that regulate their growth, division and death (Hanahan and Weinberg 2000). In other words, tumor cells act like normal cells, following their intrinsic program of proliferation and survival – but without the stringent control that governs normal cell propagation. Preceding division the genetic information in a cell is copied, and is then divided between daughter cells. Cancer cells thus accumulate genetic abnormalities that are subsequently spread by cell division. These lesions occur in individual cells, bestowing them with growth advantages over other cells. In many ways tumor progression can be likened to Darwinian evolution, in which an acquired trait might confer a growth or survival

advantage. (Weinberg 2007) The progeny of the affected cell will grow and divide at a faster rate than surrounding cells, while at the same time avoiding eradication by tumor suppressive mechanisms. The fastest growing subpopulation will eventually become the dominant component of the tumor mass. Hence, a tumor is under constant transformation, and in fact might be composed of a large number of heterogeneous clones that each derive from cells that have developed in different ways (Figure 1).

1.1.3 Genetic aberrations in cancer development

A number of traits need to be acquired in a full-blown cancer with metastatic potential: limitless replication potential, independence from external growth signals, desensitization to antigrowth signals, ability to induce angiogenesis, circumvention of apoptosis, and the capacity to invade tissues and metastasize (Hanahan and Weinberg 2000). The acquiring of these traits is a result of defects in the cell's genetic code, or aberrant genetic expression – as a consequence of any one of a number of genetic alterations. These include e.g. mutations in key genes (both somatic and constitutional), loss or gain of chromosomes or chromosomal loci, and chromosomal rearrangements. In addition epigenetic changes in DNA methylation or histone modifications, and suppression at the translational or post-translational level (RNA and protein) also contribute to cancer development (Knudson 2001, Goldmit and Bergman 2004) (Figure 2).

Although many gene types have bearing on cancer development, disruption of three categories of genes are central in oncogenesis. These are DNA repair genes, oncogenes and tumor suppressor genes (TSGs). DNA repair genes mend breaks in the DNA, and repair incoherent replication. Should one or more of these essential repair functions become inactivated the cell will start acquiring genetic defects that over time may lead to cancer. Proto-oncogenes have normal functions that promote cell survival, growth, and proliferation. When they are mutated, expressed in excess or are erroneously activated they may become oncogenes that accelerate cell cycle or convey resistance to apoptosis, thus advancing cancer development. TSGs have an opposite function: they arrest the cell cycle and cell growth, and promote apoptosis. According to the "two-hit hypothesis" formulated by Knudson, and since a TSG is represented in two alleles of the paired chromosomes, both copies of a given TSG have to be inactivated in order for that gene to lose its function (Knudson 1971, 1996, 2001). This hypothesis has been slightly modified to account for *haploinsufficiency*; since some genes do require two intact alleles for proper function loss of one copy is proposed to contribute to cancer development (Payne and Kemp 2005; Smilenov 2006).

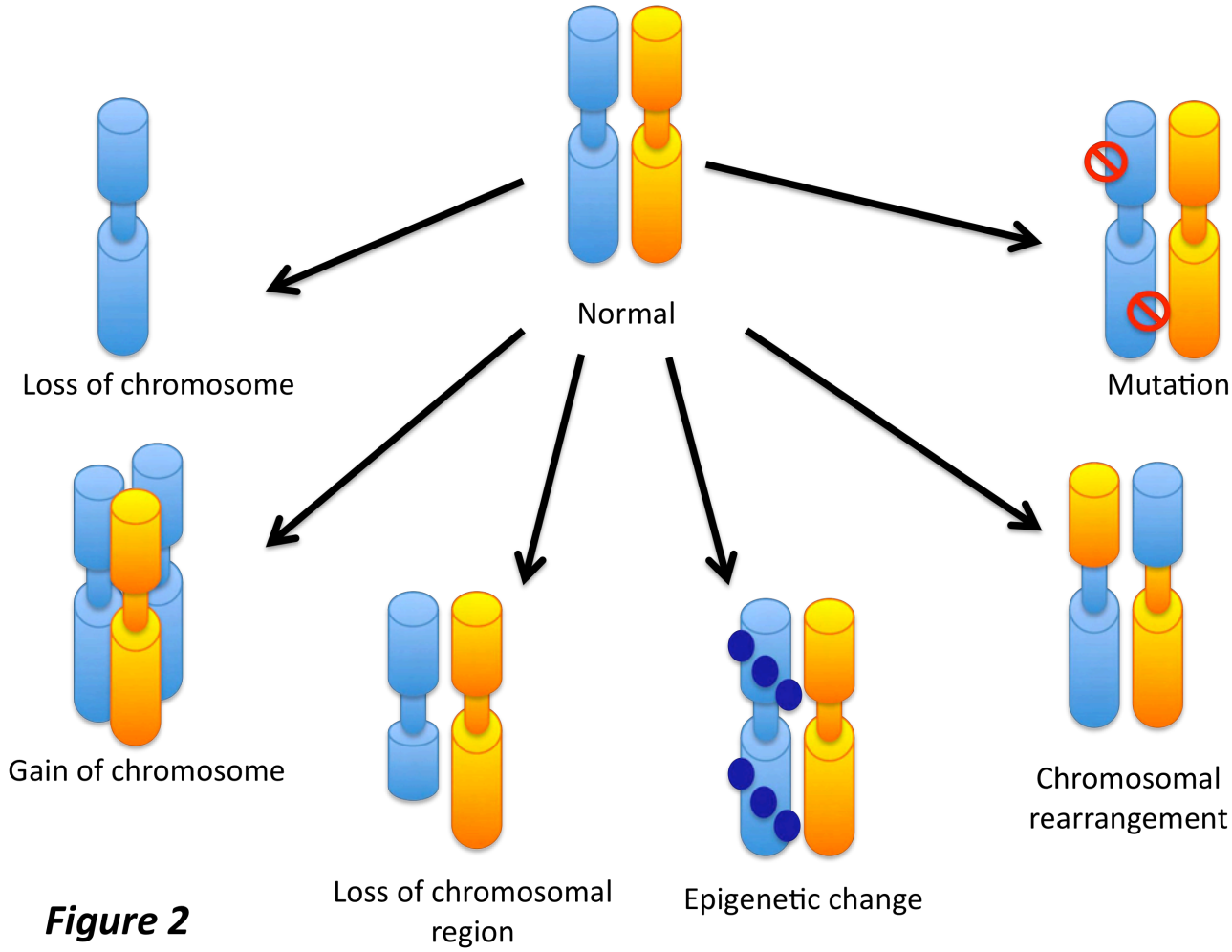

Figure 2

Figure 2. Schematic illustration of common genetic and epigenetic alterations contributing to aberrant activation or inactivation of cancer genes.

1.1.4 The cellular lifespan and its balance

The human body is composed of a fairly constant number of around 10^{14} cells (Weinberg 2007). As specialized cells wear out and accumulate damage they are constantly removed, and replaced by other cells in a balanced process (Kerr *et al.* 1972, Whyllie *et al.* 1980). Cells grow and divide upon activation by mitogenic signals in a process known as the *cell cycle*. This growth is central in normal development, maintenance of tissues, and wound healing. Dysregulated cell growth results in tumor formation, and is therefore carefully maintained. Consequently, cell growth is balanced by the *apoptotic process*, programmed cell death, which enforces equilibrium between nascent and dying cells (Kerr *et al.* 1972, Whyllie *et al.* 1980). Through apoptosis, defective or unnecessary cells can be removed from the system in an orderly fashion causing them to break down into components that can be reabsorbed by surrounding tissue (Alison and Saraff 1992, Maiuri *et al.* 2007). Cells can be induced to apoptosis acting upon an array of extrinsic or intrinsic signals (Reidl and Shi 2004). Naturally, the core processes of cell proliferation and apoptosis are

central targets in tumor formation, and therefore represent focal points for the actions of TSGs. These concepts will be explored in more detail in the following sections.

Cells can also perish through *necrosis*, which is accidental cell death by means other than apoptosis. This type of cell death occurs through damage or other processes that, unlike apoptosis, do not allow for a cell to expire in a pre-programmed, undisruptive manner. Instead necrosis is unintentional and usually a result of fast processes (Fink and Cookson 2005). Necrosis typically includes the disruption of the cell membrane, releasing potentially toxic cell contents into the affected area – which might cause inflammation and other adverse effects. Necrosis often occurs in late-stage tumors where the anomalous tissue architecture causes deficiencies in nutrients and oxygen in the tumor core (Weinberg 2007).

1.1.5 Key concepts of the cell cycle

Cells proliferate through division, *mitosis*. Before division a cell needs to grow to a sufficient size, to produce enough organelles to endow both daughter cells, and to duplicate its DNA. The entire process can be completed in the order of hours. These events are controlled by, and coordinated through the mechanisms of the cell cycle. Central in the control of the cell cycle are the cyclin-dependent kinases (CDKs), a family of proteins that sequentially activate the execution of different stages of the cell cycle. The catalytic activities of CDKs are in turn induced by the different cyclins, and specific cyclin-CDK dimers activate cascades of other proteins that initiate the different stages of the cell cycle. As the name implies, CDKs are inert without their cyclin counterparts, and can only function as a cyclin-CDK dimer. Once mitotic signals tip the balance of the intracellular milieu towards proliferation, and the cell passes through a “point of no return” (the restriction point, or *R-point*) in its cell cycle progress, it is committed to finish the cycle and to proliferate. Beyond the R-point the cell cycle becomes a chain of pre-programmed events independent from extrinsic signals. At critical points of the cell cycle a number of *checkpoints* are present that ensure that no flaws have occurred in DNA replication or other important elements. If such flaws are present at checkpoints the cell cycle transiently stops to allow for repairs, or for destruction through apoptosis. Unsurprisingly, the various aspects of the cell cycle are subject to disruption in cancer (Weinberg 2007).

1.1.6 Mitotic signaling and proliferative pathways

Depending on a variety of factors, such as tissue of origin, cells are inspired to proliferate through a wide spectrum of mitogenic signals, or *growth factors*. They attach to a diverse array of receptor molecules on the outside of the cell membrane that trigger intracellular response cascades. One

such central cascade is the MAPK (mitogen activated protein kinase) pathway in which a growth factor ligand attaches to a cell surface receptor, causing activation of the key component in this proliferative pathway, RAS (Figure 3). Active RAS proceeds to activate a series of downstream effectors that activate each other through phosphorylation. In order of involvement these are RAF, MEK and ERK. The latter translocates from the cytoplasm to the nucleus where it activates various transcription factors through phosphorylation. These in turn start the transcription of genes needed for the first phases of the cell cycle. Similar cascades are common motifs in intracellular communication, where they ensure the exponential propagation of a mitogenic stimulus and allow cells to mobilize massive resources in response to relatively faint signals. (Frame and Balmain 2000, Weinberg 2007)

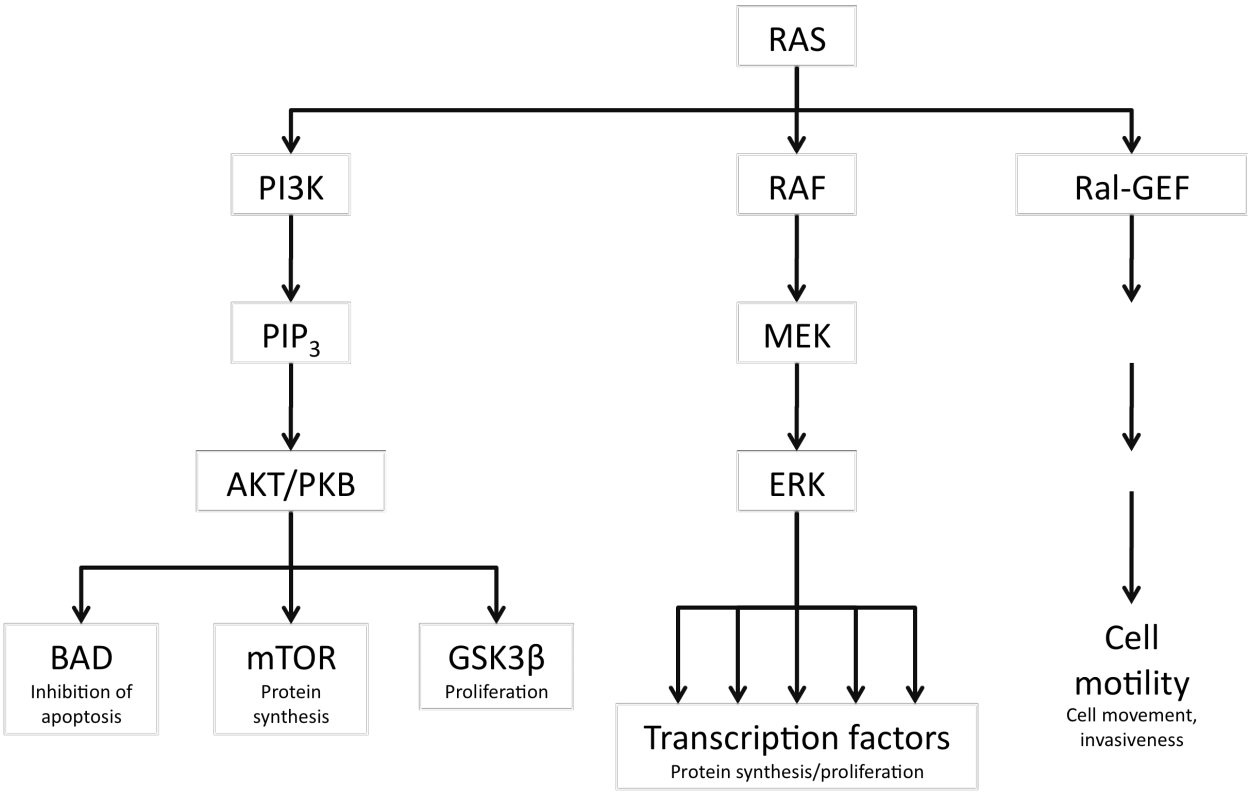

Figure 3. Summary of three pathways radiating from RAS involving PI3K, RAF and Ral-GEF. Adapted from Weinberg 2007.

At least two other mitotic signaling pathways radiate from RAS that both have important implications for cell survival and proliferation (Figure 3). The first of these is the PI3K pathway, in which RAS activates phosphatidylinositol 3-kinase (PI3K), leading to further phosphorylation of membrane-bound phosphatidylinosytol, and formation of PIP_3 (phosphatidylinosytol trihposphate) that binds and activates AKT/PKB. This kinase activates a number of downstream effectors, leading to stimulation of growth and proliferation, and, importantly, inhibition of BAD, a pro-apoptotic molecule. The second pathway is the RAL-GEF pathway whose downstream effects impact cell morphology and motility (Frame and Balmain 2000, Weinberg 2007).

Activated RAS also trigger antiproliferative downstream effectors, thereby creating a negative feedback loop: notable among these are the tumor suppressors RASSF1A and NORE1A, to be discussed in later sections (Feig and Buchsbaum 2002).

It is not difficult to appreciate that perturbance of the three RAS genes (NRAS, HRAS and KRAS) and their pathways have profound effects on cell proliferation. RAS is a true oncogene whose dysregulation affects cell proliferation and motility and counters apoptotic signals. Activating mutations of RAS are frequent in cancer. Other important mitotic pathways are the Wnt-pathway that triggers genes governing proliferation and differentiation, and the JAK-STAT pathway whose effects influence cell proliferation and survival.

1.1.7 The cell cycle and its four main phases

The cell cycle is recognized to have four specific phases in proliferating cells. A fifth, inactive stage, G_0 *(quiescence)*, is reserved for cells that are transiently removed from the cell cycle. From growth to mitosis the stages are in order: G_1-phase, the first growth phase at the end of which the cell passes the "point of no return" (restriction point; R-point) and commits to replicate; S-phase, where the DNA is replicated; G_2-phase, during which the cell grows further and doubles its macromolecular components to enable a full endowment of these passing on to daughter cells; and M-phase, where the newly doubled chromosomes are separated, and the cell is finally split in two (Weinberg 2007). Let us delve into these phases separately:

The G_1 phase: Since cells normally form a certain part of precisely structured tissue architectures, they carefully consult the neighboring environmental signals before committing to proliferation. Throughout most of the G_1-phase opposing external and internal signals dispute whether mitosis should occur, or whether the cell should remain quiescent. If the pro-mitotic signals are adequate they tip the balance in favor of proliferation and the cell dedicates itself to complete the cell cycle and divide. After this decision the cell is largely impervious towards external mitotic and anti-proliferative signals throughout the rest of the cell cycle. During the G_1-phase the D-cyclins (Cyclin D1-3) are expressed, controlled largely by extracellular signals and the mitotogenic pathways mentioned above. They now associate with two similar CDKs, CDK 4 and 6, forming a functional complex. This cyclin D-CDK4/6 complex phosphorylates the retinoblastoma protein, pRb, and ushers the cell to the aforementioned restriction point – the point of no return. (Cyclin D-CDK4/6 is inhibited by the $p16^{INK4A}$ tumor suppressor, discussed later.) pRb functions as a extremely important negative regulator of cell cycle progression by inducing closed chromatin structures though histone deacetylase (HDAC) recruitment, and by binding and inhibiting the

cell's stock supply of E2F1-3 – transcription factors that in their unbound form induce a large number of cell cycle genes. Dysregulation of pRb leads to a perpetually active cell cycle and is an important component in most cancers. (Weinberg 2007).

The initial phosphorylation by cyclin D-CDK4/6 partially inactivates pRb. It becomes fully phosphorylated by cyclin E-CDK2-complexes that accumulate around the R-point – releasing more E2Fs, which leads to transcription and subsequent translation of multiple genes. Among these are cyclin E and CDK2 themselves, resulting in a positive feedback loop that leads to phosphorylation of more pRb, release of more E2F, and increased transcription. In fact the triggering of this feedback loop constitutes the R-point in G_1. A normal cell cannot enter S-phase if DNA damage is present at the end of G_1. Detection of DNA damage comprises a checkpoint at the end of G_1 and leads to a halt in the cell cycle until the lesions have been repaired. (Weinberg 2007).

The S phase: After entry into the S-phase the cells are dedicated to complete the cycle following an autonomous set of events. The cell cycle might be halted to allow for repairs or aborted through entry into apoptosis, but cells cannot backtrack into earlier stages. During the S-phase (synthetic) the DNA in the cell nucleus becomes duplicated – in a manner of hours the synthesis of some 6.5 billion bases must be performed flawlessly. Several cyclin-CDK complexes drive the progression through the S-phase into the G_2-phase. DNA synthesis becomes halted upon detection of damage in S-phase until repairs are performed, forming a second checkpoint. (Weinberg 2007).

The G_2 phase: The second gap phase prepares the cell for mitosis; the intrinsic processes during G_2 are still poorly understood. A third checkpoint at the end of G_2 delays entry into M-phase if DNA replication is incomplete. (Weinberg 2007).

The M phase: In M-phase the cell undergoes mitosis, cell division. A carefully synchronized machinery coordinates the different steps involved. During the *prophase* of mitosis chromosomes become condensed into tightly packed structures. In the following *metaphase* the nuclear membrane disperses, and microtubules radiating from centrosomes bind and line up the chromosomes into a central plane. During *anaphase* the microtubules pull the replicated chromosomes apart to two opposite poles of the cell. The cell division concludes with the *telophase*, during which the nuclear membrane is re-formed around the separated chromosomes, and the cell is separated into two daughter cells. (Weinberg 2007).

The different phases of the cell cycle are natural focal points for the actions of oncogenes and TSGs. The mechanisms that lead up to the R-point, and indeed pRb inactivation are frequently affected. Likewise the checkpoints and the various mechanisms of DNA repair are often inactivated in cancer. A panel of TSGs is put in place to combat the actions of oncogenes at various points of the cycle.

1.1.8 Apoptosis as a regulator of cell death

As previously mentioned the apoptotic process is a means to remove excess cells from a biologic system in homeostasis. Apoptosis is not just a central aspect of tissue development (Baehrecke 2002), but is also of paramount importance for eliminating emerging morbidities (Young *et al.* 1997, Lowe and Lin 2000). Abnormalities in mitogenic pathways, for instance deregulation of oncogenes due to activating mutations or viral infections, can trigger cells to commit suicide through apoptosis (Young *et al.* 1997, Lowe and Lin 2000, Reidl and Shi 2004). The binding of extracellular signaling molecules to membrane receptors can also induce the apoptotic process (Reidl and Shi 2004). Apoptosis is mediated through positive feedback cascades of a group of proteins, *caspases*. These are constitutively present in the cell in inert forms called *procaspases*. Upon activation they are cleaved and become enzymatically active. Their function is the cleavage and destruction of other proteins, as well as activation of additional caspases. It is important for the survival of cancer cells to circumvent the TSGs that enforce apoptosis – indeed, evasion of this process is considered one of the central characteristics of cancer (Hanahan and Weinberg 2000).

When apoptosis is triggered the cell achieves distinct changes in morphology accompanied by the methodic degradation of internal components. Apoptotic cells lose their general morphology in response to this process; their surface becomes covered in bubble-like protrusions in a process called “blebbing”, cells shrink, the chromatin condenses and becomes fragmented, and the cells eventually break up into smaller components called apoptotic bodies. These are then undisruptively removed through phagocytosis by macrophages and neighboring cells (Alison and Saraff 1992, Maiuri *et al.* 2007).

Apoptosis can be triggered through two distinct signaling pathways. The intrinsic apoptotic program is mediated by caspase activation downstream of mitochondrial permeabilization by proapoptotic BCL-2 family proteins, discussed below. The extrinsic apoptotic program is mediated through transmembrane receptors on the cell surface (Reidl and Shi 2004). Prominent among the latter are the death receptors that bind death ligands – arrays of extracellular signaling

molecules that induce apoptosis. Upon ligand binding these receptors oligomerize and induce activation of initiator caspases 8 and 10. These caspases themselves activate the executioner caspases, triggering a chain reaction of caspase activation and protein degradation. A slightly different mode of activation is used by NK-cells; upon detecting aberrant (possibly cancerous) cells they inject granzyme B molecules into cells to be destroyed. These then cleave procaspases 3 and 8, thereby triggering the apoptotic cascade (Reidl and Shi 2004). Caspase 8 (CASP8) is one of the TSGs assessed in this work. Most importantly CASP8 also assists the apoptotic process by activating BID, which starts an amplification loop by engaging the intrinsic apoptotic pathway.

TP53 is involved in delaying the cell cycle upon detection of DNA damage and inducing DNA damage repair, as well as being a potent inducer of apoptosis (Weinberg 2007). It is a vital tumor suppressor frequently inactivated in cancers. TP53 is present in cell nuclei as a homotetramer that goes through a rapid cycle of synthesis and degradation known as a "futile cycle", during which the fate of TP53 is governed by the presence of the HDM2 protein that binds to it and blocks its role as a transcription factor. HDM2 also ubiquitylates TP53, targeting it for destruction in the proteasome – a large protein complex whose main function is degradation of superfluous peptides. In response to a variety of stress conditions, such as anoxia, DNA damage and disruption of the normal growth regulating machinery, TP53 loses HDM2 inhibition, becomes stabilized and starts to accumulate in the nucleus. $p14^{ARF}$, a TSG that will be presented in more detail later, responds to excessive E2F activity (a result from aberrantly regulated pRb pathway) by binding and inactivating HDM2, thereby allowing the accumulation of TP53. Amassed TP53 starts the transcription of a large array of genes, including some that directly participate in the intrinsic apoptotic pathway, such as BCL2 family members PUMA, NOXA, BID, BAX and BAD (Jiang *et al.* 2006). These proteins, when induced by TP53, open channels in the outer mitochondrial membrane, releasing stored cytochrome c proteins from the mitochondrial intermembrane space into the cytosol. Here cytochrome c assembles with procaspase 9 and the APAF-1 protein to form the apoptosome, that initiates apoptosis through activation of caspase 9 (Reidl and Shi 2004).

1.2 ADRENOMEDULLARY TUMORS

1.2.1 The sympathetic nervous system (SNS)

The autonomic nervous system can be divided into two functional parts: The parasympathetic nervous system located largely in the head and pelvic region, and the sympathetic nervous system (SNS), found in the thorax and abdomen proximal to the spinal cord (Figure 4). The tissues of the

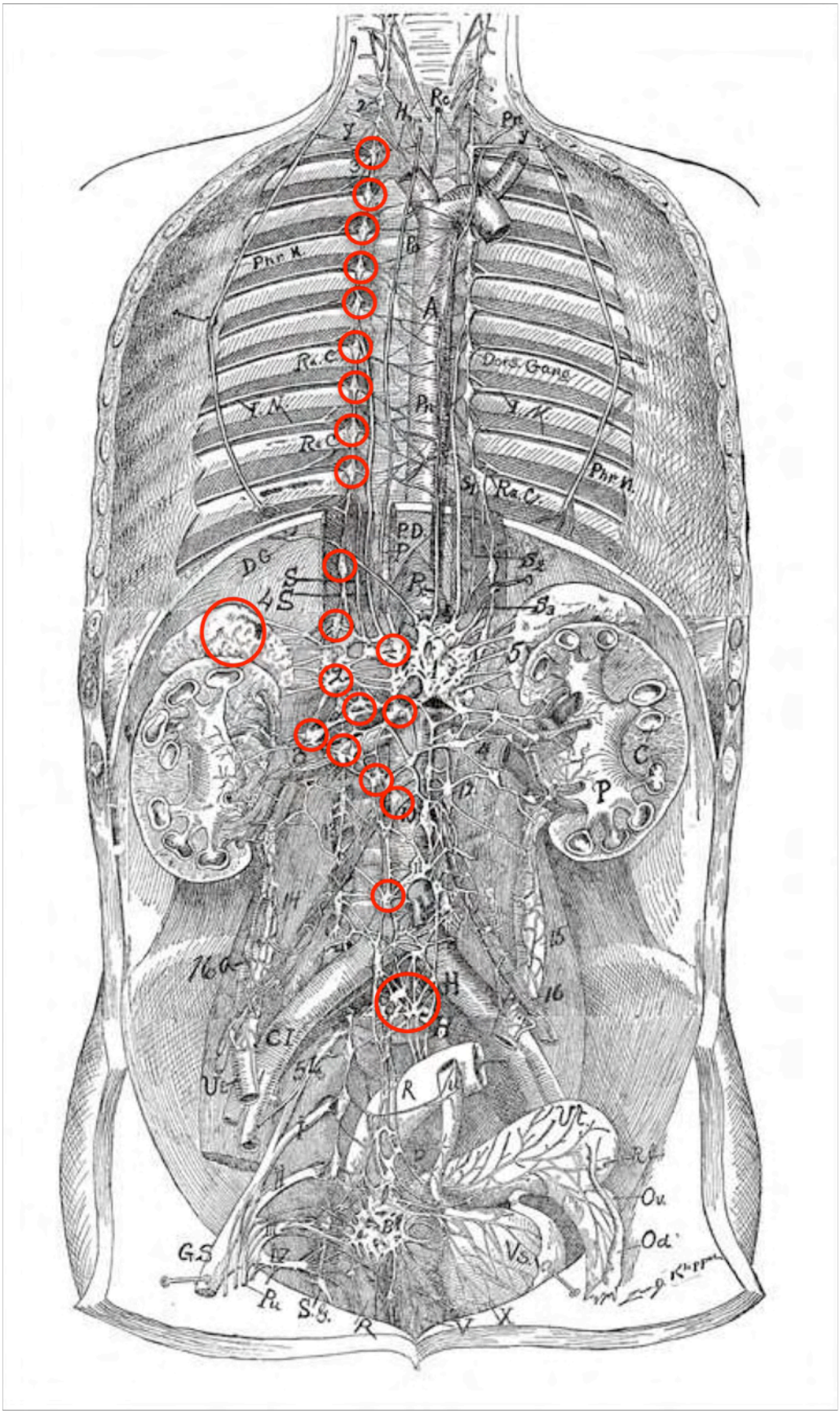

Figure 4: Red circles show ganglia in the abdomen. The adrenals are located atop of the kidneys; the sympathetic ganglia can be seen as a mesh-like structure in the central abdominal cavity and lining the spine. Robinson, Byron, M.D. The Abdominal and Pelvic Brain. Hammond, Ind.: Frank S. Betz, 1907. Figure 33 in the original publication.

SNS have a neuroectodermal origin (Langman 1981). Ganglia (clusters of sympathetic nerve cell bodies) are arranged segmentally along both sides of the vertebral column and are connected by longitudinal nerve fibers, forming the *sympathetic trunk*. Sympathetic ganglia are present in heart, lungs and the gastrointestinal tract, and are scattered throughout the abdomen, clustering around the urinary bladder and forming the organs of Zuckerkandl proximally to the bifurcation of the aorta. Nerve fibers extending from the spinal cord form synapses with the neurons of the ganglia in the sympathetic trunk and in the abdominal and thoracic cavities. These fibers convey signals that activate the sympathetic ganglion cells, relaying the signals via their axons to the heart, lungs and intestinal tract. (Sobotta 1975, Langman 1981, Lack 1997).

The adrenal glands are roughly pyramid-shaped organs about 4-6 grams in weight (Kreiner 1982) (Figure 5). They are located atop the kidneys, and are composed of the cortex, that has a mesodermal origin, and the medulla that originates from the neuroectoderm (Langman 1981). Both components have important endocrine functions, the cortex being a chief producer of corticosteroid hormones that regulate stress- and immune responses, inflammation, electrolyte levels, and metabolism. However, it is the medulla that is of chief interest here; its main functional components are the catecholamine-producing chromaffin cells, also found in sympathetic ganglia, that synthesize the bulk of the circulating adrenaline and noradrenaline. These cells give rise to pheochromocytomas and paragangliomas discussed below (Hoehner *et al.* 1998). Paragangliomas can also arise in non-chromaffin cells in the parasympathetic ganglia in head and neck (DeLellis *et al.* 2006) often referred to in the literature as head and neck paragangliomas. These tumors very seldom produce catecholamines and are not a subject for this publication.

Upon stimulation from preganglionic neurons in the spinal cord the cells of the sympathetic ganglia release noradrenaline. Extended sympathetic activation can induce release of adrenaline from the adrenal medulla. Circulating adrenaline and noradrenaline bind adrenergic receptors in peripheral tissues, eliciting the “fight-or-flight” responses that are the visceral manifestations of SNS activation. These include, among others, increases in heart rate and blood pressure, muscle tone, sweating and glucose and fatty acid metabolism. While noradrenaline is synthesized in chromaffin cells in both adrenal medulla and sympathetic paraganglia, the production of adrenaline is predominantly in adrenal medullary cells that have the requisite enzyme, phenylethanolamine N-methyltransferase, for adrenalin synthesis (Karagiannis *et al.* 2006).

1.2.2 Pheochromocytoma and paraganglioma – tumors of the SNS

1.2.2.1 Clinical presentation

Pheochromocytomas and paragangliomas are related tumors that arise in the neural crest-derived chromaffin cells of the autonomous nervous system (Hoehner *et al.* 1998, DeLellis *et al.* 2006). While pheochromocytoma usually denotes catecholamine-secreting tumors of the adrenal medulla the term paraganglioma is reserved for tumors in extra-adrenal sympathoadrenal and parasympathetic ganglia. Some confusion exists regarding the nomenclature of these tumors: the term "extra-adrenal pheochromocytoma" is sometimes used to describe paragangliomas, while "paraganglioma" may refer to tumors of the parasympathetic ganglia in head and neck. Pheochromocytomas are also occasionally referred to as "adrenal paragangliomas". For the sake of simplicity, henceforth the term "pheochromocytoma" only refers to chromaffin cell tumors in the adrenal medulla, while "paraganglioma" is only used for tumors arising in the ganglia throughout the abdomen (unless otherwise stated). (Figure 5)

The prevalence of pheochromocytomas and paragangliomas is reported to be 0.8 in 100,000 person-years (DeLellis *et al.* 2006), although this number is obscured by the fact that many tumors are nonsymptomatic or present with discrete symptoms, and therefore are only recorded at autopsy (Sutton *et al.* 1981, Sibal *et al.* 2006). Tumors occur at comparable frequencies in men and women, in all age categories, but are most prevalent at mid-life (Bravo and Tagle 2003, Karagiannis 2007). Hereditary forms of the disease present at younger age, although there is a large overlap with sporadic tumors (Elder *et al.* 2005, Karagiannis 2007). Around 10% occur in children. Benign pheochromocytomas are usually unilateral and restricted to the adrenal, although familial tumors could be bilateral and multicentric (DeLellis *et al.* 2006).

The clinical manifestation of these tumors is largely related to their endocrine function. The systemic release of adrenaline and noradrenaline results in hypertension and related cardiovascular complications. Other symptoms include headache, sweating, palpitations and anxiety, and are all SNS-response related. About 8% of the patients are asymptomatic (Karagiannis 2007).

Pheochromocytomas and paragangliomas are typically treated by surgical removal. Secondary symptoms, like hypertension and tachycardia, are usually treated using diuretic drugs and ß-blockers respectively (Bravo and Tagle 2003, Karagiannis *et al.* 2007). Chemotherapy is reserved for rapidly metastasizing tumors and radiotherapy is used in palliative treatments to relieve pain from metastases (Karagiannis *et al.* 2007).

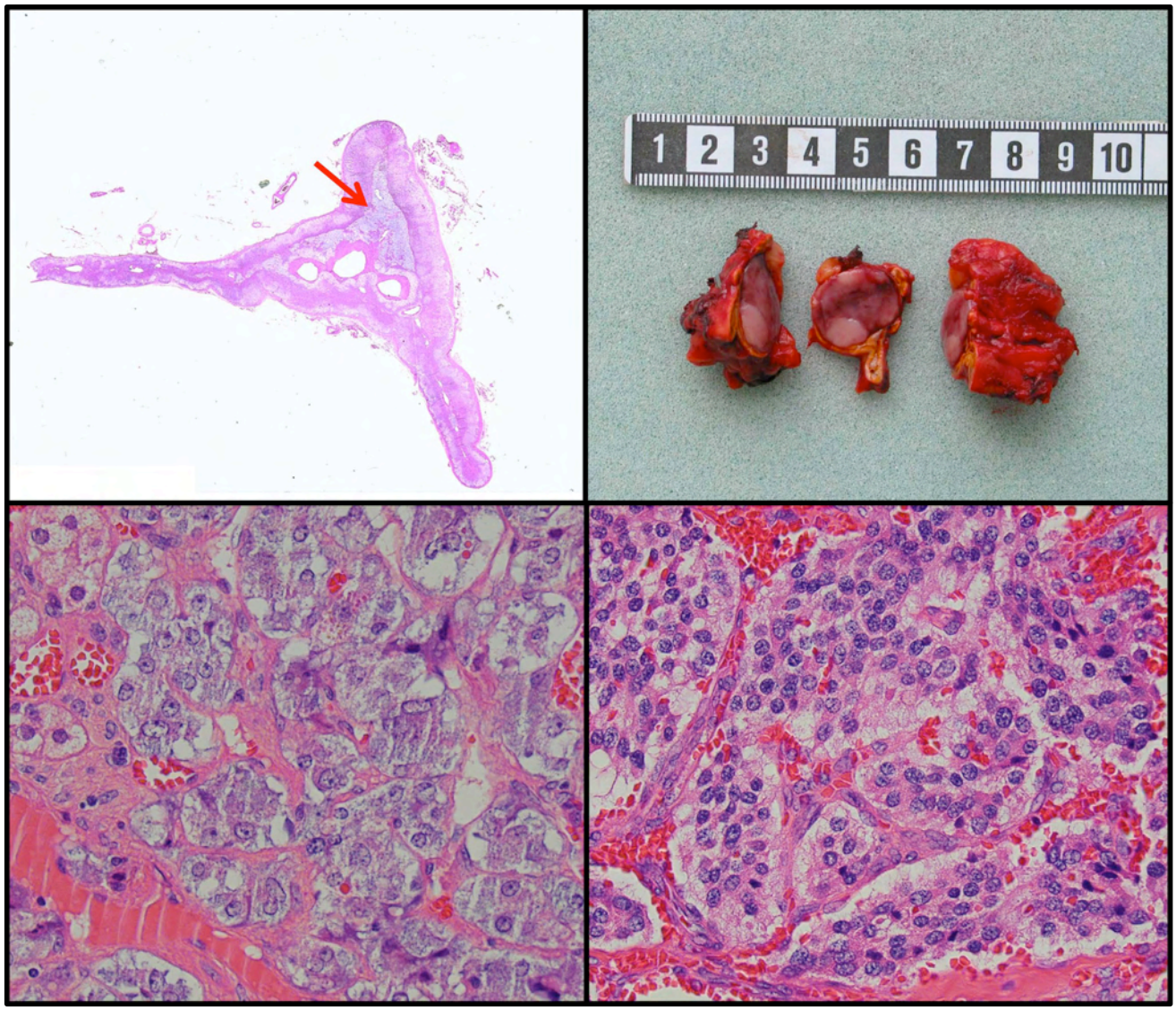

Figure 5. Top: Section of normal adrenal medulla (left). The arrow indicates the border between adrenal cortex and medulla. A macroscopic image of pheochromocytoma (right). Cortex and residual medulla can be seen along the edge of the tumor. Bottom: Microscopic images of normal adrenal medulla (left) and pheochromocytoma (right). The tumor is highly vascularized and displays the characteristic "zellballen" structure, where cells are arranged in small ball-like lobules.

1.2.2.2 Identification of malignant disease

Depending on the study population between 3-13% of all pheochromocytoma/paraganglioma cases are malignant - paragangliomas having the highest rate of malignancy (Lack 1997, DeLellis *et al.* 2006). However, the prediction of malignant potential is uncertain, and metastases can occur years after removal of the primary tumor (Karagiannis 2007). The common metastatic sites are bones, lungs, liver and lymph nodes (DeLellis *et al.* 2006). Metastasis carries a dismal 5-year survival rate of around 50% (Lenders *et al.* 2005, DeLellis *et al.* 2006). Given the distinct risk of recurrent disease it is discussed whether patients should be followed up for the remainder of their

lives (Lenders *et al.* 2005). These circumstances are also reflected in the classification systems. In the WHO system malignancy requires the identification of metastasis (DeLellis *et al.* 2006), while the Armed Forces Institute of Pathology (AFIP, Lack 1997) also recognizes extensive local invasion as a sign of malignancy. The AFIP criteria have been used in this thesis.

1.2.2.3 Genetic predisposition to pheochromocytoma and paraganglioma

Historically known as "The 10% tumor", it is now generally recognized that a wide spectrum of pheochromocytomas and abdominal paragangliomas indeed have a hereditary background. A number of inherited mutations are associated with these tumors, and represent at least 25% of the cases (Neumann *et al.* 2002, Elder *et al.* 2005).

Von Hippel – Lindau disease (VHL)

VHL is a common autosomal dominant disorder with an incidence of about 1 in 3600 births. The affected gene, VHL (3p25-26), encodes for the pVHL tumor suppressor that regulates the activity of the hypoxia inducible factor 1a (HIF1a) transcription factor. Abolishment of pVHL leads to stabilization of HIF1a and the consequent transcription of angiogenesis-related factors (Maxwell *et al.* 1999). A VHL gene mutation predisposes to multiple tumor forms including hemangioblastomas in the retina, cerebellum and spine; clear cell renal cell carcinoma, pancreatic islet cell tumors, and pheochromocytoma. The VHL syndrome is subdivided into categories based on clinical manifestation: VHL type I patients are at low risk for pheochromocytoma, instead developing clear cell renal cell carcinoma and CNS hemangioblastoma. VHL type II patients develop pheochromocytomas and also have low (type IIA) or high risk (type IIB) for clear cell renal cell carcinoma (Eng *et al.* 1995, Elder *et al.* 2005, Karagiannis *et al.* 2007).

Neurofibromatosis type 1 (NF1)

NF1 (von Recklinghausen syndrome) is an autosomal dominant disorder caused by an inactivating mutation of the neurofibromin tumor suppressor gene (17q11.2). The prevalence is around 1 in 3000 individuals (Friedman 1999). Manifestation of the phenotype includes neurofibromas (benign Schwann cell tumors), "café-au-lait spots" (patchy discolorations of the skin), axillary or inguinal freckling, iris hamartomas, optic-nerve glioma and bowing of long bones (usually tibia). These also aid the clinical diagnosis. NF1 is associated to a number of endocrine neoplasias, including primary hyperparathyroidism, medullary thyroid carcinoma, and has a pheochromocytoma/paraganglioma involvement in around 2% of the cases (Elder *et al.* 2005, Opocher *et al.* 2005, Bausch *et al.* 2007, Karagiannis *et al.* 2007).

Multiple endocrine neoplasia type 2 (MEN2)

MEN2 is caused by activating mutations in the RET proto-oncogene. The vast majority of patients (95%) develop medullary thyroid carcinoma. In MEN2A 50% of patients also present with pheochromocytoma, and 20% with primary hyperparathyroidism. In addition, patients with MEN2B develop marfanoid habitus (a group of defects otherwise encountered in Marfan syndrome that affecs multiple organ systems including heart, lungs and eyes) and mucosal and gastrointestinal ganglioneuromatosis (Elder *et al.* 2005, Marini *et al.* 2006, Karagiannis *et al.* 2007). Patients with MEN2A and MEN2B present constitutional mutations in different parts of the RET gene, with a high occurrence of *de novo* mutations in MEN2B patients (Marsh *et al.* 1997, Lodish and Stratakis 2008).

SDH mutations – The Familial Paraganglioma Syndromes

The familial paraganglioma syndromes are a group of disorders caused by mutations in the various subunits of succinate dehydrogenase – a mitochondrial enzyme complex (Complex II) involved in the citric acid cycle and the electron transport chain (Figure 6). SDHD mutations (chromosome 11q21-23) cause the autosomal dominantly inherited familial paraganglioma syndrome type 1 typically manifested by benign parasympathetic head and neck paragangliomas (Baysal *et al.* 2000). SDHD mutations are rarely associated with sympathetic paraganglioma/pheochromocytoma. Mutations in SDHB (1p35-36) cause the familial paraganglioma syndrome type 4 characterized by sympathetic paragangliomas with strong malignant tendencies (Astuti *et al.* 2001). There is also an increased risk for renal cell carcinoma and papillary thyroid cancer. SDHC is involved in familial paraganglioma syndrome type 3, characterized by benign parasympathetic head and neck paragangliomas (Elder *et al.* 2005, Müller *et al.* 2005, Karagiannis *et al.* 2007). Recently, mutations of SDH5 at the 11q13 region were identified in familial paraganglioma syndrome 2 (Hao *et al.* 2009, Kaelin 2009).

1.2.2.4 Gross chromosomal alterations in pheochromocytoma/paraganglioma

Using conventional and array CGH techniques several common chromosomal aberrations have been found in pheochromocytomas and paragangliomas. These include deletions in 1p, 3p, 3q, 11p, 11q and 22q, and gains in 17q and chromosome 19 (Edström *et al.* 2000, Cascón *et al.* 2005, Jarbo *et al.* 2006). By means of CGH and LOH studies our group has identified candidate regions for TSGs on 1p, and established that observed 1p and 3q losses were highly similar in pheochromocytomas and paragangliomas (Edström *et al.* 2000, Edström-Elder *et al.* 2002).

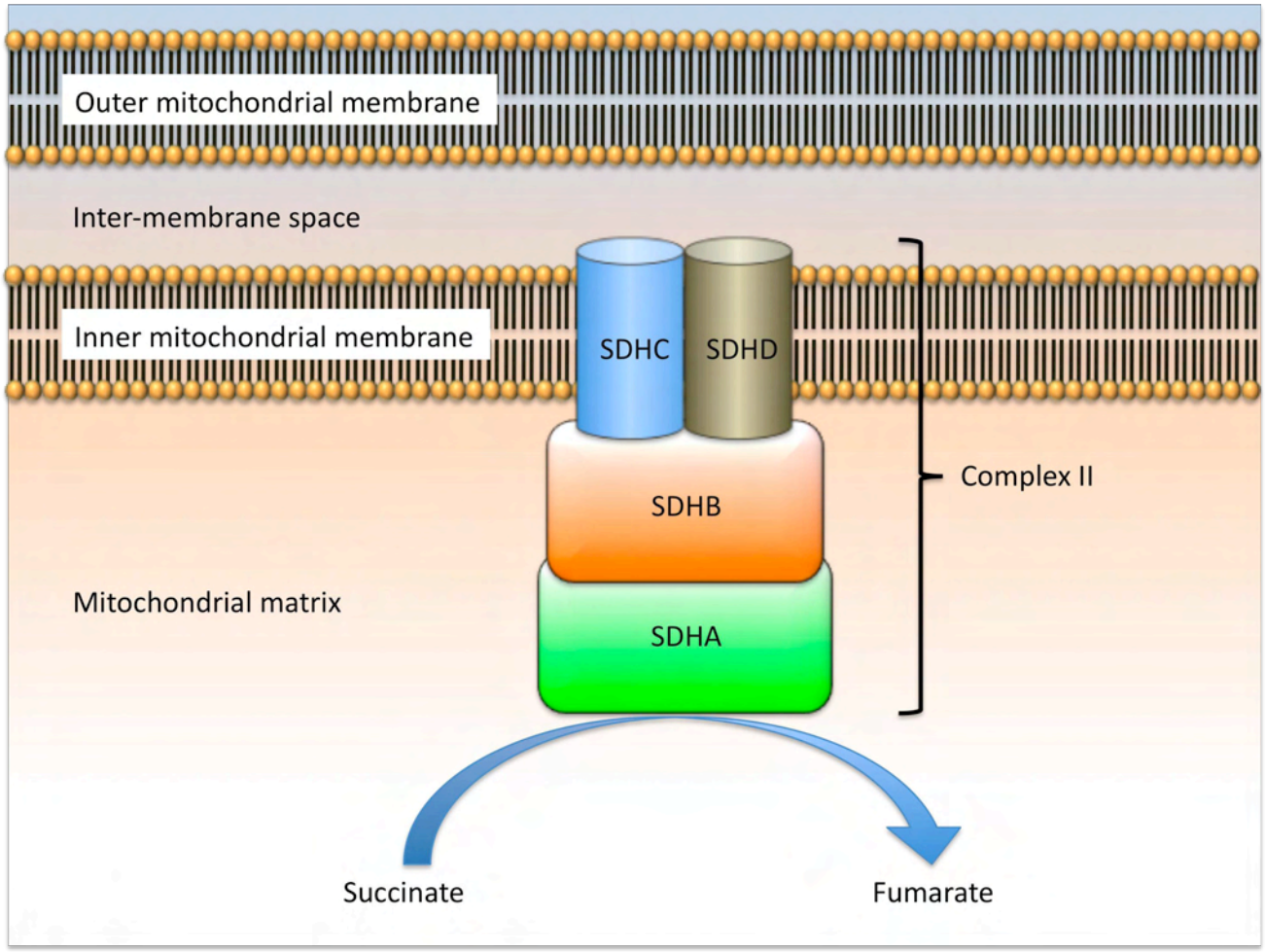

Figure 6. The four subunits (A-D) of the succinate dehydrogenase complex (Complex II) of the inner mitochondrial membrane

1.2.3 Neuroblastoma

Neuroblastomas are the most common extracranial solid tumors in childhood, and account for approximately 15% of cancer deaths in children (Grovas *et al.* 1997, Maris *et al.* 2007). They originate from an unidentified subset of neural crest-derived cells (Hoehner *et al.* 1998). Neuroblastoma patients are characterized by wide heterogeneity in clinical presentation, disease progression, responsiveness to treatment and outcome. Most tumors arise in the abdomen, with over 50% of primary tumors localized in the adrenal medulla. Roughly half of the patients have metastasized disease at detection; frequent locations for metastasis being lymph nodes, liver and bone marrow. Symptoms vary depending on primary tumor location and presence of metastasis. Children with metastases often exhibit extensive tumor burden and usually present with severe symptoms (Maris *et al.* 2007).

Neuroblastomas are treated by a wide array of procedures based on observations of clinical manifestation and assessment of the above prognostic factors. Treatment modalities include

surgery, radiotherapy and chemotherapy, and in a subset of cases, observation alone (Maris *et al.* 2007).

1.2.3.1 Clinical staging of neuroblastoma tumors

Disease progression and responsiveness to treatment is predicted based on observations of a number of clinical features, the most important being tumor stage, histopathological classification and patient age. Neuroblastomas are by agreement categorized according to the International Neuroblastoma Staging System (INSS) based on disease characteristics at time of surgery (Brodeur *et al.* 1993). The INSS has four incremental stages, with stage 1 describing low-risk, localized tumors, and stage 4 representing disseminated disease featuring distant organ metastases. Category 4S represents a special subset of stage 4 tumors. These are defined as localized primary tumors in children less than 1 year, with dissemination limited to skin, liver or bone marrow. Contrasting stage 4 neuroblastomas, cases classified as 4S usually have excellent prognosis. Concerning age patients younger than 1 year have better outcome than patients with similar clinical features of older age (Breslow and McCann 1971, Evans *et al.* 1987, London *et al.* 2005).

1.2.3.2 Molecular features of prognostic importance

Neuroblastomas are associated with a number of molecular and genetic features. Poor outcome is associated with amplification of the MYCN oncogene located in chromosomal region 2p24.1 (Brodeur and Seeger 1986, Brodeur *et al.* 1987). This occurs in around 20% of cases and is strongly correlated with limited responsiveness to treatment and advanced disease stage. MYCN amplification also negates otherwise favorable characteristics such as low INSS stage or stage 4S (Seeger *et al.* 1985, Katzenstein *et al.* 1998). MYCN is a transcription factor that complexes with MAX for transcriptional activation of genes that promote progression through the cell cycle G_1. In the absence of MYC, MAX homodimerizes and works as a transcriptional repressor (Weinberg 2007).

Deletions of the short arm of chromosome 1 (1p loss) is evident in around a third of the cases, strongly correlating with MYCN amplification and advanced disease (Gehring *et al.* 1995, Martinsson *et al.* 1995). This region is assumed to contain yet-unidentified TSG(s) involved in neuroblastoma pathogenesis. Deletion mapping studies have suggested the probable location of such a gene in 1p36 (White *et al.* 1995). Allelic loss of 11q is seen in up to 45% of neuroblastomas. This feature is rare in MYCN-amplified tumors, but associated to adverse disease progression (Planatz *et al.* 2007). Neuroblastomas also display frequent gains of 17q. This gain is

often accomplished by unbalanced translocation between chromosome 17 with chromosome 1 (Bown *et al.* 1999). The breakpoints for these translocations vary, and therefore obstruct the identification of involved genes. Rather, 17q gains likely convey a dose effect that support neuroblastoma development (Łastowska *et al.* 2002).

Neuroblastoma behavior is also dependent on expression of the TRK family of membrane receptor tyrosine kinases for neurotrophine growth factors. TRKs mediate cell differentiation and survival under normal developmental conditions. Neuroblastomas that express TRKA and TRKC usually have favorable outcome in contrast to neuroblastomas with high TRKB expression (Brodeur *et al.* 2009).

Activating somatic and germline mutations of *ALK*, a tyrosine kinase receptor located in chromosomal region 2p23, was recently implicated in neuroblastomas. The identification of germline *ALK* mutations provides insight into familial forms of the disease (Chen *et al.* 2008, Eng 2008, Mossé *et al.* 2008).

1.3 EPIGENETIC MODIFICATIONS IN CANCER

Epigenetic mechanisms constitute the control machinery that determines gene expression and activity in a heritable fashion without modification of the underlying DNA. The best example of epigenetic control in multicellular organisms is cell differentiation, the chain of events that generates the diverse cell sub-populations that makes up tissues and organs from omnipotent stem cells. Epigenetic mechanisms can either activate or inhibit gene expression, and can, when dysregulated, lead to morbidity – such as cancer.

1.3.1 Histone modifications

DNA stored in the cell nucleus is packed around octamers of small proteins called *histones*. A length of DNA corresponding to approximately 147 base pairs are wound around each octamer that consists of dual copies of histones 2A, 2B, 3 and 4 in a structure known as the *nucleosome*. Histone 1 provides linkage between two such nucleosomes and the whole structure superficially resembles a long string of beads. Histones not only provide a means for efficient packing of DNA, but also play a pivotal role in gene regulation. Key amino acids in the histone structure are subjects for an array of covalent modifications that affect how tightly DNA is bound to the histone octamer, and how closely nucleosomes are associated to each other. A condensed nucleosome structure in a given genetic allele is associated with transcriptional inactivation, while a relaxed structure spatially allows the binding of transcription factors and RNA polymerases needed for

gene transcription. Histone modifications provide very diverse and subtle means for epigenetic regulation of genes. Histones can be acetylated, methylated and phosphorylated on various sites. Acetylation is generally associated with transcriptional activation, while the effects of methylation are more diffuse and depend on which amino acid is methylated. The processes of histone modifications are dysregulated in cancer, and cancers display changes in their expression of histone-modifying enzymes compared to normal cells (Ozdag *et al.* 2006, Esteller 2008).

1.3.2 DNA methylation

DNA methylation is a central mechanism for genetic control. It is achieved through the covalent binding of methyl groups to cytosines followed by guanines in the CpG dinucleotide motif. CpGs are not randomly scattered throughout the genome but converge in "CpG islands", CpG rich regions that are located in the regulatory sequences of many genes. Such CpG islands are usually not methylated in normal tissues, although tissue-specific CpG island methylation patterns are present in normal conditions (Esteller 2008). CpG island methylation in gene promoters is strongly associated with silenced expression. Gene methylation is also an important component in controlling parental imprinting (where either the maternal or the paternal allele of a specific gene becomes transcriptionally inactivated) and the deactivation of the redundant X-chromosome in females (Jones and Takai 2001). Cancer cells frequently lose their normal methylation patterns and a common observation during tumor progression is the incremental hypomethylation of CpGs in repetive sequences, coding regions and introns. This hypomethylation may be selected for in tumorigenesis as it bestows transforming potential to developing cancers (Esteller 2008). Global hypomethylation can lead to genetic instability, transcriptional activation of transposable elements such as LINE-1 (long interspersed nuclear elements) and loss of imprinting (Esteller 2008, Ogino *et al.* 2008). An important effect of aberrant CpG methylation in cancers is the transcriptional inactivation of TSGs (Esteller 2008). DNA methylation provides cancer cells with an alternative route to abolish TSG expression beside the classically recognized schemes of mutation and deletion. Thus it can provide the "second hit" in the Knudson two-hit hypothesis (Jones and Baylin 2002). An attractive prospect stemming from this recognition is that many TSGs, though epigenetically silenced in cancers, may not be genetically lost. Thus the triggering of their reexpression emerges as a pharmacological possibility.

The cause-effect relationship between histone modifications and CpG island methylation is incompletely elucidated. Likely the two mechanisms overlap to provide intricate levels of epigenetic control.

1.4 TUMOR SUPPRESSOR GENES ASSAYED IN THIS STUDY

The papers included in this thesis explore expression and promoter methylation status of several TSGs in pheochromocytoma/paraganglioma; APC, CDH1, DAPK, DCR2, NORE1A, $p14^{ARF}$, $p16^{INK4A}$, PTEN, RARB, RASSF1A and TP73, and, additionally, BLU, CASP8, RASSF2 and RIZ in neuroblastoma. These genes, the most salient of which are described below, are implicated in many of the central pathways of cell cycle progression and apoptosis.

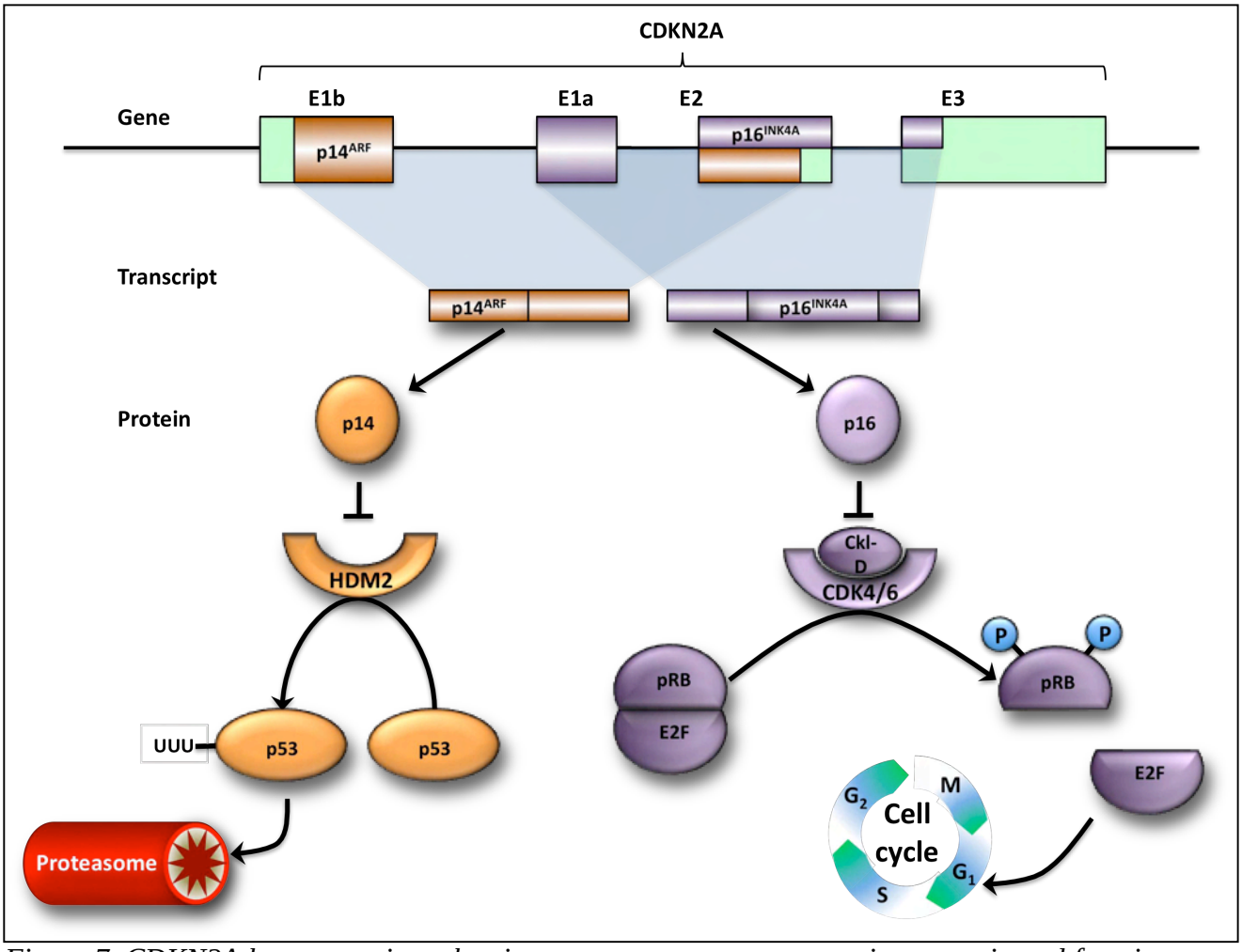

Figure 7. CDKN2A locus overview, showing gene arrangement, transcripts, protein and function for the gene products p14 and p16. Adapted from Sekulic et al. *2008*

1.4.1 $p16^{INK4A}$ and $p14^{ARF}$, TSGs of the CDKN2A locus

Two central TSGs reside in the CDKN2A gene locus at 9p21 (Sharpless and DePinho 1999) (Figure 7). These genes, $p16^{INK4A}$ and $p14^{ARF}$, that are vital modulators of the cell cycle. A perplexing feature of these true TSGs is their spatial arrangement in relation to each other. While having a dissimilar first exon (termed 1α for $p16^{INK4A}$ and 1β for $p14^{ARF}$) and separate promoters, their second and third exons occupy the same chromosomal location but are translated in different reading frames (Quelle *et al.* 1995). The protein products of these genes are completely unrelated, but both are involved in fundamental anti-mitotic pathways (Serrano *et al.* 1993). $p16^{INK4A}$ inhibits

the cyclin D-CDK4/6 complex, the key propagator of cell cycle progression, to and past the R point in the G_1 phase. Thus, $p16^{INK4A}$ action poses an early restraint, preventing entry into the autonomous part of G_1 and S phase (Sharpless 2005). $p14^{ARF}$ binds to and inhibits HDM2, an inhibitor of TP53 action. The net effect of $p14^{ARF}$ presence is the stabilization of the TP53 transcription factor, allowing it to exert its function as an inducer of cell cycle arrest, DNA repair and/or apoptosis (Sharpless 2005).

The CDKN2A locus is involved in an array of cancers. Inactivation is achieved through mutations and DNA methylation (Uchida *et al.* 1997, Kusy *et al.* 2004, Auerkari 2005, Matsuda 2008). The genes of this locus were unambiguously implicated in pheochromocytoma in a mouse knockout study, where Pten-null, neoplasia prone mice, hemizygously knocked out for the mouse CDKN2A equivalent Ink4A/Arf, frequently developed bilateral pheochromocytomas, presenting earlier and with malignant tendencies in Ink4A/Arf-/- mice (You *et al.* 2002).

While copy number loss at CDKN2A is rare in pheochromocytoma, $p16^{INK4A}$ promoter methylation has previously been reported using nonquantitative methods in small subsets of tumors (Aguiar *et al.* 1996, Dammann *et al.* 2005). The involvement of the CDKN2A locus concerning mutations and DNA methylation and expression in adrenomedullary tumors is explored in this thesis.

1.4.2 DCR2 – a death receptor enigma

DCR2 – located in 8p21 – is not commonly classified as a classical TSG. Nevertheless its inactivation has been implicated in multiple cancers, including pheochromocytomas. Promoter methylation is the frequently observed mode of inactivation (Shivapurkar *et al.* 2004, Margetts *et al.* 2005, Hornstein *et al.* 2008). DCR2 is a receptor for TRAIL, a member of the tumor necrosis factor (TNF)-type cytokines that induce apoptosis in transformed cells. Unlike the related TRAIL receptors TRAILR1 and TRAILR2 that are potent initiators of apoptosis, DCR2 only possesses a truncated death domain. While the exact function of DCR2 remains to be elucidated, it has been suggested to purvey resistance against TRAIL-induced apoptosis (Liu *et al.* 2005). Paradoxically, its frequent inactivation in cancers suggests that it instead may have a tumor suppressive function (Hornstein *et al.* 2008).

1.4.3 RIZ1, a transcriptional repressor with tumor suppressive function

The RIZ gene is located in 1p36, a region subject to deletions in multiple neoplasias, including pheochromocytomas/paragangliomas and neuroblastomas (Buyse *et al.* 1995, White *et al.* 1995,

Fang *et al.* 2001). Two RIZ isoforms have been identified, RIZ1 and RIZ2 (Figure 8). The transcripts are initiated by separate promoters. Both isoforms are identical at the 3' end, but differ in that the short isoform RIZ2 lacks a 5' SET/PR domain, present in RIZ1 (Huang *et al.* 1998, Canote *et al.* 2002). RIZ1 belongs to the family of PR/SET domain proteins that have important gene regulatory functions in development and cancer, by inducing transcriptional repression through histone 3 lysine 9 methylation (Kim *et al.* 2003). Studies have shown that RIZ1, but not RIZ2 have tumor suppressive functions (He *et al.* 1998, Huang 1999, Gazzerro *et al.* 2006). Transcription of RIZ1 is attenuated in various cancers, including pheochromocytoma (Geli *et al.* 2005).

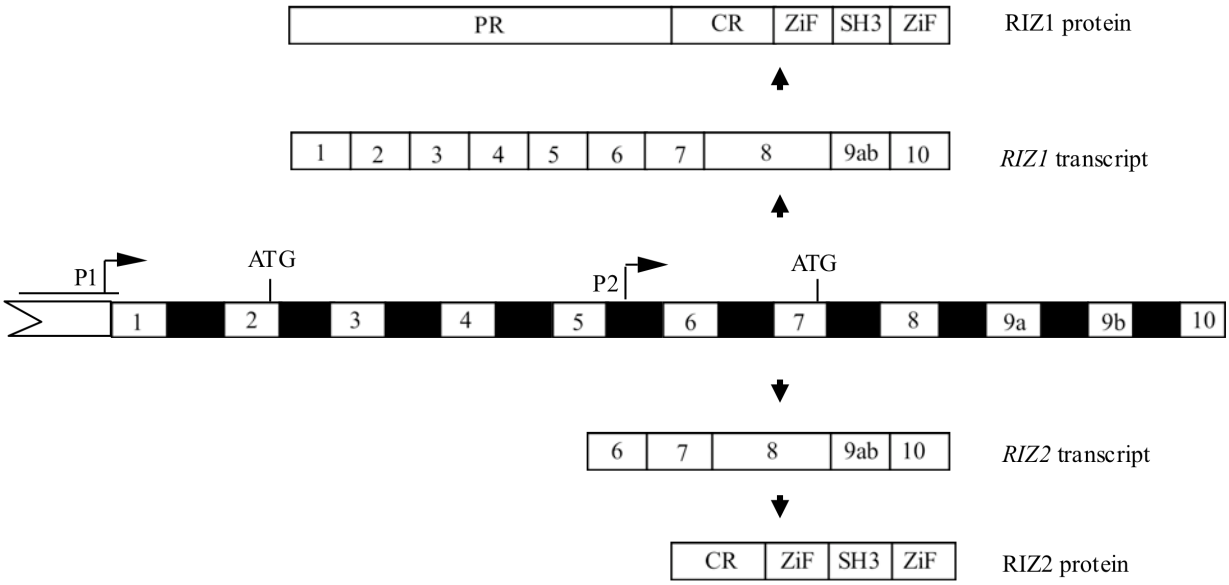

Figure 8. Shematic illustration of the RIZ locus, its two transcripts RIZ1 and RIZ2, and their encoded proteins. Adapted from Geli et al. *2005*

1.4.4 CASP8 – an initiator caspase

The protein product of the CASP8 gene (2q33), caspase 8, is an important initiator caspase in death-receptor and granzyme B-mediated apoptosis. As such, it is the first caspase to be cleaved upon death ligand binding, together with caspase 10, and is activated together with caspase 3 by granzyme B introduced into the target cell by cytotoxic cells (Muzio *et al.* 1996, Weinberg 2007). Active caspase 8 initiates the caspase cascade that results in cell termination through apoptosis. CASP8 is frequently deleted or silenced in neuroblastoma. Its promoter hypermethylation is a common event in neuroblastomas, and has been associated with poor outcome, and possibly MYCN amplification (Teitz *et al.* 2000, Takita *et al.* 2001 Teitz *et al.* 2001, Yang *et al.* 2007).

1.4.5 RASSF1A, RASSF2, NORE1A: TSGs of the RAS association domain family

The earlier described RAS gene family has a large number of downstream effectors. The most well characterized pathways radiating from RAS are the PI3K and the MAPK pathways that mediate survival and cell cycle progression. A contrasting effect is mediated through two members of the RAS association domain family, RASSF1 (3p21.3) and NORE1A (RASSF5; 1q32.1) (Feig and Buchsbaum 2002, Agathanggelou *et al.* 2005). These heterodimerize upon RAS activation and propagate apoptosis through recruitment of mammalian Ste20-related kinase (MST1; Khokhlatchev *et al.* 2002, Feig and Buchsbaum 2002, Praskova *et al.* 2004) (Figure 9). RASSF1 and NORE1A have ~60% sequence similarity (Tomassi *et al.* 2002).

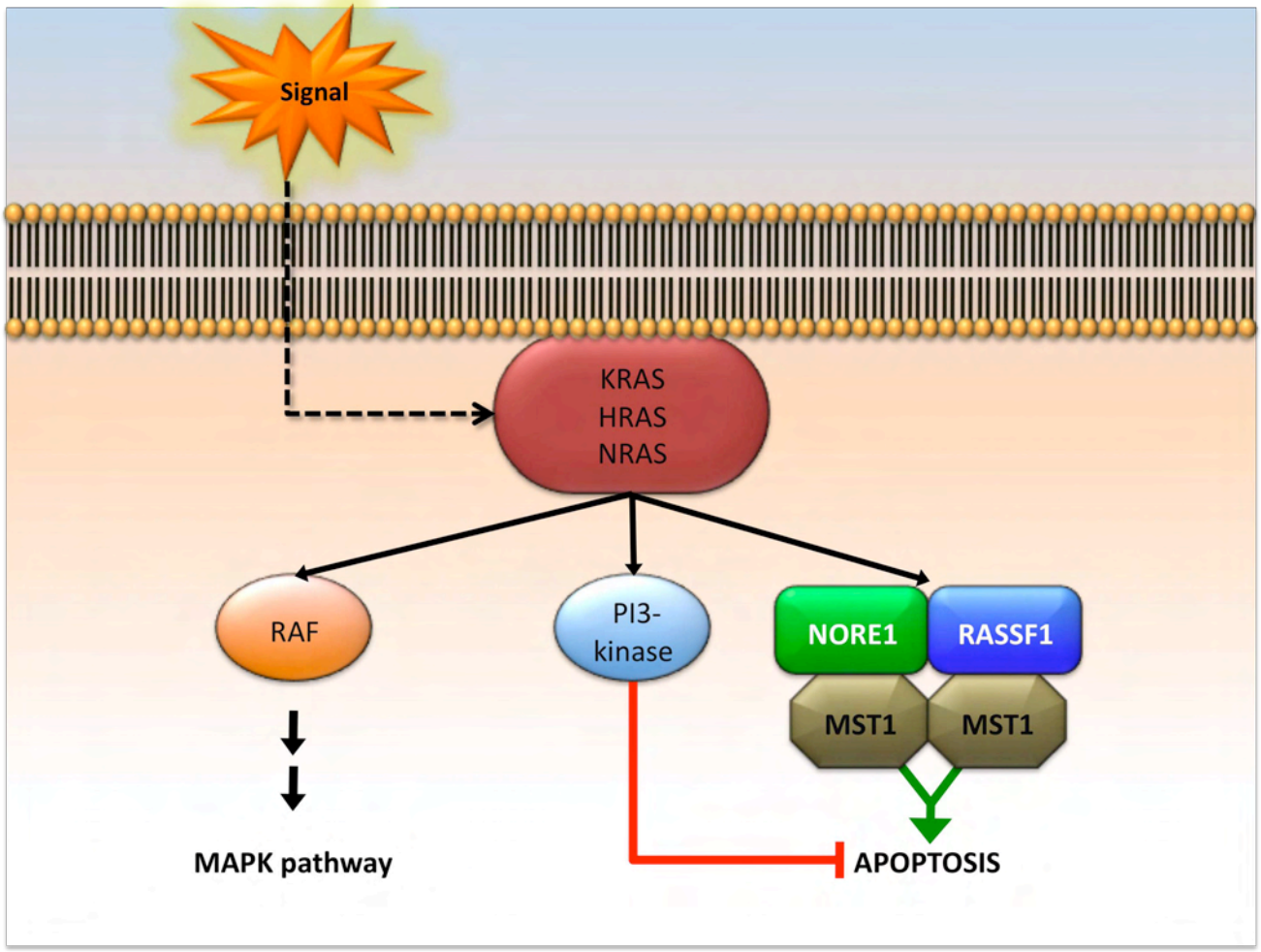

Figure 9. Active RAS mediates apoptotic downstream effects through RASSF1A and NORE1A. Adapted from Feig and Buchsbaum 2002.

RASSF1A is the most comprehensively defined out of several RASSF1 isoforms. It is an unequivocal tumor suppressor often lost through deletion, though rarely mutated. Promoter methylation is the major silencing factor for RASSF1A, frequently occurring in neuroblastoma

and other cancers (Agathanggelou *et al.* 2005, Dammann *et al.* 2005, Hesson *et al.* 2007, Liao *et al.* 2008, Michalowski *et al.* 2008, Huang *et al.* 2009).

NORE1A is the best characterized of four known NORE1 transcripts. It can act as a tumor suppressor, and is frequently silenced in neoplasias through promoter hypermethylation (Hesson *et al.* 2003, Vos *et al.* 2003, Avruch *et al.* 2006, Moshnikova *et al.* 2006, Geli *et al.* 2008). We provide the first epigenetic and expressional characterization of NORE1A in pheochromocytoma and paraganglioma.

RASSF2 (20p13) is a less explored member of the RAS association domain gene family. The protein product of this gene purveys anti-tumorigenic effects through caspase 3-BID and MST2-mediated apoptosis and cell cycle arrest, an effect that is enhanced by activated KRAS (Cooper *et al.* 2009). RASSF2 silencing through promoter hypermethylation is involved in several neoplasias (Liao *et al.* 2008, Huang *et al.* 2009, Nagasaka *et al.* 2009), and we here present the first assessment of RASSF2 methylation status in neuroblastomas.

2 AIMS

2.1 PAPER I

To assess the involvement of the two TSGs at the CDKN2A gene locus, $p16^{INK4A}$ and $p14^{ARF}$ in the pathogenesis of pheochromocytomas and paragangliomas. These genes were originally implicated in pheochromocytoma and paraganglioma development in a knockout mouse study (You *et al.* 2002).

2.2 PAPER II

To assess global and gene specific methylation levels in pheochromocytomas and paragangliomas in relation to clinical phenotype.

2.3 PAPER III

To verify concerted TSG promoter hypermethylation in a subset of malignant paragangliomas with SDHB mutation – as demonstrated in Papers I and II, and to elucidate the timely occurrence of genetic and epigenetic events that precede tumor formation.

2.4 PAPER IV

To evaluate the involvement of two RAS effector proteins, RASSF1A and NORE1A in pheochromocytomas and paragangliomas.

2.5 PAPER V

To assess global and gene specific methylation levels in neuroblastomas in relation to clinical phenotype, in equivalent fashion to Papers II and III.

2.6 PAPER VI

To assess expression and gene methylation status of RIZ in neuroblastomas. The gene is situated in 1p36, a region subject to allelic loss in neuroblastomas with adverse outcome.

3 MATERIALS AND METHODS

3.1 TUMOR SAMPLES - PHEOCHCHROMOCYTOMAS, PARAGANGLIOMAS AND NEUROBLASTOMAS (PAPERS I-VI)

Tumor samples included in this thesis were surgically removed from patients operated at the Department of Surgery at the Karolinska University Hospital, Stockholm, Sweden. Tissues were dissected by a pathologist immediately upon surgery, snap frozen in liquid nitrogen, and stored at -70 ℃. Sections from each tumor were reviewed histopathologically to ensure representativity, by verifying >70% tumor cell content. The AFIP criteria were used to assign malignancy in pheochromocytomas and paragangliomas (Lack 1997). We used blood samples from a subset of paraganglioma patients to verify the constitutional nature of SDHB mutations. Tumors and blood samples were collected with informed consent and with approval from a local ethical review board. The tumors of Paper III Series A were collected at the Department of Surgery at the Sahlgrenska University Hospital, Göteborg, Sweden, following local and international ethical guidelines.

Neuroblastoma tumors were collected at Karolinska University Hospital with consent from parents or legal guardians, and with ethical approval. Samples were routinely screened for MYCN amplification, 1p loss and DNA ploidy (Hedborg *et al.* 1992, Martinsson *et al.* 1995, 1997). Tumors were staged according to the INSS (Brodeur *et al.* 1993).

3.2 NORMAL REFERENCE SAMPLES (PAPERS I-VI)

Extracts from normal adrenomedullary samples were commercially available. Non-tumorous normal adrenal was collected with ethical approval and informed consent at the Karolinska University Hospital.

3.3 CELL LINES (PAPERS I, IV-VI)

Seven well-established neuroblastoma cell lines were used in Papers V and VI: IMR-32, SH-SY5Y, SK-N-AS, SK-N-BE, SK-N-DZ, SK-N-FI and SK-N-S for TSG methylation analyses. An established rat pheochromocytoma cell line, PC12, was used in Paper IV for functional assays. SAOS-2 osteosarcoma and MCF-7 breast cancer cell lines were used as positive and negative controls for p16 expression respectively in Paper I.

3.4 APOPTOSIS ANALYSIS THROUGH FLICA ASSAY (PAPER IV)

As described earlier apoptosis is mediated through caspases, a group of proteases that have central roles in degrading various cellular components. The action of caspases is executed through coordinated cascades upon activation of the apoptotic mechanisms, and results in DNA fragmentation and proteolysis. Activated caspases indicate ongoing or concluded apoptosis, and can be detected in a population of cells using the Fluorescent Labeled Inhibitor of CAspases (FLICA) assay (Bedner *et al.* 2000). In the FLICA-assay a fluorochrome-bound peptide inhibitor of caspases is added to the cell population. The molecules permeate the cell membrane, and the peptide moiety binds covalently to the active site of activated caspases. Unbound marker molecules are washed away in subsequent steps, and apoptotic cells can be detected visually by their fluorescence in a microscope, or through FACS.

3.5 BRDU PULSE LABELING FOR PROLIFERATION ANALYSIS (PAPER IV)

Bromodeoxyuridine (BrdU) is an analogue of thymidine and is used in cell proliferation assays to detect newly synthesized DNA strands. BrdU competes with thymidine for incorporation into the forming DNA, and can be detected using a reporter molecule-coupled antibody (most often a fluorescent molecule). Pulse labeling is inoculating cells with BrdU (such an inoculation is termed a pulse). This can be done at different time points, for instance before and after treatment, to determine cell cycle kinetics. A pulse is sufficiently short only to affect cells in the S-phase (when DNA replication occurs), preventing BrdU to be inserted into cells in other stages of their cycle. Immediately after a pulse cells are harvested and can be subjected to flow cytometry where their fluorescent properties are determined. Naturally, cells can be analyzed cytometrically for a variety of parameters. (Dolbeare 1995, 1996)

3.6 COMBINED BISULFITE RESTRICTION ASSAY (COBRA) FOR METHYLATION ANALYSIS (PAPER IV)

In Combined Bisulfite Restriction Assay (COBRA), the target DNA is first treated with bisulfite, introducing methylation-dependent sequence differences. Conversion of unmethylated cytosine residues to uracil and ultimately tymine can lead to the methylation-dependent creation of new restriction sites, or the disruption of existent sites. For COBRA, PCR primers are designed around the region of interest so as not to include CpG sites, and therefore they amplify the target DNA regardless of its methylation state. The targeted region must contain one or more methylation-dependent restriction sites that are either created or destroyed following bisulfite conversion. Following PCR the product is digested using a restriction enzyme that targets these restriction

sites. Presence of PCR product cleavage, as visualized on agarose gels, discloses the methylation status of the methylation-dependent restriction sites. (Xiong and Laird 1997)

3.7 DNA SEQUENCING (PAPERS I-III)

Dye terminator sequencing is the preferred method for determining the sequence of nucleotides in DNA. The region to be assessed is first amplified using a standard PCR, producing many copies of the sequence of interest. A second PCR, termed the sequencing reaction, is then performed, this time using only a single primer (either the forward or reverse primer.) The sequencing master mix contains the four normal deoxynucleotides (dNTPs), dATP, dCTP, dGTP and dTTP, which in the presence of a template strand and DNA polymerase will form a growing complementary DNA strand. Additionally, the sequencing master mix contains a small portion of chain-terminating dideoxynucleotides (ddNTPs), ddATP, ddCTP, ddGTP and ddTTP. These are similar to (and completely interchangeable with) standard deoxynucleotides, but lack a hydroxyl group essential for coupling to the phosphate group of the next nucleotide in the growing DNA chain. Therefore, incorporation of a dideoxynucleotide effectively terminates further DNA elongation. Only a fraction of the nucleotides are ddNTPs, and they are incorporated randomly, thus terminating the synthesis of DNA fragments at various lengths. Following the sequencing reaction the fragments are separated in a capillary electrophoresis system according to length: shortest fragments migrate first. Each of the four ddNTPs, the last nucleotide to appear in each fragment, are marked with different fluorescent dyes. As the fragments emerge through the capillary one by one according to their length, the fluorescence type and intensity of the terminating nucleotide is read and recorded by the electrophoresis system, ultimately giving the full sequence of the amplified region. (Dovichi and Zhang 2001)

3.8 FLUORESCENCE-ACTIVATED CELL SORTING (PAPER IV)

Fluorescence-Activated Cell Sorting (FACS) is a form of flow cytometry, where cells pass through a laser in a hydrodynamically focused jet of fluid. As the cells pass through the light beam, they scatter the light depending on the physical and chemical characteristics of the cell. These characteristics are recorded for each cell by detectors and provide a range of information about the observed cells. Cells marked with fluorescent labels emit light when passing through the beam, and can be sorted into different containers based on the presence or absence of fluorescent signals. Sorting is done though breaking the hydrodynamically focused fluid jet containing the cells into droplets immediately following the measuring station. A charge is placed on each droplet depending on its fluorescent status, and differentially charged droplets are deflected in an

electrostatic field, thereby sorting them into different fractions. FACS can have a variety of applications, and was here used for assessment and sorting of GFP~Nore1a-transfected cells. (http://www.invitrogen.com/site/us/en/home/support/ Tutorials.html)

3.9 LINE-1 ASSAY OF GLOBAL METHYLATION LEVELS (PAPERS II, III, V)

Long Interspersed Nuclear Elements (LINEs) are retrotransposons, transposable DNA sequences that code for a reverse transcriptase. LINEs are extremely abundant in mammalian genomes, and the most plentiful LINE element is LINE-1 (Han and Boeke 2005). LINE-1s themselves make up about 15% of the human DNA. Their exact function and evolutionary origin is not fully known, but they seem to play a role in regulating gene expression, and may have an important evolutionary role in forming new combinations of genes – there is also a risk of LINEs inserting into existing genes, disrupting their function. (Kazazian and Goodier 2002, Han and Boeke 2005). The reverse transcriptase coded by LINEs has an increased affinity for the LINE RNA itself, making a DNA copy of it that can be incorporated into the genome at other sites. LINEs move by copying themselves, and therefore extend the genome when active. CpG sites in the LINE promoters are normally heavily methylated, indicating that the transposons are inactive. Aberrant methylation, such as a general loss of methylation in malignancies, could affect LINE promoters, leading to LINE activation and ultimately to genomic instability. (Kazazian and Goodier 2002, Schulz *et al.* 2006) The promoter methylation of LINE-1 has here been assessed using Pyrosequencing as one surrogate measure of the global methylation status in pheochromocytomas, paragangliomas and neuroblastomas.

3.10 LUMINOMETRIC METHYLATION ASSAY (LUMA) (PAPER II)

LUMA provides another approach to measuring global methylation levels different from the LINE-1 promoter CpG assessment. The LUMA technique is based on the differential activity of two isoschizomeric restriction enzymes, *Hpa*II and *Msp*I. The target sequence for both is CCGG, but contrary to *Msp*I, *Hpa*II cannot cleave this site if the internal CpG is methylated. Cleavage by *Eco*RI is used as an internal control. *Eco*RI cleaves the GAATTC sequence regardless of methylation, producing A and T overhangs. In contrast, *Hpa*II and *Msp*I both produce C and G overhangs. In short, genomic DNA is cleaved in two separate reactions; *Hpa*II + *Eco*RI, and *Msp*I + *Eco*RI. The overhangs thus created are quantified in a pyrosequencing reaction (See **Pyrosequencing** below). Incorporated A and T nucleotides correspond to the overhangs created by *Eco*RI cleavage, and resulting peak heights allow for intra- and intersamplar normalization. C and G peak heights relate to *Hpa*II or *Msp*I cleavage. The peaks generated by *Msp*I cleavage correspond to the total number of CCGG sites in the target DNA, and peaks resulting from *Hpa*II

cleavage represent the amount of unmethylated CCGG sites. After normalization using *Eco*RI, the degree of CCGG methylation can be calculated thusly: Normalized *Hpa*II peak height divided by normalized *Msp*I peak height. The quotient is inversely correlated to methylation: Completely unmethylated DNA would have a *HpaII*/*MspI* ratio around 1, and in fully methylated DNA the *HpaII*/*MspI* ratio would approach zero. (Karimi *et al.* 2006)

3.11 METHYLATION-SPECIFIC PCR (MSP) (PAPER I)

Sodium bisulfite is used to introduce a sequence difference between methylated and unmethylated DNA. PCR primers are designed to encompass CpG sites where a bisulfite-induced sequence difference is expected depending on the methylation state. One set of primers is specific towards methylated DNA, in which CpG C-s remain unchanged, and another primer set targets the same stretch of DNA, but assumes a conversion of all C-s to T-s. (Herman *et al.* 1996). A PCR is performed with each of these primer sets, and in theory will only give a product if the methylation status of the DNA corresponds to the primer set used. In practice, however, the DNA is frequently a composite of unmethylated and methylated DNA, therefore often yielding a product in both reactions. One of the main strengths of MSP is also its major drawback. The method is non-quantitative and extremely sensitive, and can, with a well-optimized PCR, yield products even though the targeted methylation state is present only in <1% of the total DNA. False positives are a problem using this methodology. On the other hand, some cancers can be detected very early using the MSP methodology (Honorio *et al.* 2003).

3.12 PYROSEQUENCING (PAPERS I-VI)

Pyrosequencing is a sensitive, quantitative method for detecting DNA methylation. Originally developed for interrogating SNPs, this method was found to be versatile enough for other applications, including assessment of DNA methylation, and recently, full genome sequencing using the 454 technology (Morozova and Marra 2008). When used for DNA methylation analyses the Pyrosequencing methodology relies on sodium bisulfite treatment to introduce sequence differences between methylated and unmethylated DNA. These differences are detected in a quantitative manner, and give an accurate and highly reproducible evaluation of the methylation level. The Pyrosequencing reaction is preceded by a PCR on the bisulfite treated DNA using primers that encompass, but do not include, the CpGs of interest. The reverse primer is biotinylated, allowing the PCR fragment to be immobilized on streptavidin-coated sepharose beads following the PCR. The PCR product is washed and denatured while being subjected to vacuum suction. This removes the forward DNA strand, leaving only the immobilized reverse strands attached to the sepharose beads. A sequencing primer is added that attaches near the 3’

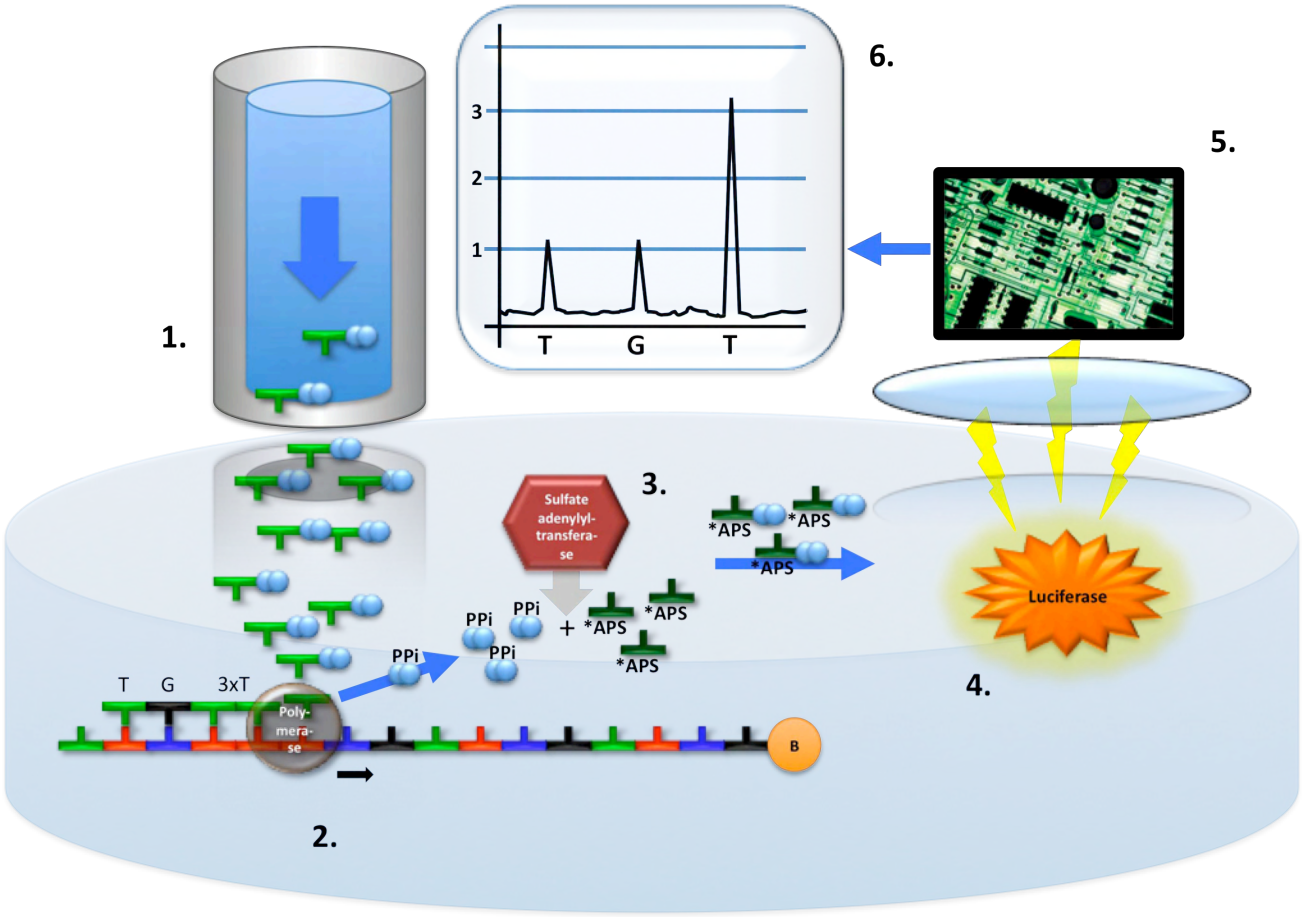

Figure 10. The pyrosequencing process. (1) Nucleotides are dispensed through a capillary. (2) Some nucleotides incorporate into the newly synthesized strand. A proportionate amount of pyrophosphates (PPi) are cleaved off. (3) PPi becomes attached to APS, forming an ATP homologue, that fuels luciferase. (4) Light, proportional to the incorporated nucleotides, is emitted. (5) The light emission is quantified electronically. (6) A pyrogram output is generated.

end of the reverse strand template. Here the Pyrosequencing reaction itself commences, and nucleotides (ATP, CTP, GTP and TTP) are dispensed by the instrument one by one into the sample wells containing template (Figure 10). If a nucleotide is complementary to the next nucleotide in the template sequence the DNA polymerase present in the reaction mix incorporates it in the growing chain. Simultaneously a pyrophosphate group (PP_i) is cleaved off; sulfurylase attaches it to adenosine 5' phosphosulphate (APS). This ATP homologue fuels luciferase, with the result that a flash of light is given off each time a successful incorporation occurs. Remaining nucleotides are degraded by apyrase. The intensity of the light is directly proportional to the amount of incorporated nucleotides, and is registered by the Pyrosequencing instrument. By analyzing which nucleotides are incorporated, and the relative amount of the incorporation, the instrument is able to accurately determine the sequence. Polymorphisms, such as SNPs or methylation-specific CpGs/TpGs are detected through dispensation of both possible nucleotides (e.g. C and T). For a methylated and bisulfite treated CpG the C/T peak ratio mirrors the proportion of methylated vs. unmethylated allele.

In-house designed primer sequences were tested against preferential amplification bias towards either the methylated or unmethylated fraction. A dilution series between methylated and unmethylated bisulfite treated DNA was subjected to Pyrosequencing (100% methylated, 0% unmethylated, to 0% methylated, 100% unmethylated in 25% increments). Only assays where the experimentally determined C/T ratio matched the methylated/unmethylated ratio of the diluted DNA on all measured points were used for further analysis. The main drawbacks of Pyrosequencing are the relatively short sequences that are obtainable; in practice around 200-300 bases; and the difficulty of optimizing newly designed assays.
(http://www.pyrosequencing.com/DynPage.aspx?id=7454)

3.13 QUANTITATIVE REAL-TIME PCR (QRT-PCR; PAPERS I, IV, VI)

Allowing both detection and quantification of target sequences, qRT-PCR has become an indispensible tool in contemporary genetic research. This method is often used to assess levels of RNA expression, in which case cDNA (complementary DNA) is first constructed from a pool of mRNA templates trough the action of reverse transcriptase. The use of fluorescent dyes is central for this method, and the real-time measurement of the fluorescence generated during the PCR provides the means for precisely calculating the starting amount of template: throughout the exponential phase of the PCR the amount of newly generated PCR product is proportionate to the starting template amount. Measurements of the generated fluorescence in this stage allow extrapolation of the data back in order to determine the starting quantity of the template. This is done in relation to a serially diluted standard sample, allowing relative comparisons between different tissues. Simultaneous assessment of housekeeping gene expression levels provide a means for normalizing the data. Two common methods exist for generating fluorescence during a qRT-PCR reaction: (1) fluorescent dyes that adhere to double-stranded DNA and then emit light (SYBR-green is representative for this group); and (2) oligonucleotide probes that hybridize between standard PCR primers, and use a fluorescent molecule and a quencher as a reporting system (e.g. TaqMan). In the latter system the fluorescent molecule only emits light after it has been separated from the quencher; i.e. after the 5' exonuclease activity of the Taq degrades the probe during the elongation.
(http://pathmicro.med.sc.edu/pcr/realtime-home.htm)

3.14 SODIUM BISULFITE TREATMENT (PAPERS I-VI)

Sodium bisulfite treatment of DNA is a commonly used method in epigenetic research. Bisulfite chemically induces a sequence difference between methylated and unmethylated DNA by

converting unmethylated cytosines to uracils (ultimately thymidines after PCR). Conversely, methylated cytosine residues (5-methylcytosine) do not react with sodium bisulfite, and the sequence remains unchanged after PCR amplification. This induced sequence difference between methylated and unmethylated DNA can be detected using a variety of techniques, including COBRA, MSP and Pyrosequencing as was done in the current studies.

3.15 STATISTICAL ANALYSIS (PAPERS I-VI)

Statistical calculations were carried out in the Statsoft Inc. STATISTICA software, versions 7 and 8. Additionally, Microsoft Excel was used for simpler computations and data management. In all papers results were considered significant if $p \leq 0.05$. Mann-Whitney U test and Kruskal-Wallis one way analysis of variance was used to compare two- and multiple groups of continuous data respectively. Fischer's exact test was used for comparing categorical data (2X2 test). Spearman rank order correlation was used to evaluate correlations between continuous data sets. In Paper III we used log-rank test and Kaplan-Meier plots to illustrate survival in relation to $p16^{INK4A}$ and RASSF1A promoter methylation.

3.16 WESTERN BLOT ANALYSIS (PAPER I)

Western blot is a technique for detecting the presence of protein expression. This technique employs gel electroporesis to separate proteins in solution. In its most common application Western blot separates proteins based on the length of the polypeptide chain on a denaturing gel, typically containing acrylamide, SDS (sodium dodecyl sulfate) and a buffering solution. SDS is a denaturing detergent that disbands secondary and tertiary protein structures, and supplements the polypeptide with a negative charge proportionate to its molecular weight. When subjected to an electric field the polypeptides migrate through the acrylamide gel matrix towards the positive electrode. Smaller peptides move faster inside the acrylamide mesh, while long polypeptides are retarded. This results in a separation of peptides according to size. After the size separation the proteins are transferred (blotted) onto a nitrocellulose or PVDF (Polyvinylidene fluoride) membrane. A protein staining method, most frequently Ponceau staining, is used to temporarily visualize the proteins transferred to the PVDF membrane. This has the dual purpose of visualizing a successful transfer and giving a reference of loading. Ideally, the level of staining is comparable for all wells, showing that equal amounts of sample was loaded. The membrane is made inert towards unspecific binding of proteins (antibodies) in the subsequent step. This is done through blocking with nonspecific proteins from powdered milk. Next, primary antibodies are added specific towards the protein of interest, followed by addition of secondary antibodies that adhere to the primary antibodies. The secondary antibodies are marked with a reporter molecule, usually

horseradish peroxidase (HRP), that cleaves a chemiluminescent compound, thereby producing luminescence in the vicinity of the antibody binding site. This is recorded by light-sensitive film or in CCD detection systems, and is proportionate to the amount of bound antibody – thereby facilitating quantitative assessment. This is usually followed by the application of a second, hosekeeping gene specific antibody set. Comparisons with housekeeping gene signals allow for normalizing.
(http://www.biology.arizona.edu/immunology/activities/western_blot/w_main.html)

3.17 Z-SCORE CALCULATION (PAPERS II, III, V)

Calculating Z-scores is a statistical tool to normalize and compare groups of variables that might differ from each other by orders of magnitude. It is essentially a means to assign to *each* sample its difference, measured in units of standard deviation, from the population mean. Here it was used to illustrate with continuous values the methylation difference between a given sample and a population mean in a given TSG promoter. A Z-score of 0 would thus indicate that the sample has the same mean methylation as the population average; a Z-score of +2 indicates that the sample has two standard deviations *larger* methylation than the population average. Z-scores are calculated as follows: (mean CpG methylation density of the assessed promoter for each sample – mean of methylation density for the tumor panel)/standard deviation of methylation density of the assessed promoter.

4 RESULTS AND DISCUSSION

4.1 PAPER I: METHYLATION OF THE P16INK4A PROMOTER IS ASSOCIATED WITH MALIGNANT BEHAVIOR IN ABDOMINAL EXTRA-ADRENAL PARAGANGLIOMAS BUT NOT PHEOCHROMOCYTOMAS.

In this paper we aimed to assess whether the TSGs of the CDKN2A locus were involved in human pheochromocytomas and paragangliomas, and whether there was an epigenetic element to their inactivation. This study involved analyses at the RNA, DNA and protein levels. We extracted DNA, RNA and protein from a panel of 55 primary tumors and 2 metastatic lesions. We further used a number of normal specimens for the different experiments: a commercially available pooled sample (normal adrenal pool, NAP) consisting of RNA extracts from 61 normal adrenal glands served as a baseline indicator during the expressional studies; commercially obtained normal adrenal RNA and DNA from 8 distinct individuals provided information about $p16^{INK4A}$ and $p14^{ARF}$ status in normal individuals, and 2 normal adrenals resected at the Karolinska Institute were used to compare p16 potein expression.

Promoter methylation levels in $p16^{INK4A}$ and $p14^{ARF}$ were tested using two separate techniques, MSP and Pyrosequencing. As expected both genes had very low levels of methylation in the 8 normal controls. With complete agreement between MSP and pyrosequencing we found substantial hypermethylation in the $p16^{INK4A}$ promoter of 5 primary tumors and two paired metastases. All the tumors with $p16^{INK4A}$ promoter hypermethylation were paragangliomas, and 4/5 primary tumors were malignant. Aberrant $p16^{INK4A}$ hypermethylation was thus strongly associated with malignant behavior. In two primary tumors methylation was similar to their paired metastases, indicating that this alteration was established prior to the metastatic event. Contrasting $p16^{INK4A}$, methylation levels for $p14^{ARF}$ were overall very low – although initial MSP indicated partial methylation in the promoters of 42 out of 57 tumors a quantitative assessment using Pyrosequencing established that the $p14^{ARF}$ methylation levels are within the same low range as the normal adrenomedullary controls.

$p16^{INK4A}$ and $p14^{ARF}$ expression was assessed using TaqMan qRT-PCR. Serial NAP dilutions provided a standard curve for relative quantification. We normalized our samples against the geometric average of two housekeeping genes, ACTB and B2M. Overall no significant difference was observed in $p16^{INK4A}$ or $p14^{ARF}$ expression between tumor panel and normal adrenal medulla. However, $p16^{INK4A}$ expression was lower in malignant tumors compared to benign tumors and showed a strong trend ($p=0.0502$) to being suppressed in malignant tumors compared to normal

adrenal medulla. This finding underpins the study in knock-out mice where loss of Cdkn2a promoted malignancy (You *et al.* 2002), and indicates that $p16^{INK4A}$ inactivation may be a component in the malignification of human paragangliomas.

After observing promoter hypermethylation in $p16^{INK4A}$ and expressional silencing at the transcriptional level we decided to assess p16 protein expression levels using Western blot. p16 band intensities were measured, and quantified against ACTB. Normal adrenal extracts provided a p16 expression reference for comparison. Since the sensitivity of Western blot is relatively low, arbitrary values were accounted instead of exact figures: reduced (<50% of normal adrenal p16), unaltered (50-150% of normal adrenal p16) and increased (>150% of normal adrenal p16). Expression was lost or reduced in 16 tumors, unaltered in 33, and increased in 7. Reduced p16 expression was reflected by $p16^{INK4A}$ mRNA silencing in all observed cases. However, in some instances where p16 protein and $p16^{INK4A}$ expression did not correlate well; unaltered protein levels were detected despite of silenced mRNA expression. This phenomenon has been documented previously (Gygi *et al.* 1999, Chen *et al.* 2002, Brownhill *et al.* 2007), and can be an effect of varying transcript stability or heterogeneity in the tumor.

$p16^{INK4A}$ was also sequenced to elucidate whether mutation of this gene is an important component in human pheochromocytoma. Four tumors had sequence alterations; one novel missense mutation, and 3 previously characterized SNPs associated with malignant melanoma, breast and lung cancer (https://biodesktop.uvm.edu/perl/p16). However, there was no correlation with malignancy or other tumor features, indicating that while $p16^{INK4A}$ mutations may contribute to pheochromocytoma development they do not commonly promote malignancy.

SDHB mutation predisposes to the familial paraganglioma syndrome 4, which is characterized by malignant paragangliomas (Elder *et al.* 2005). Since SDHB mutation screening was not performed in our tumor series we decided to test all malignant cases, all paragangliomas, and cases with early age of presentation (<30 years) for SDHB mutations. Of the 21 primary tumors thus analyzed, 5 exhibited SDHB mutations, including deletions, frameshift-causing insertions and splice mutations. Most notably, these samples were all paragangliomas, 4/5 were malignant, and all cases with SDHB mutation featured $p16^{INK4A}$ promoter hypermethylation. Thus we linked SDHB mutation to malignancy as well as to silencing of the $p16^{INK4A}$ promoter.

We thus conclude that epigenetic inactivation of $p16^{INK4A}$, but not DNA sequence variations, is a significant event in paraganglioma development, but dispute the involvement of $p14^{ARF}$ in these

tumors. As malignancy in pheochromocytomas and paragangliomas are notoriously difficult to diagnose novel biomarkers are urgently needed. We here report an epigenetic change in a central TSG pathway, and its association with malignancy, that may provide a diagnostic tool as well as a target for future therapies.

Our results are in agreement with observations of $p16^{INK4A}$ hypermethylation and reduced expression (Dammann *et al*. 2005, Muscarella *et a*l. 2008). Our findings corroborate the mouse study that indicated that components of the Cdkn2a gene locus are involved in malignancy (You *et al.* 2002). However, loss of p16/p14 was not enough to prompt pheochromocytoma/paraganglioma development in the mouse model. Thus, to confirm our findings and to further elucidate the epigenetic background in pheochromocytoma and paraganglioma we assessed methylation status in multiple tumor suppressor genes in Paper II and in an expanded tumor series in Paper III.

4.2 PAPER II: GLOBAL AND REGIONAL CPG METHYLATION IN PHEOCHROMOCYTOMAS AND ABDOMINAL PARAGANGLIOMAS: ASSOCIATION TO MALIGNANT BEHAVIOR

Alongside mutation, deletion and rearrangement promoter hypermethylation provides an alternative way to TSG silencing. The phenomenon that aberrant hypermethylation occurs in a coordinated fashion in the promoters of multiple genes was observed some time ago (Toyota *et al.* 1999) and was termed CpG island methylator phenotype, CIMP. In this study we set out to interrogate promoter methylation in a number of TSGs as well as global methylation levels, and put them in relation to clinical characteristics in pheochromocytoma and paraganglioma. 53 primary tumors were included, alongside 2 metastases derived from two of the paired tumors. Using pyrosequencing we quantitatively assessed promoter methylation in a number of TSG promoters. Our results were supplemented with data from Paper I and Paper IV to finally include quantitative methylation data for the following genes: APC, CDH1, DAPK, DCR2, NORE1A, $p14^{ARF}$, $p16^{INK4A}$, PTEN, RARB, RASSF1A and TP73. Promoters were considered methylated if their mean methylation substantially surpassed methylation levels observed in normal adrenal medulla commercially obtained from 4 healthy subjects. For all genes except DCR2 we observed very low levels of promoter methylation in normal samples – thus we regarded samples to be methylated if their mean methylation exceeded 10%. The DCR2 cutoff level was set at 30% since the methylation range reached ≤21% in one normal reference sample. Using these criteria we found promoter hypermethylation of the following genes: APC, CDH1, DCR2, NORE1A,

$p16^{INK4A}$, RARB and RASSF1A. 14 primary tumors had hypermethylation in the promoter of at least one gene – five out of 53 tumors displayed promoter hypermethylation in three or more genes. This concordant hypermethylation was notable, suggested a CpG island methylator phenotype (CIMP). CIMP refers to concerted hypermethylation across several genes, as was first described in colon cancers (Toyota *et al*. 1999). Interestingly, four of the tumors with CIMP were diagnosed as malignant paragangliomas; three had distant metastases. The tumor carrying the CIMP phenotype that did not exhibit malignant features had an unusually early age of presentation; 14 years. Tumors with paired metastases displayed very similar patterns in both primary lesion and metastasis, suggesting that methylation precedes the metastatic spread. CIMP was associated both with malignancy and younger age at presentation, even though malignant tumors as a whole did not present earlier than benign tumors. To further ascertain our observations Z-scores were calculated for all genes that showed hypermethylation in at least one sample: CDH1, DCR2, NORE1A, $p16^{INK4A}$, RARB and RASSF1A. As expected, tumors with CIMP had significantly higher Z-scores than non-CIMP tumors, and Z-scores higher than 0 were associated with malignancy. Global methylation was quantified using two Pyrosequencing-based methods; LINE-1 retrotransposon promoter methylation, and LUMA. Tumors had lower LINE-1 methylation than normal adrenomedullary samples, however, there was no significant difference in methylation between CIMP and non-CIMP tumors. Global hypomethylation of the LINE-1 retrotransposon element therefore seems to be a general element in pheochromoctomas and paragangliomas. Using LUMA we found no difference between tumors and normal samples, nor any correlation between global CCGG methylation levels and particular tumor phenotypes. We also screened our samples for the BRAF mutation GTG→GAG at nucleotide 1799 that causes the V600E missense mutation. This mutation is associated with CIMP in colorectal cancer (Kambara *et al.* 2004), but was absent in our tumor panel. However, when compared with the SDHB mutation analysis in Paper I we found an almost complete overlap – of the four primary tumors with SDHB mutation all exhibited CIMP, all were paragangliomas, and all except one was malignant. Only in one CIMP tumor and its metastasis did we not detect SDHB mutation, although a mutation could still be present in an unsequenced, intronic region.

The current study, in concert with Paper I, suggests that SDHB mutation is associated with concerted hypermethylation in TSG promoters in malignant paragangliomas. This aberrant hypermethylation might be a key contributing factor to the development of malignant paragangliomas. However, a number of questions require further elucidation: A) Is CIMP a cause of malignant transformation, or an effect thereof? The finding of a benign sample with CIMP and SDHB mutation indicates that CIMP precedes malignant spread, further strengthened by the

observation of CIMP in the paired primary tumors and metastases. B) The concerted hypermethylation of several TSGs seem to indicate a disruption of pathways directly involved in DNA methylation. However, it is here linked to mutation of SDHB, the B subunit of the mitochondrial succinate dehydrogenase (SDH) complex – a component of the respiratory chain. Is this an etiological or an incidental link? C) By what mechanisms could SDHB mutation induce promoter-specific methylation in TSGs? We attempt to address some of these issues in Paper III.

In summary we here report the existence of concerted hypermethylation of multiple tumor suppressor genes and a strong association of this epigenetic phenotype with malignant behavior in abdominal paragangliomas. Furthermore, TSG hypermethylation is strongly linked to SDHB mutation. This study suggests the use of demethylating drugs for a subset of paraganglioma patients that display the CIMP phenotype.

4.3 PAPER III: ACQUIRED HYPERMETHYLATION OF THE P16^{INK4A} PROMOTER IN ABDOMINAL PARAGANGLIOMA: RELATION TO ADVERSE TUMOR PHENOTYPE AND PREDISPOSING MUTATION

In Paper II we described concerted hypermethylation of p16^{INK4A}, CDH1, DCR2, ECAD, RASSF1A and RARB, a condition termed CIMP, in a group of paragangliomas. CIMP was strongly associated with SDHB mutation and malignancy. The purpose of the present paper was to corroborate our findings in an independent tumor series, with the added goal of elucidating temporally the chain of events leading up to CIMP manifestation and malignant transformation.

Our expanded tumor series (Series A) consisted of 20 primary tumors and 2 metastases from patients with known or suspected mutations in pheochromocytoma/paraganglioma susceptibility genes. The tumors from Paper II (Series B) were included for comparison.

DNA promoter methylation was quantified in tumors from Series A and matching normal samples from Series B. We assessed TSGs that were significantly hypermethylated in Paper III; p16^{INK4A}, CDH1, DCR2, ECAD, RASSF1A and RARB. A methylation index (MetI) was calculated for each gene for each sample, indicating the average methylation for all assessed CpGs in the gene. Three tumors from Series A fulfilled the criteria for CIMP, i.e. they had MetI above the normal variation in 3 or more genes. Increased methylation at single CpGs was also observed. p16^{INK4A}, DCR2 and RASSF1A were most highly and frequently methylated. Methylation Z-scores were also calculated for all genes in all samples. Mean Z-scores were significantly higher in

metastasizing and malignant samples, in cases with poor outcome and samples with SDHB mutation.

SDHB was sequenced in 12 Series A tumors. 4 tumors with SDHB mutations were found, giving a total of 10 cases with SDHB mutation in Series A+B. Altogether 26 cases from series A+B had a hereditary disease background. The SDHB-related cases had, with very few exceptions, pronounced hypermethylation in $p16^{INK4A}$, DCR2 and RASSF1A – with MetIs above cutoff. Hypermethylation with MetIs above cutoff was also evident for CDH1, NORE1A and RARB. In sharp contrast, none of the NF1/MEN2/VHL-related cases exhibited hypermethylation above cutoff in any of the assessed genes. $p16^{INK4A}$ promoter hypermethylation was very strongly associated to SDHB mutation and short survival. LINE-1 methylation levels in tumors overlapped those of normal adrenal medulla.

We further verified constitutional (non-tumorous) SDHB mutations in DNA from all patients where such samples were available. Further, we assessed methylation levels for the TSGs in non-tumorous DNA in Series B, demonstrating that constitutional SDHB mutation was present prior to tumor formation, and that TSG methylation was an acquired trait in tumors. Taken together with 1p deletions observed in SDHB/CIMP tumors, and the almost complete absence of TSG methylation in non-SDHB-related tumors these data indicated that biallelic loss of SDHB function may be a causative factor in TSG promoter hypermethylation at tumorigenesis.

It was recently shown that SDH loss plays a role in epigenetic alterations through histone modifications (Smith *et al.* 2007, Cervera *et al.* 2009). However, these mechanisms do not seem to account fully for the specific TSG DNA methylation we observe in conjunction with SDHB mutation.

In summary we have here consolidated the co-occurrence of malignant SDHB-related paraganglioma phenotype and concerted TSG DNA hypermethylation. Based on these findings we forsee the use of $p16^{INK4A}$ as an indicator of germline SDHB mutations in patients with paraganglioma, and suggest the investigation of demethylating agents as an adjuvant treatment modality for malignant paragangliomas.

4.4 PAPER IV: THE RAS EFFECTORS NORE1A AND RASSF1A ARE FREQUENTLY INACTIVATED IN PHEOCHROMOCYTOMA AND ABDOMINAL PARAGANGLIOMA

RAS has diverse downstream functions mediated by a variety of effectors. Two such effectors are NORE1A and RASSF1A, both of which have anti-tumorigenic effects. They both integrate in a pathway that mediates apoptosis through MST1 recruitment. While NORE1A is frequently silenced as a result of promoter hypermethylation it is rarely mutated in cancers, and maps to a chromosomal region, 1q32.1, that is rarely lost in pheochromocytomas and paragangliomas. RASSF1A, an authentic TSG, is located at 3p21.3, a region commonly deleted in these tumors, and is often silenced as a result of promoter hypermethylation in pheochromocytomas. The aim of this study was to interrogate the involvement of RASSF1A and NORE1A in chromaffin cell tumors using a panel of 54 primary pheochromocytomas and 2 paired metastases, with emphasis on expression levels, promoter methylation status, and in addition the ability of Nore1a to induce apoptosis and reduce tumorigenic features upon transfection into a rat pheochromocytoma cell line lacking Nore1a expression.

In addition to our tumor panel we used normal adrenal medullary RNA from 8 healthy individuals to provide a baseline for expression. TaqMan qRT-PCR was used to assess NORE1A and RASSF1A mRNA expression levels. We normalized our results against the geometric average of three housekeeping genes, HRPT1, ACTB and B2M, which were stably expressed across the samples. Both NORE1A and RASSF1A expression was substantially reduced in tumors in comparison with normal adrenal medulla. However, we did not find any significant correlation between reduced expression and any particular tumor phenotype, hence we hypothesize that silencing of NORE1A and RASSF1A has a general role in the development of these tumors. Next we wanted to evaluate the extent to which promoter hypermethylation affected NORE1A and RASSF1A mRNA expression in pheochromocytomas and paragangliomas.

NORE1A promoter methylation status was assessed using COBRA. Promoter hypermethylation proved a rare event in pheochromocytomas; only a single metastasis displayed evidence of partial hypermethylation. Its primary tumor was devoid of promoter methylation, indicating that NORE1A promoter hypermethylation is a late-stage event in pheochromocytomas/ paragangliomas. Nore1a was re-introduced into PC12 rat pheochromocytoma cell line to assess its efficacy to act as a TSG in pheochromocytoma by inducing apoptosis and countering anchorage-independent growth. Transfection was through two separate vectors, pcDNAF~Nore1a and GFP~Nore1a, following which cells with the different constructs were analyzed for apoptosis

using FACS flow cytometry and the FLICA assay – and ability to grow anchorage-independently in soft agar. Indeed, reintroduction of Nore1a induced apoptosis in ~20% of the cell population, compared to 0.82% in untransfected cells. This was >2x higher than the apoptotic rate induced by GFP transfection alone. Nore1a re-introduction also markedly reduced anchorage-independent growth, which serves as a good analogue to *in vivo* tumorigenic potential: Nore1a reintroduction via GFP~Nore1a reduced colony formation by 73% (59 vs. 163 colonies), and pcDNAF~Nore1a by 90% (17 vs 163 colonies) when compared to GFP alone. These results indicate that loss of NORE1A in pheochromocytoma cells may reduce the apoptotic ability, and contribute to their neoplastic potential.

Pyrosequencing was used to assess the promoter methylation of RASSF1A in 5 separate CpG sites. Methylation levels were substantially higher in primary malignant tumors than benign tumors, and were also reflected in the two metastases; 5 primary tumors, 4 malignant and one benign, had methylation levels exceeding 20%[2]. In accordance with their primary tumors the two paired metastases also showed substantial promoter hypermethylation, which might suggest that RASSF1A promoter hypermethylation precedes metastasis. Although promoter hypermethylation was accompanied by reduced RASSF1A expression it does not fully account for the silencing we observed across the tumor panel compared to expression in normal adrenal medulla. Likely, other mechanisms are involved, such as LOH – the 3p21.3 region is frequently lost in pheochromocytomas.

In conclusion, both NORE1A and RASSF1A are significantly under-expressed in pheochromocytomas and paragangliomas. Re-introduction of Nore1a into rat pheochromocytoma cell line induced a reversal of its neoplatic features. While promoter hypermethylation is rare in NORE1A, RASSF1A promoter hypermethylation might be a component of the malignification process in these tumors.

[2] For arbitrary reasons the cut-off for RASSF1A hypermethylation was set at 20% in this paper. In later papers, a cutoff of 30% was used, which is well above the range observed in our panel of normal adrenal medullary samples.

4.5 PAPER V: GLOBAL AND GENE-SPECIFIC PROMOTER METHYLATION IN RELATION TO BIOLOGICAL PROPERTIES OF NEUROBLASTOMAS

Concerted aberrant promoter methylation, previously referred to as CIMP, has previously been described in neuroblastomas (Abe *et al.* 2005, 2007). However, many studies assessed CIMP in non-TSG promoters, and few have utilized a quantitative approach for assessing methylation. This concordant hypermethylation may be selected for during tumorigenesis, as the epigenetic inactivation of certain genes may bestow the transforming cells with a growth advantage. Global hypomethylation and gene-specific hypermethylation is a commonly observed phenomenon in cancer (Feinberg and Vogelstein 1983, Esteller 2008), and global methylation patterns has proven to be well analogized by LINE-1 methylation (Yang *et al.* 2004, Weisenberger *et al.* 2005). It has recently been suggested that LINE-1 methylation is inversely correlated with CIMP (Ogino *et al.* 2008), but the conducive factors need further clarification. Encouraged by interesting findings in paragangliomas, another adrenomedullay tumor type, we here undertook the quantitative mapping of promoter methylation in a number of TSGs in neuroblastoma, as well as estimating global methylation levels through assessment of the LINE-1 promoter element.

Our tumor panel consisted of 35 neuroblastomas and 3 ganglioneuromas. Four commercially obtained normal adrenal medullary DNA samples provided normal control. A Pyrosequencing approach was used to assess promoter methylation levels in 14 TSGs; APC, BLU, CASP8, DAPK, DCR2, CDH1, NORE1A, $p14^{ARF}$, $p16^{INK4A}$, p73, PTEN, RARB, RASSF1A, and RASSF2. Furthermore we compared methylation of two separate regions of the CASP8 gene, each implicated in different publications (Teitz *et al.* 2000, Banelli *et al.* 2002). Of the 14 analyzed genes we found 6 that had at least one case with promoter hypermethylation: BLU, CASP8, DCR2, CDH1, RASSF1A and RASSF2. Only these genes were considered for statistical analysis. As previously (Paper II), CIMP was defined as tumors showing TSG hypermethylation in 3 or more genes. Six neuroblastomas fulfilled these criteria. Z-scores were calculated for each gene, and a mean Z-score was computed for each sample. Methylation in CASP8 and RASSF1A proved the most pronounced in our neuroblastoma panel. We found no significant correlation between CIMP and other clinical features, however, mean Z-scores were higher in CIMP tumors. Mean Z-scores were also higher in patients that died of their disease or those who are alive with disease. We found no association between CIMP or CASP8 methylation and MYCN amplification as was indicated in Abe *et al.* (2005, 2006) and Teitz *et al.* (2000) respectively.

LINE-1 promoter methylation was assessed. We found higher levels of methylation in stage 4 tumors. In comparison with previous studies where LINE-1 proved hypomethylated in advanced

tumors these results are unexpected, but corroborate that aberrant epigenetic profile is a feature of neuroblastoma.

The current study provided insight into a debate concerning CASP8. Teitz *et al.* (2000) found methylation in a region close to the CASP8 transcription start site, which they associated with silenced gene expression and MYCN amplification, hypothesizing that the inactivation of the Fas pathway through silencing of CASP8 was necessary in MYCN-amplified tumors. This was later challenged by Benelli *et al.* (2002) who found no correlation of CASP8 silencing together with MYCN amplification, and implicated another region as responsible for gene regulation. Here we assessed both regions, calling the region covered by Teitz *et al.* "CASP8 A1", and the Banelli *et al.* region "CASP8 A2". We thus found that CASP8 A1 is abundantly methylated in neuroblastomas, but not in normal adrenal medulla. Conversely CASP8 A2 methylation is inherently high in normals (on average 36%, vs. 42% in tumors). Therefore, contrasting CASP8 A1, CASP8 A2 methylation proved to be a poor marker for morbidity in neuroblastomas.

Interestingly, in neuroblastoma cell lines we observed extensive methylation in the same genes as we did in our tumor panel. Moreover, the relative methylation was comparable; genes that were highly methylated in cell lines also had high levels of methylation in tumors.

In conclusion – TSG promoter hypermethylation is a prominent feature of neuroblastoma tumors and cell lines. Concerted TSG methylation, CIMP, while common, is not significantly associated with adverse clinical features in neuroblastoma using the criteria here applied.

4.6 PAPER VI: SUPPRESSION OF RIZ IN BIOLOGICALLY UNFAVOURABLE NEUROBLASTOMAS

RIZ is preferentially expressed in neuroendocrine tissues (Buyse *et al.* 1995), and are frequently suppressed in cancer including pheochromocytoma (Geli *et al.* 2005). Reconstitution of RIZ reduced cancer growth in various cell lines and xenograft models (He *et al.* 1998, Chadwick *et al.* 2000) Furthermore RIZ is located at the distal 1p, a region frequently deleted in neuroblastomas. Hence RIZ is an attractive candidate gene for involvement in these tumors. This study had dual purposes: A) to establish whether RIZ1 expression is suppressed in a panel of well-characterized neuroblastomas, and B) to determine whether such a suppression is a result of promoter hypermethylation as was suggested in one of two previous studies (Alaminos *et al.* 2004,

Hoebeeck et al. 2009). The tumor panel was compiled from 33 neuroblastomas and 3 ganglioneuromas, giving a good representation of the Swedish neuroblastoma population with regards to disease phenotype. Furthermore, an array of 7 neuroblastoma cell lines was assessed. Using TaqMan qRT-PCR we characterized mRNA expression levels of RIZ1 and RIZ1+2 – the latter assay targeted both transcripts, since RIZ2 is devoid of uniquely assessable regions. We found that RIZ1 and RIZ1+2 had lower expression in A) tumors with 1p loss than in tumors without such loss; B) tumors with MYCN amplification, C) neuroblastomas classified as INSS Stage 4, and D) in tumors that presented at later age. (These parameters largely overlapped in the series.) Neuroblastoma cell lines had low RIZ1 and RIZ1+2 expressions overall. Pyrosequencing was used to interrogate whether hypermethylation of the RIZ promoter P1 (governing RIZ1) is a factor in RIZ1 silencing. We found that, contrasting other tumor types (Carling *et al.* 2003, Hasegawa *et al.* 2007, Akahira *et al.* 2007) RIZ1 promoter hypermethylation is a rare event in neuroblastomas. Taken together our evidence show that RIZ1 and RIZ1+2 expression is reduced in neuroblastomas with adverse clinical features such as 1p deletion, MYCN amplification and advanced stage. However, this is likely a dose-effect resulting from 1p loss, and a co-occurrence of the other adverse factors. RIZ1 promoter hypermethylation is not a major factor in neuroblastoma development. Our data supports that RIZ1 loss may contribute unfavorably to the neuroblastoma phenotype, perhaps as a composite effect of several deleted genes on 1p.

5 CONCLUSIONS AND FUTURE PROSPECTS

5.1 PAPER I

Epigenetic and expressional silencing of $p16^{INK4A}$ but not $p14^{ARF}$ is evident in human malignant paraganglioma, corroborating results from previous knockout mouse studies. $p16^{INK4A}$ hypermethylation overlaps with SDHB mutation and may serve as a marker for malignancy.

5.2 PAPER II

We describe CIMP, an epigenetic phenotype, in malignant paragangliomas with early onset. CIMP overlaps with $p16^{INK4A}$ hypermethylation and SDHB mutation described in Paper I suggesting a functional link. These findings indicate a use for demethylating agents as adjuvant therapy in the treatment of malignant paragangliomas

5.3 PAPER III

In Paper III we confirm the association between TSG promoter hypermethylation and SDHB mutation in an expanded tumor series. We also present evidence supporting a temporal order of events where constitutional SDHB mutations are followed by allelic loss of SDHB, TSG hypermethylation, tumor formation and subsequent metastasis.

5.4 PAPER IV

Paper IV puts forward evidence that two members of the RASSF family, both with proven tumor suppressive functions, are markedly silenced in pheochromocytoma and paraganglioma. RASSF1A hypermethylation was associated with malignancy. The reconstitution of Nore1a into rat pheochromocytoma cell lines induced apoptosis and reduced anchorage-independent growth, further acknowledging the anti-tumorigenic function of Nore1a in pheochromocytoma.

5.5 PAPER V

In this paper we describe epigenetic involvement in neuroblastomas and find a correlation between increased overall TSG methylation and poor disease outcome. A CIMP phenotype is described in neuroblastomas, but is not unequivocally correlated to adverse tumor features. This contrasts the situation in paragangliomas, and may indicate that TSG promoter hypermethylation is a more general and subtle effect of neuroblastoma formation.

5.6 PAPER VI

RIZ emerges as a possible target tumor suppressor gene in the frequently lost 1p36 chromosomal location in unfavorable neuroblastomas.

5.7 WHERE TO GO NEXT?

Our collective data shows that TSG hypermethylation is a prominent feature of the malignant paraganglioma syndrome characterized by SDH mutations. This raises interesting prospects for future inquiries. Above all, subsequent works on pheochromocytoma/paraganglioma and SDH should strive to elucidate the following:

1. What other TSGs are hypermethylated in SDHB-mutant paragangliomas? Is methylation in conjunction with SDHB-mutation specific for a subset of genes, or more random event targeting multiple genes? These inquiries are best approached by means of methylation arrays. Indeed, such experiments are scheduled in the near future.

2. What histone modifications occur in conjunction with DNA methylation, and what is the temporal and causative relation between histone modifications and DNA methylation? A mechanism has been described where histone modification marks a gene for hypermethylation (Viré *et al.* 2006). This particular mechanism has been implicated in $p16^{INK4A}$ silencing (Bachman et. al. 2003) and may provide a link between the histone modifications associated with SDH abolishment observed by Smith *et al.* 2007 and Cervera *et al.* 2009, and the TSG promoter hypermethylation described in this thesis. ChIP on Chip could prove a viable approach for assessing the epigenetic changes that occur in conjunction with functional inactivation of SDH.

3. Does SDHB mutation/abolishment directly effect DNA methylation? We propose functional studies of the different subunits of the SDH complex in cell line models in combination with pyrosequencing/array techniques, to establish the effects of SDH elimination/reintroduction.

TSG methylation, while present in neuroblastomas, did not have a specific distribution as was evident in paragangliomas. Expanded analyses on multiple genes, preferably on methylation arrays, could elucidate whether specific patterns of promoter methylation do occur in specific subsets of neuroblastoma, or whether it is a random effect of the tumorigenic process.

6 ACKNOWLEDGEMENTS

My supervisors and senior colleagues. Thank you…

Catharina Larsson. In character you parallel Marcus Aurelius, brilliantly combining a sense of tactics with deep wisdom, kindness, and stoic resilience. Your ability to pick out the most significant kernel of information in any given data set and to subtly make your point never ceases to amaze me. You are never too busy to give support or advice, and you seem to make every concern magically disappear. Thank you for your unending cheerfulness and all of your generous endorsement during the last 4,5 years.

János "Guest Star" Geli, my cosupervisor and good friend. Also known as the MD with the Midas Touch, or so the saying goes… ☺ Thanks for great friendship, including thaiboxing- and shisha sessions, talks, ideas, and all the support – technical and otherwise – throughout the years. By the way, I still haven't gotten the recipe for the sajtos csirkepaprikás…

Tomas "Teejay" Ekström, my mentor both on paper and when it comes to epigenetics. You are one of the most approachable professors around, and an inspiration regarding your personal integrity and upfront style. Thank you for many great interactions regardless of topic.

Marin Bäckdahl, for constructive input regarding publications, and your dignified kindness. Thank you also for your support with my grant application!

Anders Höög for most excellent management of samples and prompt responses to all pathology-related questions. Thank you for the splendid histological and macroscopical photographs that grace this thesis!

My nearest cancer research colleagues and office neighbors ("cell mates" :-) Thank you…

Christofer "Chribbs" Juhlin, for keeping my spirit up during long hours of tedious work with hilarious "presents" and profound discussions about everything under the sun (and below the belt). Thank you also for the introduction to Blues music, your broad knowledge about history, literature and medicine, and your "evil genius" whit that made my day so many times. Folks, this man has more strings on his guitar than what most who know him just superficially would believe! Congrats on soon becoming a "fascha"!

Felix "JD" Haglund, AHOA! Joined by our aversion for a certain Capt. B., we understand abstract aspects of life that only a select few have had the privilege (?) to encounter. Thank you for your excellent sense of humor that made me laugh so many times, for many great stories, humongous hamburgers, and for a plethora of other activities in the lab and outside.

Stefano "Steff" Caramuta for keeping a jolly and cheerful atmosphere with entertaining streams of esoteric Italian. You are indeed a true friend and most welcome to visit me in NZ for diving, beach-bumming, beers, or whatever. Best of luck to you, Cecilia and your lovely little baby girl Isabella.

… my eminent summer student **Hedvig Kvanta**, for being a great lab buddy and an overall sharp cookie!

"Co-Larssoners": Thank you…

Svetlana Lagercrantz for being one of the kindest and warmest persons at the Karolinska Institute. **Weng-Onn "King Louie" Lui** for your caring and social personality, and for all the great grant ideas! **Davíd "Quetzalcoatl" Velazques** for excellent wines with funny names and introduction into the subtleties of Mexican cooking (chicken with chocolate and chilli, anyone?). **Jamileh Hashemi** for the homely atmosphere you spread with your presence, and for the unselfish help you give to everyone. **Jia-Jing "Jaded Buddhist" Lee** for rewarding discussions about Life, the Universe and Everything. **Emma "The Lander" Flordal-Thelander** for being a solid rock of calm in a raging sea of lab chaos. **Luqman "Luke" Sulaiman** for your pleasant demeanor and for being a great asset in the lab (+I like your iPhone… ;-). **Andrea "Casablanca" Villablanca** for teaching me that size does matter☺. **Andrei "KGB" Alimov** for objective opinions on subjective labwork, and for inspiring me to "paint". All is forgotten, please come back... **Theodoros "Theus" Foukakis** for being a Greek God in the Dionysian arts of eating and drinking, and for insights into antique culture between lifting sets at the gym. **Mattias "Matte" Berglund**, for the fishing trip that never happened, and for many great chats. **Lasse "Foppa" Forsberg** for being the ultimate lab "Sleeper". **Andrii "S.T.A.L.K.E.R." Dinetz** for your cheerful and relaxed style. **Pinar Akcakaya** for being a promising aspirant in our lab. **Hong Xie** for the excellent dumplings and stimulating discussions. **Kawa "Super Mario" Ali**, for trying to learn aCGH with me. **Anastasios Sofiadis** for interesting lunch discussions. **Dashti Mustafa** for teaching me to appreciate a real Swedish Christmas table… So long y'all!

Oh, I almost forgot **Mr. X**, a.k.a. **"Ollie"**, the labmate that nobody knew. He's out there, somewhere. In the basement… In the bathroom… Waiting… Watching… Anyway, thanks for great stories and side-splitting pranks!

Other CMM colleagues: Thank you…

Caterina Zannin, for your sharp sense of humor and great kindness. You are one of the most generous persons I know, and your company and a few of your stories can lift a dull day like nothing else. A big thank you for all the last- or first minute autoclaves, mid-day pick-me-up chats, and thoughtful sweets that kept my energy high. **Agneta Gunnar**, the true CMM Swiss Army Knife. You solve every arising problem, know the answer to every tricky question, and you're always willing to lend a pen, a post-it, or a smile. **Jonas Lantz** for making stuff work here at CMM. Also a big thank you for many cool conversations on all manner of topics around the lunchroom waterhole: the coffee machine! **Delphi Post** for excellent management of administrative tasks at CMM, and for being a most good-hearted person. **Tzvetomia Philipova** and **Tatiana Yakovleva** for all the help with Western Blots. **Han Solo** for making a great wall ornament while frozen in carbonite. **Yu Ming** for many cheerful corridor greetings, and **Vladana Vukojevic** for your friendly and pleasant style.

Anna-Maria "Mrs. X" Marino, for your good-hearted spirit and mischievous demeanor. I greatly enjoyed our many verbal sparring sessions. Thanks for always being in good mood and having a few friendly words to spare. **Mohsen Karimi**, my brilliant colleague, for many enlightening discussions. I especially appreciated our non-scientific interactions, with topics that ranged from relationships to religion and history, and everything in between. **Sofia Johansson** for introduction into the Alcoholics project (Related Publication #6) and pleasant interactions. **Zahidul Khan** and **Monira Akhtar** for pleasing chats about cricket and research.

Yin-Choi Chuan, The CMM Zorro. This suave guy encouraged us all to "trabaja", while he himself lounged around CMM on the prowl for labmates to harass, and innocent girls to sweep off their feet. Thank you for many great corridor chats, and for your endless good humor. **Mattias "Matte" Vesterlund** for taking good care of "the apple of my eye", the floor 1 developing machine, and for being a very agreeable chap overall! **Hamilcar Flores** for pleasant talks in the CMM hallways. I just noticed: you look exactly like Javier Bardem in No Country For Old Men! "CALL IT!!" **John Flanagan** for arranging the CMM pub with me – one of the definitive highlights during my time at KI. If you think this guy is great on a weekday just wait until he's slammed down a few cold ones! **Louisa Cheung** for expanding my musical horizons by inviting me to Spotify. **Carolina Gustavsson**, the Western blot Lucia, bringing light to many a Hyperfilm™ ECL membranes! Thanks for the introduction to a bunch of techniques and machines. **Roxana Merino Martinez** for very insightful talks about your life and adventures in an obsolete dictatorship. The recognition factor was high. Your appetite for life is inspiring beyond words… **Ananesia "Ani" Andrade** for many entertaining discussions. Our wrangle about "Hillary vs. Barak" is a classic! **"Don" Diego Iglesias** and **Jin Hu** for pleasant chats.

The group of **Thomas Renné**, whom I unfortunately didn't have time to get to know. **Daniel X Johansson** for showing me the ropes (Literally!) when I was new at CMM. Thanks also for splendid climbing sessions, and for teaching me to rappel Aussie style. **Amennai "Amme" Beyeen** for great talks about Kilimanjaro and other faraway places. **Anna "Multisport Guru" Aminoff** for being a good friend since Biomedicine class. **Tianling "Ling-Ling" Wei** for your inviting style and for being a perfect partner for verbal jousts. Just don't drink and drive! **Simone "The Marathon Man" Picelli** for being my running coach, and for making the world's best tiramisu. **Fredrik Lundberg** for a lot of help with sequencing, and many nice chats. **Annika Eriksson** and the other diligent KIGENE people for very pleasing interactions and for fast, and above all *good* results! Special thanks to **Selim Sengul** for excellent lunches and conversations throughout my time at CMM.

The PIs at CMM L8:01: **Lars Terenius**, **Gunnar Nordstedt**, **Clas Johansson**, **Kristina Broliden**, **Mats Persson** and **Per Svenningsson**.

…and (m)any other good people at Circus MaxiMus who I might have forgotten.

The **IT department**: Ehrm… Thank you for your kind and speedy assistance, and for your expert maintenance of the CMM network. Toodaloo lalala!

Colleagues outside CMM: Thank you…

Dan Grandér for an exceptional introduction to cell biology when I still was a Biomedical student, and for many interesting conversations in the gym. **Janne Zedenius** for arranging the excellent Molecular Wine seminar series. **Maria Rogdaki** and **Joëlle Rüegg**: I seem to attract redheads, especially if they have a Pyrosequencing problem! Thanks for many great interactions, even though the pyro results weren't always to par. **Dr. Oliver Twinkle** for insights into the revolutionary industrial aspects of cellular cultivation. **Monica Pettersson** and **Cecilia Johansson** at Qiagen, formerly Biotage. Thank you for patiently and swiftly answering all my inquires about methylation and pyrosequencing while I still was a greenhorn (and a few times afterwards too…). The giggly girls at restaurant MF for making my lunches enjoyable. **Joe dePierre** and **Janet Holmén** for inspiration to "emprov me vrytting an speeling".

My friends: Thank you…

My brothers **Ismael "Smokey" Gritli** & **Dali "Kim Zong Il" Zong**: I have you to thank for a great deal of things, most of which are outside the scope of this thesis… Smokey, amongst many-many other things, big thanks for saving my fanny at the Syrian border. Things might have turned out real ugly that time... Dali, thanks for being my patient roommate pushing 1 ½ years now, and thanks for the introduction to Asian cooking. I can't even begin to thank you for the rest. LJBF forever, gentlemen.

Maya "Fru Troll" Vergara, You are the best (and – when your bloodsugar is low – worst) travel mate a former archaeologist can share GI-related anecdotes with! See you in Norrland around 2045! I'll bring the distillator if you bring them shotguns…

Alexei "Dr. Alex" Sergeiovich Leonov for many vodka-cranberries, insightful discussions and anecdotes about the fairer sex, and a newfound (and totally unrelated) curiosity towards midgets. Above all thank you for the most profound and powerful quote of my life.

Ninib "OG" Baryawno, my "astrological wingman". Thanks buddy for three terrific weeks in Peru. I admire your integrity and your steadfast commitment to whatever enterprises you partake in. (And your plethora of principles – although I wouldn't follow a bunch of 'em… Meeh… ;-)

Davor "Peewee" Petrovic for many great post-exam pizzas, movies and far-reaching talks about electronics, mechanics and biology (Especially XX-phenotypes!). Word 2 yo' motha! ;-)

Betzhabe "Betzy" Chavez for professional jelly shots (you got the hang of it eventually!) and for trailblazing in Peru. That mushroom soup in Cusco was yummy!

Maeh-Long Wang for standing for what's right and real in times of need. Thanks for all the fun. Keep up the hard work dude!

Natalie Luna for arranging the CCK pubs – sorry I couldn't join more!

Tobbe "Biff #2" Rudholm for awesome training sessions – there's no doubt about it – you ARE The Red Hulk!

David "Banner" Sica, **Micke "Oxus" Malmquist**, **Simon "Blondie" Sundberg**, **Leo "Leostenes" Derkert**, **Johan "Oldsmobile" Post**, **Andreas "Pettson" Petterson** and **Ivo "Meloonen" Palm** for good old times, and for motivation in the future!

Special thanks to:

Clive Evans, for having fate in me. Looking forward to a highly interesting and fruitful collaboration, sir!

Inger Andersson, my old kindergarten-teacher, for teaching me proper Swedish. This is a gift that I benefit from every day and that cannot be measured, or thanked enough.

Drs. **Birgit Sköldenberg** and **Anders Hjalmarsson** for medical expertise and unending patience in handling my pre-Antarctic checkups.

Lasse, **Mattias**, **Mattias**, **David**, **Jesper**, **Tobias** and the other good people at SKMC who beat me up regularly over the past 7 years; the arrangers of **Stockholm Toastmasters International**, **My Gudmundsdotter** at Stockholms Improvisatinsteater, **Owen K**, **Dave dA**, **Eric v S**, **Tony R**, and **Ernie E**.

Brothers-in-arms: It was an honor serving with you. Thank you…

Hans-Christian "MacIver" I; dude, for some reason you always remind me of Duke Nukem. We've been through stuff both "back then" and out in the Civilian Darkness. There's no one else I'd rather have keeping my back when the SHTF, and I'm proud to call you a friend. If there are skulls to be bashed or APCs to be fixed, u tha man to call. Thanks for keeping me alive through Basic Training, brother. Best of luck to you and Lojs!

Edmund "Sherminator" S, and his lovely wife Catherine, for teaching me not to judge in advance, for great philosophy sessions, and of course for all the fine champagne. I'll always remember our conversation down by the pier about what's important in life, surrounded by thick puffs of smoke from exceptional Cuban cigars. Ed, you enthuse me with your drive, and your multi-facetted personality and appreciation for the finer things in life. It was a special honor to be your Best Man. May you and Catherine live long and happy, and may at least your kids be lesser MÖP-s!

Tommy "Rez" R, my Master and Commander in all things musical. Thank you for finding the paths to and in NZ, and for the reindeer jerky. Looking back at those days I have especially fond memories from the time when you broke out the smoked elk heart at the barracks one late evening resulting in a veritable feast of dry meat and good stories. Your impervious good humor and easygoing style got me through at times when the weather and snow gave its best... ÅSA February 2011: 39˚09'25.74" S, 175˚37'57.17" W.

Johnny "Ironman" L; dude, did you escape from some US Army research lab, or are you just a cyborg? Ladies and gentlemen, this here individual won all individual squadron PT championships! (Well, except one… ;-) Thank you for great friendship when the going was not-so-great and for inspiration ever after!

* * *

Hélène "Prinsessan" Englund – Others come and go, but you will always be in my heart. Thank you for everything.

* * *

My second family; **Tahani** and **Inger Gritli**, who always treated me with unequalled warmth and generosity. Thank you for great stories, meals, magic and experiences. It is a privilege to call your boy a brother. **Wa'el** and **Iman**, thank you for great friendship, many expertly made "hababababas" and unforgettable memories from Jordan and Petra. Wa'el: \m/ \m/ forever!

Hanga, my personal shopper and legal counselor, it's always fun to have you around, come visit me in NZ!

Ferenc (**"Ferry"**), thank you for your wonderful sense of humor, for lessons in linoleum carving and doghouse designing, as well as many inspiring discussions on a range of topics.

Judit, my aunt and GP all baked into the same person. Many thanks for short-notice medical checkups for a wide spectrum of end goals, including ESA, NSF and so on…

Rebeka, my sweet little sister with an incredible esthetic talent and a heart of gold. When the time comes heed these words: follow it.

Mózsi, my evil twin (or is it the other way around? ;-) We have always chosen different paths, but they might still take us just as far… Wouldn't it be cool if we both ended up to be polar researchers?

Sándor, thanks for help with the homework, for teaching us how to drive, for taking us on vacations throughout Europe… For introduction into music and art, and for many-many other things. In short, for raising us…

My dad **Botond**, for instilling in me a great love for nature and the outdoors, and for giving me that restless spirit that always wants to see what's beyond the next horizon.

My mom **Biborka**, who sacrificed four years without her children, and endured all manners of harassment and persecution in Europe's most severe dictatorship to give us the gift of freedom. Without you I wouldn't have ended up in research.

My grandparents **Zoltan (Tati)** and **Magdalena (Mami)**, you are like parents to me. Thank you for setting me on the right path when I was a kid.

7 REFERENCES

Abe M, Ohira M, Kaneda A, Yagi Y, Yamamoto S, Kitano Y, Takato T, Nakagawara A, Ushijima T. CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. *Cancer Res.* **65**: 828-34. 2005

Abe M, Westermann F, Nakagawara A, Takato T, Schwab M, Ushijima T. Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas. *Cancer Lett.* **247**: 253-8. 2007

Agathanggelou A, Cooper WN, Latif F. Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. *Cancer Res.* **65**: 3497-508. 2005

Aguiar RC, Dahia PL, Sill H, Toledo SP, Goldman JM, Cross NC. Deletion analysis of the p16 tumour suppressor gene in phaeochromocytomas. *Clin Endocrinol (Oxf).* **45**: 93-6. 1996

Akahira J, Suzuki F, Suzuki T, Miura I, Kamogawa N, Miki Y, Ito K, Yaegashi N, Sasano H. Decreased expression of RIZ1 and its clinicopathological significance in epithelial ovarian carcinoma: correlation with epigenetic inactivation by aberrant DNA methylation. *Pathol Int.* **57**: 725-33. 2007

Alison MR, Sarraf CE. Apoptosis: a gene-directed programme of cell death. *J R Coll Physicians Lond.* **26**: 25-35. 1992

Astuti D, Latif F, Dallol A, Dahia PL, Douglas F, George E, Sköldberg F, Husebye ES, Eng C, Maher ER. Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *Am J Hum Genet.* **69**: 49-54. 2001

Auerkari EI. Methylation of tumor suppressor genes p16(INK4a), p27(Kip1) and E-cadherin in carcinogenesis. *Oral Oncol.* **42**: 5-13. 2006

Avruch J, Praskova M, Ortiz-Vega S, Liu M, Zhang XF.Nore1 and RASSF1 regulation of cell proliferation and of the MST1/2 kinases. *Methods Enzymol.* **407**: 290-310. 2006

Bachman KE, Park BH, Rhee I, Rajagopalan H, Herman JG, Baylin SB, Kinzler KW, Vogelstein B. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell.* **3**: 89-95. 2003

Baehrecke EH. How death shapes life during development. *Nat Rev Mol Cell Biol.* **3**: 779-87. 2002

Banelli B, Casciano I, Croce M, Di Vinci A, Gelvi I, Pagnan G, Brignole C, Allemanni G, Ferrini S, Ponzoni M, Romani M. Expression and methylation of CASP8 in neuroblastoma: identification of a promoter region. *Nat Med.* **8**: 1333-5 2002

Bausch B, Borozdin W, Mautner VF, Hoffmann MM, Boehm D, Robledo M, Cascon A, Harenberg T, Schiavi F, Pawlu C, Peczkowska M, Letizia C, Calvieri S, Arnaldi G, Klingenberg-

Noftz RD, Reisch N, Fassina A, Brunaud L, Walter MA, Mannelli M, MacGregor G, Palazzo FF, Barontini M, Walz MK, Kremens B, Brabant G, Pfäffle R, Koschker AC, Lohoefner F, Mohaupt M, Gimm O, Jarzab B, McWhinney SR, Opocher G, Januszewicz A, Kohlhase J, Eng C, Neumann HP; European-American Phaeochromocytoma Registry Study Group. Germline NF1 mutational spectra and loss-of-heterozygosity analyses in patients with pheochromocytoma and neurofibromatosis type 1. *J Clin Endocrinol Metab.* **92**: 2784-92. 2007

Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, van der Mey A, Taschner PE, Rubinstein WS, Myers EN, Richard CW 3rd, Cornelisse CJ, Devilee P, Devlin B. Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science.* **4**: 848-51. 2000

Bedner E, Smolewski P, Amstad P, Darzynkiewicz Z.
Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. *Exp Cell Res.* **259**: 308-13. 2000

Bown N, Cotterill S, Lastowska M, O'Neill S, Pearson AD, Plantaz D, Meddeb M, Danglot G, Brinkschmidt C, Christiansen H, Laureys G, Speleman F, Nicholson J, Bernheim A, Betts DR, Vandesompele J, Van Roy N. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N Engl J Med.* **340**: 1954-61. 1999

Bravo EL, Tagle R. Pheochromocytoma: state-of-the-art and future prospects. *Endocr Rev.* **24**: 539-53. 2003

Breslow N, McCann B: Statistical estimation of prognosis for children with neuroblastoma. *Cancer Res.* **31**: 2098-2103, 1971

Brodeur GM, Seeger RC. Gene amplification in human neuroblastomas: basic mechanisms and clinical implications. *Cancer Genet Cytogenet.* **19**: 101-11. 1986

Brodeur GM, Hayes FA, Green AA, Casper JT, Wasson J, Wallach S, Seeger RC. Consistent N-myc copy number in simultaneous or consecutive neuroblastoma samples from sixty individual patients. *Cancer Res.* **47**: 4248-53. 1987

Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castelberry RP, De Bernardi B, Evans AE, Favrot M, Hedborg F, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol.* **11**: 1466-77. 1993

Brodeur GM, Minturn JE, Ho R, Simpson AM, Iyer R, Varela CR, Light JE, Kolla V, Evans AE. Trk receptor expression and inhibition in neuroblastomas. *Clin Cancer Res.* **15**: 3244-50. 2009

Brownhill SC, Taylor C, Burchill SA. Chromosome 9p21 gene copy number and prognostic significance of p16 in ESFT. *Br J Cancer.* **96**: 1914-23. 2007

Buyse IM, Shao G, Huang S. The retinoblastoma protein binds to RIZ, a zinc-finger protein that shares an epitope with the adenovirus E1A protein. *Proc Natl Acad Sci U S A.* **92**: 4467-71. 1995

Canote R, Du Y, Carling T, Tian F, Peng Z, Huang S. The tumor suppressor gene RIZ in cancer gene therapy. *Oncol Rep.* **9**: 57-60. 2002

Carling T, Du Y, Fang W, Correa P, Huang S. Intragenic allelic loss and promoter hypermethylation of the RIZ1 tumor suppressor gene in parathyroid tumors and pheochromocytomas. *Surgery.* **134**: 932-40. 2003

Cascón A, Ruiz-Llorente S, Rodríguez-Perales S, Honrado E, Martínez-Ramírez A, Letón R, Montero-Conde C, Benítez J, Dopazo J, Cigudosa JC, Robledo M. A novel candidate region linked to development of both pheochromocytoma and head/neck paraganglioma. *Genes Chromosomes Cancer.* **42**: 260-8. 2005

Cervera AM, Bayley JP, Devilee P, McCreath KJ. Inhibition of succinate dehydrogenase dysregulates histone modification in mammalian cells. *Mol Cancer*. **8**: 89. 2009

Chadwick RB, Jiang GL, Bennington GA, Yuan B, Johnson CK, Stevens MW, Niemann TH, Peltomaki P, Huang S, de la Chapelle A. Candidate tumor suppressor RIZ is frequently involved in colorectal carcinogenesis. *Proc Natl Acad Sci U S A.* **97**: 2662-7. 2000

Chen G, Gharib TG, Huang CC, Taylor JM, Misek DE, Kardia SL, Giordano TJ, Iannettoni MD, Orringer MB, Hanash SM, Beer DG. Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics.* **1**: 304-13. 2002

Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M, Wang L, Soda M, Kikuchi A, Igarashi T, Nakagawara A, Hayashi Y, Mano H, Ogawa S. Oncogenic mutations of ALK kinase in neuroblastoma. *Nature.* **455**: 971-4. 2008

Cooper WN, Hesson LB, Matallanas D, Dallol A, von Kriegsheim A, Ward R, Kolch W, Latif F. RASSF2 associates with and stabilizes the proapoptotic kinase MST2. *Oncogene.* **28**: 2988-98. 2009

Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet.* **25**: 315-9. 2000

Dammann R, Schagdarsurengin U, Seidel C, Strunnikova M, Rastetter M, Baier K, Pfeifer GP. The tumor suppressor RASSF1A in human carcinogenesis: an update. *Histol Histopathol.* **20**: 645-63. 2005

Dammann R, Schagdarsurengin U, Seidel C, Trümpler C, Hoang-Vu C, Gimm O, Dralle H, Pfeifer GP, Brauckhoff M. Frequent promoter methylation of tumor-related genes in sporadic and men2-associated pheochromocytomas. Exp Clin *Endocrinol Diabetes.* **113**: 1-7. 2005

DeLellis RA, Lloyd RV, Heitz PU, Eng C. Pathology and genetics of the tumors of endocrine organs. In: *WHO classification of tumors.* Lyon: IARC Press: 135-166. 2006

Dolbeare F. Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part I: Historical perspectives, histochemical methods and cell kinetics. *Histochem J.* **27**: 339-69. 1995

Dolbeare F. Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part II: Oncology, chemotherapy and carcinogenesis. *Histochem J.* **27**: 923-64. 1995

Dolbeare F. Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part III. Proliferation in normal, injured and diseased tissue, growth factors, differentiation, DNA replication sites and in situ hybridization. *Histochem J.* **28**: 531-75. 1996

Dovichi NJ, Zhang J. DNA sequencing by capillary array electrophoresis. *Methods Mol Biol.* **167**: 225-392001

Edström E, Mahlamäki E, Nord B, Kjellman M, Karhu R, Höög A, Goncharov N, Teh BT, Bäckdahl M, Larsson C. Comparative genomic hybridization reveals frequent losses of chromosomes 1p and 3q in pheochromocytomas and abdominal paragangliomas, suggesting a common genetic etiology. *Am J Pathol.* **156**: 651-9. 2000

Edström Elder E, Nord B, Carling T, Juhlin C, Bäckdahl M, Höög A, Larsson C. Loss of heterozygosity on the short arm of chromosome 1 in pheochromocytoma and abdominal paraganglioma. *World J Surg.* **26**: 965-71. 2002

Elder EE, Elder G, Larsson C. Pheochromocytoma and functional paraganglioma syndrome: no longer the 10% tumor. J *Surg Oncol.* **89**: 193-201. 2005

Eng C, Crossey PA, Mulligan LM, Healey CS, Houghton C, Prowse A, Chew SL, Dahia PL, O'Riordan JL, Toledo SP, et al. Mutations in the RET proto-oncogene and the von Hippel-Lindau disease tumour suppressor gene in sporadic and syndromic phaeochromocytomas. J *Med Genet.* **32**: 934-7. 1995

Eng C. Cancer: A ringleader identified. *Nature.* **455**: 883-884. 2008

Esteller M. Epigenetics in cancer. *N Engl J Med.* **358**: 1148-59. 2008

Evans AE, D'Angio GJ, Propert K, Anderson J, Hann HW. Prognostic factor in neuroblastoma. *Cancer.* **59**: 1853-9. 1987

Fang W, Piao Z, Buyse IM, Simon D, Sheu JC, Perucho M, Huang S. Preferential loss of a polymorphic RIZ allele in human hepatocellular carcinoma. *Br J Cancer.* **84**: 743-7. 2001

Feig LA, Buchsbaum RJ. Cell signaling: life or death decisions of ras proteins. *Curr Biol.* **12**: 259-61. 2002

Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature.* **301**: 89-92. 1983

Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun.* **73**: 1907-16. 2005

Frame S, Balmain A. Integration of positive and negative growth signals during ras pathway activation in vivo. *Curr Opin Genet Dev.* **10**: 106-13. 2000

Friedman JM. Epidemiology of neurofibromatosis type 1. *Am J Med Genet.* **89**: 1-6. 1999

Gazzerro P, Abbondanza C, D'Arcangelo A, Rossi M, Medici N, Moncharmont B, Puca GA. Modulation of RIZ gene expression is associated to estradiol control of MCF-7 breast cancer cell proliferation. *Exp Cell Res.* **312**: 340-9. 2006

Gehring M, Berthold F, Edler L, Schwab M, Amler LC. The 1p deletion is not a reliable marker for the prognosis of patients with neuroblastoma. *Cancer Res.* **55**: 5366-9. 1995

Geli J, Nord B, Frisk T, Edström Elder E, Ekström TJ, Carling T, Bäckdahl M, Larsson C. Deletions and altered expression of the RIZ1 tumour suppressor gene in 1p36 in pheochromocytomas and abdominal paragangliomas. *Int J Oncol.* **26**: 1385-91. 2005

Geli J, Kogner P, Lanner F, Natalishvili N, Juhlin C, Kiss N, Clark GJ, Ekström TJ, Farnebo F, Larsson C. Assessment of NORE1A as a putative tumor suppressor in human neuroblastoma. *Int J Cancer.* **123**: 389-94. 2008

Goldmit M, Bergman Y. Monoallelic gene expression: a repertoire of recurrent themes. *Immunol Rev.* **200**: 197-214. 2004

Grovas A, Fremgen A, Rauck A, Ruymann FB, Hutchinson CL, Winchester DP, Menck HR. The National Cancer Data Base report on patterns of childhood cancers in the United States. *Cancer.* **80**: 2321-32. 1997

Gygi SP, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol.* **19**: 1720-30. 1999

Han JS, Boeke JD. LINE-1 retrotransposons: modulators of quantity and quality of mammalian gene expression? *Bioessays.* **27**: 775-84. 2005

Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* **100**: 57-70. 2000

Hao HX, Khalimonchuk O, Schraders M, Dephoure N, Bayley JP, Kunst H, Devilee P, Cremers CW, Schiffman JD, Bentz BG, Gygi SP, Winge DR, Kremer H, Rutter J. SDH5, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma. *Science.* **325**: 1139-42. 2009

Hasegawa Y, Matsubara A, Teishima J, Seki M, Mita K, Usui T, Oue N, Yasui W. DNA methylation of the RIZ1 gene is associated with nuclear accumulation of p53 in prostate cancer. *Cancer Sci.* **98**: 32-6. 2007

He L, Yu JX, Liu L, Buyse IM, Wang MS, Yang QC, Nakagawara A, Brodeur GM, Shi YE, Huang S. RIZ1, but not the alternative RIZ2 product of the same gene, is underexpressed in breast cancer, and forced RIZ1 expression causes G2-M cell cycle arrest and/or apoptosis. *Cancer Res.* **58**: 4238-44. 1998

Hedborg F, Lindgren PG, Johansson I, Kogner P, Samuelsson BO, Bekassy AN, Olsen L, Kreuger A, Påhlman S. N-myc gene amplification in neuroblastoma: a clinical approach using ultrasound guided cutting needle biopsies collected at diagnosis. *Med Pediatr Oncol.* **20**: 292-300. 1992

Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A.* **93**: 9821-6. 1996

Hesson L, Dallol A, Minna JD, Maher ER, Latif F. NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. *Oncogene.* **22**: 947-54. 2003

Hesson LB, Cooper WN, Latif F. The role of RASSF1A methylation in cancer. *Dis Markers.* **23**: 73-87. 2007

Hoebeeck J, Michels E, Pattyn F, Combaret V, Vermeulen J, Yigit N, Hoyoux C, Laureys G, De Paepe A, Speleman F, Vandesompele J. Aberrant methylation of candidate tumor suppressor genes in neuroblastoma. *Cancer Lett.* **273**: 336-46. 2009

Hoehner JC, Hedborg F, Eriksson L, Sandstedt B, Grimelius L, Olsen L, Påhlman S. Developmental gene expression of sympathetic nervous system tumors reflects their histogenesis. *Lab Invest.* **78**: 29-45. 1998

Honorio S, Agathanggelou A, Schuermann M, Pankow W, Viacava P, Maher ER, Latif F. Detection of RASSF1A aberrant promoter hypermethylation in sputum from chronic smokers and ductal carcinoma in situ from breast cancer patients. *Oncogene.* **22**: 147-50. 2003

Hornstein M, Hoffmann MJ, Alexa A, Yamanaka M, Müller M, Jung V, Rahnenführer J, Schulz WA. Protein phosphatase and TRAIL receptor genes as new candidate tumor genes on chromosome 8p in prostate cancer. *Cancer Genomics Proteomics.* **5**: 123-36. 2008

Huang KH, Huang SF, Chen IH, Liao CT, Wang HM, Hsieh LL. Methylation of RASSF1A, RASSF2A, and HIN-1 is associated with poor outcome after radiotherapy, but not surgery, in oral squamous cell carcinoma. *Clin Cancer Res.* **15**: 4174-80. 2009

Huang S, Shao G, Liu L. The PR domain of the Rb-binding zinc finger protein RIZ1 is a protein binding interface and is related to the SET domain functioning in chromatin-mediated gene expression. *J Biol Chem.* **273**: 15933-9. 1998

Huang S. The retinoblastoma protein-interacting zinc finger gene RIZ in 1p36-linked cancers. *Front Biosci.* **4**: D528-32. 1999

Jarbo C, Buckley PG, Piotrowski A, Mantripragada KK, Benetkiewicz M, Diaz de Ståhl T, Langford CF, Gregory SG, Dralle H, Gimm O, Bäckdahl M, Geli J, Larsson C, Westin G, Akerström G, Dumanski JP. Detailed assessment of chromosome 22 aberrations in sporadic pheochromocytoma using array-CGH. *Int J Cancer.* **118**: 1159-64. 2006

Jiang P, Du W, Heese K, Wu M. The Bad guy cooperates with good cop p53: Bad is transcriptionally up-regulated by p53 and forms a Bad/p53 complex at the mitochondria to induce apoptosis. *Mol Cell Biol.* **26**: 9071-82. 2006

Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. *Science.* **293**: 1068-70. 2001

Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet.* **3**: 415-28. 2002

Kaelin WG Jr. SDH5 mutations and familial paraganglioma: somewhere Warburg is smiling. *Cancer Cell.* **16**: 180-2. 2009

Kambara T, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, Barker MA, Arnold S, McGivern A, Matsubara N, Tanaka N, Higuchi T, Young J, Jass JR, Leggett BA. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut.* **53**: 1137-44. 2004

Karagiannis A, Mikhailidis DP, Athyros VG, Harsoulis F. Pheochromocytoma: an update on genetics and management. *Endocr Relat Cancer.* **14**: 935-56. 2007

Karimi M, Johansson S, Ekström TJ. Using LUMA: a Luminometric-based assay for global DNA-methylation. *Epigenetics.* **1**: 45-8. 2006

Katzenstein HM, Bowman LC, Brodeur GM, Thorner PS, Joshi VV, Smith EI, Look AT, Rowe ST, Nash MB, Holbrook T, Alvarado C, Rao PV, Castleberry RP, Cohn SL. Prognostic significance of age, MYCN oncogene amplification, tumor cell ploidy, and histology in 110 infants with stage D(S) neuroblastoma: the pediatric oncology group experience--a pediatric oncology group study. *J Clin Oncol.* **16**: 2007-17. 1998

Kazazian HH Jr, Goodier JL. LINE drive. retrotransposition and genome instability. *Cell.* **110**: 277-80. 2002

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer.* **26**: 239-57. 1972

Khokhlatchev A, Rabizadeh S, Xavier R, Nedwidek M, Chen T, Zhang XF, Seed B, Avruch J. Identification of a novel Ras-regulated proapoptotic pathway. *Curr Biol.* **12**: 253-65. 2002

Kim KC, Geng L, Huang S. Inactivation of a histone methyltransferase by mutations in human cancers. *Cancer Res.* **63**: 7619-23. 2003

Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A.* **68**: 820-3. 1971

Knudson AG. Hereditary cancer: two hits revisited. J Cancer Res *Clin Oncol.* **122**: 135-40. 1996

Knudson AG. Two genetic hits (more or less) to cancer. *Nat Rev Cancer.* **1**: 157-62. 2001

Kreiner E. Weight and shape of the human adrenal medulla in various age groups. *Virchows Arch A Pathol Anat Histol.* **397**: 7-15. 1982

Kusy S, Larsen CJ, Roche J. p14ARF, p15INK4b and p16INK4a methylation status in chronic myelogenous leukemia. *Leuk Lymphoma.* **45**: 1989-94. 2004

Lack EE. Tumors of the adrenal gland and extra-adrenal paraganglia. Washington D.C.: *Armed Forces Institute of Pathology.* 1997

Langman, Jan. Medical embryology. *Williams & Wilkins.* 349-353, 1981.

Łastowska M, Cotterill S, Bown N, Cullinane C, Variend S, Lunec J, Strachan T, Pearson AD, Jackson MS. Breakpoint position on 17q identifies the most aggressive neuroblastoma tumors. *Genes Chromosomes Cancer.* **34**: 428-36. 2002

Lenders JW, Eisenhofer G, Mannelli M, Pacak K. Phaeochromocytoma. *Lancet.* **366**: 665-75. 2005

Liao X, Siu MK, Chan KY, Wong ES, Ngan HY, Chan QK, Li AS, Khoo US, Cheung AN. Hypermethylation of RAS effector related genes and DNA methyltransferase 1 expression in endometrial carcinogenesis. *Int J Cancer.* **123**: 296-302. 2008

Liu X, Yue P, Khuri FR, Sun SY. Decoy receptor 2 (DcR2) is a p53 target gene and regulates chemosensitivity. *Cancer Res.* **65**: 9169-75. 2005

Lodish MB, Stratakis CA. RET oncogene in MEN2, MEN2B, MTC and other forms of thyroid cancer. *Expert Rev Anticancer Ther.* **8**: 625-32. 2008

London WB, Castleberry RP, Matthay KK, Look AT, Seeger RC, Shimada H, Thorner P, Brodeur G, Maris JM, Reynolds CP, Cohn SL. Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. *J Clin Oncol.* **23**: 6459-65. 2005

Lowe SW, Lin AW. Apoptosis in cancer. *Carcinogenesis.* 21: 485-95. 2000

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol.* **8**: 741-52. 2007

Margetts CD, Astuti D, Gentle DC, Cooper WN, Cascon A, Catchpoole D, Robledo M, Neumann HP, Latif F, Maher ER. Epigenetic analysis of HIC1, CASP8, FLIP, TSP1, DCR1, DCR2, DR4, DR5, KvDMR1, H19 and preferential 11p15.5 maternal-allele loss in von Hippel-Lindau and sporadic phaeochromocytomas. *Endocr Relat Cancer.* **12**: 161-72. 2005

Marini F, Falchetti A, Del Monte F, Carbonell Sala S, Tognarini I, Luzi E, Brandi ML. Multiple endocrine neoplasia type 2. *Orphanet J Rare Dis.* **1**: 45. 2006

Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *Lancet.* **369**: 2106-20. 2007

Marsh DJ, Mulligan LM, Eng C. RET proto-oncogene mutations in multiple endocrine neoplasia type 2 and medullary thyroid carcinoma. *Horm Res.* **47**: 168-78. 1997

Martinsson T, Sjöberg RM, Hedborg F, Kogner P. Deletion of chromosome 1p loci and microsatellite instability in neuroblastomas analyzed with short-tandem repeat polymorphisms. *Cancer Res.* **55**: 5681-6. 1995

Martinsson T, Sjöberg RM, Hallstensson K, Nordling M, Hedborg F, Kogner P. Delimitation of a critical tumour suppressor region at distal 1p in neuroblastoma tumours. *Eur J Cancer.* **33**: 1997-2001. 1997

Matsuda Y. Molecular mechanism underlying the functional loss of cyclindependent kinase inhibitors p16 and p27 in hepatocellular carcinoma. *World J Gastroenterol.* **14**: 1734-40. 2008

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature.* **399**: 271-5. 1999

Michalowski MB, de Fraipont F, Plantaz D, Michelland S, Combaret V, Favrot MC. Methylation of tumor-suppressor genes in neuroblastoma: The RASSF1A gene is almost always methylated in primary tumors. *Pediatr Blood Cancer.* **50**: 29-32. 2008

Morozova O, Marra MA. Applications of next-generation sequencing technologies in functional genomics. *Genomics.* **92**: 255-64. 2008

Moshnikova A, Frye J, Shay JW, Minna JD, Khokhlatchev AV. The growth and tumor suppressor NORE1A is a cytoskeletal protein that suppresses growth by inhibition of the ERK pathway. *J Biol Chem.* **281**: 8143-52. 2006

Mossé YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, Laquaglia MJ, Sennett R, Lynch JE, Perri P, Laureys G, Speleman F, Kim C, Hou C, Hakonarson H, Torkamani A, Schork NJ, Brodeur GM, Tonini GP, Rappaport E, Devoto M, Maris JM. Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature.* **455**: 930-5. 2008

Müller U, Troidl C, Niemann S. SDHC mutations in hereditary paraganglioma/pheochromocytoma. *Fam Cancer.* **4**: 9-12. 2005

Muscarella P, Bloomston M, Brewer AR, Mahajan A, Frankel WL, Ellison EC, Farrar WB, Weghorst CM, Li J. Expression of the p16INK4A/Cdkn2a gene is prevalently downregulated in human pheochromocytoma tumor specimens. *Gene Expr.* **14**: 207-16. 2008

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell.* **85**: 817-27. 1996

Nagasaka T, Tanaka N, Cullings HM, Sun DS, Sasamoto H, Uchida T, Koi M, Nishida N, Naomoto Y, Boland CR, Matsubara N, Goel A. Analysis of fecal DNA methylation to detect gastrointestinal neoplasia. J *Natl Cancer Inst.* **101**: 1244-58. 2009

Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Altehoefer C, Zerres K, Januszewicz A, Eng C, Smith WM, Munk R, Manz T, Glaesker S, Apel TW, Treier M, Reineke M, Walz MK, Hoang-Vu C, Brauckhoff M, Klein-Franke A, Klose P, Schmidt H, Maier-Woelfle M, Peçzkowska M, Szmigielski C, Eng C; Freiburg-Warsaw-Columbus Pheochromocytoma Study Group. Germ-line mutations in nonsyndromic pheochromocytoma. *N Engl J Med.* **346**: 1459-66. 2002

Ogino S, Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, Fuchs CS. LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Int J Cancer.* **122**: 2767-73. 2008

Opocher G, Conton P, Schiavi F, Macino B, Mantero F. Pheochromocytoma in von Hippel-Lindau disease and neurofibromatosis type 1. *Fam Cancer.* **4**: 13-6. 2005

Ozdağ H, Teschendorff AE, Ahmed AA, Hyland SJ, Blenkiron C, Bobrow L, Veerakumarasivam A, Burtt G, Subkhankulova T, Arends MJ, Collins VP, Bowtell D, Kouzarides T, Brenton JD, Caldas C. Differential expression of selected histone modifier genes in human solid cancers. *BMC Genomics.* **7**: 90. 2006

Payne SR, Kemp CJ. Tumor suppressor genetics. *Carcinogenesis.* **26**: 2031-45. 2005

Pearse AM, Swift K. Allograft theory: transmission of devil facial-tumour disease. *Nature.* **439**: 549. 2006

Plantaz D, Vandesompele J, Van Roy N, Lastowska M, Bown N, Combaret V, Favrot MC, Delattre O, Michon J, Bénard J, Hartmann O, Nicholson JC, Ross FM, Brinkschmidt C, Laureys G, Caron H, Matthay KK, Feuerstein BG, Speleman F. Comparative genomic hybridization (CGH) analysis of stage 4 neuroblastoma reveals high frequency of 11q deletion in tumors lacking MYCN amplification. *Int J Cancer.* **91**: 680-6. 2001

Praskova M, Khoklatchev A, Ortiz-Vega S, Avruch J. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. *Biochem J.* **381**: 453-62. 2004

Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell.* **83**: 993-1000. 1995

Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol.* **5**: 897-907. 2004

Robinson, B. The Abdominal and Pelvic Brain. Hammond, Ind.: *Frank S. Betz,* 1907.

Schulz WA, Steinhoff C, Florl AR. Methylation of endogenous human retroelements in health and disease. *Curr Top Microbiol Immunol.* **310**: 211-50. 2006

Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N Engl J Med.* **313**: 1111-6. 1985

Sekulic A, Haluska P Jr, Miller AJ, Genebriera De Lamo J, Ejadi S, Pulido JS, Salomao DR, Thorland EC, Vile RG, Swanson DL, Pockaj BA, Laman SD, Pittelkow MR, Markovic SN; Melanoma Study Group of Mayo Clinic Cancer Center. Malignant melanoma in the 21st century: the emerging molecular landscape. *Mayo Clin Proc.* **83**: 825-46. 2008

Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature.* **366**: 704-7. 1993

Sharpless NE, DePinho RA. The INK4A/ARF locus and its two gene products. *Curr Opin Genet Dev*. **9**: 22-30. 1999

Sharpless NE. INK4a/ARF: a multifunctional tumor suppressor locus. *Mutat Res.* **576**: 22-38. 2005

Shivapurkar N, Toyooka S, Toyooka KO, Reddy J, Miyajima K, Suzuki M, Shigematsu H, Takahashi T, Parikh G, Pass HI, Chaudhary PM, Gazdar AF. Aberrant methylation of trail decoy receptor genes is frequent in multiple tumor types. *Int J Cancer.* **109**: 786-92. 2004

Sibal L, Jovanovic A, Agarwal SC, Peaston RT, James RA, Lennard TW, Bliss R, Batchelor A, Perros P. Phaeochromocytomas presenting as acute crises after beta blockade therapy. *Clin Endocrinol (Oxf)* . **65**: 186-90. 2006

Smilenov LB. Tumor development: haploinsufficiency and local network assembly. *Cancer Lett.* **240**: 17-28, 2006

Smith EH, Janknecht R, Maher LJ 3rd. Succinate inhibition of alpha-ketoglutarate-dependent enzymes in a yeast model of paraganglioma. *Hum Mol Genet.* **16**: 3136-48. 2007

Sobotta J Atlas of human anatomy / Vol. 3, Central nervous system, autonomic nervous system, sense organs and skin, peripheral nerves and vessels. München: *Urban & Schwarzenberg*. 1975

Sutton MG, Sheps SG, Lie JT. Prevalence of clinically unsuspected pheochromocytoma. Review of a 50-year autopsy series. *Mayo Clin Proc.* **56**: 354-60. 1981

Takita, J. et al. Allelic imbalance on chromosome 2q and alterations of the caspase 8 gene in neuroblastoma. *Oncogene.* **20**: 4424–4432. 2001

Teitz T, Wei T, Valentine MB, Vanin EF, Grenet J, Valentine VA, Behm FG, Look AT, Lahti JM, Kidd VJ. Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nat Med.* **6**: 529-35. 2000

Teitz, T. , Lahti, J. M. & Kidd, V. J. Aggressive childhood neuroblastomas do not express caspase-8: an important component of programmed cell death. *J. Mol. Med.* **79**: 428–436. 2001

Tommasi S, Dammann R, Jin SG, Zhang XF, Avruch J, Pfeifer GP. RASSF3 and NORE1: identification and cloning of two human homologues of the putative tumor suppressor gene RASSF1. *Oncogene.* **21**: 2713-20. 2002

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A.* **96**: 8681-6. 1999

Uchida T, Kinoshita T, Saito H, Hotta T. CDKN2 (MTS1/p16INK4A) gene alterations in hematological malignancies. *Leuk Lymphoma.* **24**: 449-61. 1997

Viré E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit Y, Fuks F. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature.* **439**: 871-4. 2006

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med.* **319**: 525-32. 1988

Vos MD, Martinez A, Ellis CA, Vallecorsa T, Clark GJ. The pro-apoptotic Ras effector Nore1 may serve as a Ras-regulated tumor suppressor in the lung. *J Biol Chem.* **278**: 21938-43. 2003

Weinberg, RA. The biology of cancer. New York: *Garland Science.* 2005

Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW. Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res.* **33**: 6823-36. 2005

White PS, Maris JM, Beltinger C, Sulman E, Marshall HN, Fujimori M, Kaufman BA, Biegel JA, Allen C, Hilliard C, et al. A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3. *Proc Natl Acad Sci U S A.* **92**: 5520-4. 1995

Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol.* **68**: 251-306. 1980

Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* **25**: 2532-4. 1997

Yang AS, Estécio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res.* **32**: 38. 2004

Yang Q, Kiernan CM, Tian Y, Salwen HR, Chlenski A, Brumback BA, London WB, Cohn SL. Methylation of CASP8, DCR2, and HIN-1 in neuroblastoma is associated with poor outcome. *Clin Cancer Res.* **13**: 3191-7. 2007

You MJ, Castrillon DH, Bastian BC, O'Hagan RC, Bosenberg MW, Parsons R, Chin L, DePinho RA. Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice. *Proc Natl Acad Sci U S A.* **99**: 1455-60. 2002

Young LS, Dawson CW, Eliopoulos AG. Viruses and apoptosis. Br Med Bull.;**53**: 509-21. 1997

http://pathmicro.med.sc.edu/pcr/realtime-home.htm

http://www.biology.arizona.edu/immunology/activities/western_blot/w_main.html

http://www.invitrogen.com/site/us/en/home/support/ Tutorials.html

http://www.pyrosequencing.com/DynPage.aspx?id=7454