From 1p3 to PI3K

Studies of Neuroblastoma

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Gothenburg, Sweden, 2011

Cover image: Ectotopically expressed p37δ (dsRED-tagged) co-localizes with p85α (GFP-tagged) in cytoplasmatic speckles.

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ISBN: 978-91-628-8197-9
E-published: http://hdl.handle.net/2077/23820

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Printed by Geson Hylte Tryck, Göteborg, Sweden 2011

To my family

Cancer: The final frontier
These are the voyages of a PhD student
Its 4 year mission
To explore strange new splice variants
To seek out new genes and new pathways
To boldly go where no one has gone before

Adapted from Star Trek™

ABSTRACT

From 1p3 to PI3K
Studies of Neuroblastoma

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Neuroblastoma (NB) is a tumor of the sympathetic nervous system and is the most common extra-cranial tumor of childhood, accounting for 7% of all pediatric malignancies. Despite recent advances in therapeutics, outcome is still fatal for patients with aggressive NB and side-effects of treatment are severe. These are important reasons to gain further knowledge of the biology behind NB.

Aims: The objective of this thesis was to explore genes and gene products that might contribute to initiation and progression of NB and possibly also other malignancies. Main focus has been on the chromosomal region 1p36.2-3 and participants of the PI3K/Akt signaling pathway.
Results: Real-time expression analysis of 30 genes at 1p36.2-3 showed that *TNFRSF9* and *PIK3CD* were down regulated in 1p-deleted compared to non-deleted NB tumors. Studies of the same region showed four genes (*ERRFI*, *CASZ1*, *RBP7* and *PIK3CD)* possibly regulated by epigenetically means. Bisulphite sequencing of these four genes in NB cell lines and primary tumors showed that methylation probably is not involved but that histone deacetylation could be implicated in their regulation. Some rare sequence variants were also identified in *ERRFI* and *PIK3CD*. *PIK3CD* encodes a catalytic subunit of the phosphatidylinositol 3-kinase (PI3K) that is involved in activation of Akt. Analysis of mRNA levels in a set of 88 genes associated to PI3K/Akt signaling showed that *PDGFRA, PIK3R1, PIK3CD, PRKCBI, PRKCZ* and *EIF4EBP1* were differentially expressed comparing stage 1-2 to stage 4 NB. At the protein level a stage-dependent expression of the different catalytic isoforms were detected, where levels of p110α were higher in stage 4 tumors compared to stage 1-2, while the opposite was seen for p110δ. Stage 4 NB also had higher levels of phosphorylated Akt (T308 and S473) compared to low stage NB. Furthermore, levels of phosphorylated Akt T308 showed inverse correlation to protein levels of the tumor suppressor Pten.
We have also identified a novel splice variant p37δ, encoded by *PIK3CD*. Usage of an alternative donor site leads to truncation in the RAS-binding domain and loss of the catalytic domain. Despite the truncation, p37δ interact with RAS and there is a strong correlation between protein levels of p37δ and RAS in primary cells. Expression of p37δ is increased in human cancers of the ovaries and colon and ubiquitous expression of the human p37δ in *Drosophila* increased the body size of the fly. Furthermore, over-expression of p37δ in HEK-293 and mouse embryonic fibroblasts increased proliferation and invasive properties compared to controls, indicating a role in tumorgenicity.
Conclusion: Analysis of expression levels of genes and proteins could be used for pinpointing important genes and pathways. This thesis has added more knowledge about the genes at 1p36.2-3, a region commonly deleted in NB, as well as the PI3K/Akt signaling in NB. We have also described a new splice variant of p110δ that is expressed in human cancer and increases proliferation *in vitro* and *in vivo*.

Keywords; cancer, tumor, neural crest, mucosa, premalignant, neuroblastoma, tumor suppressor gene, oncogene, gene expression, epigenetics, splicing, signaling, 1p36, PI3K, Akt, Western blot, TaqMan, *PIK3CD*, *PIK3R1*, p110δ, p37δ, p85, RAS

LIST OF PAPERS

This thesis is based on the following papers that will be referred to in the text by their roman numerals:

I. **Fransson S**, Martinsson T & Ejeskär K. Neuroblastoma tumors with Favorable and Unfavorable outcome: Significant differences in mRNA expression of Genes Mapped at 1p36.2. *Genes, Chromosomes and Cancer (2007) 46:45-52*

II. Carén H, **Fransson S**, Ejeskär K, Sjöberg R-M, Kogner P & Martinsson T. Genetic and epigenetic changes in the common 1p36 deletion in neuroblastoma tumors. *Br j Cancer (2007) 19;97(10):1416-24*

III. **Fransson S**, Abel, F, Eriksson H, Kogner P, Martinsson T & Ejeskär K. Analysis of the PI3K/Akt signaling pathway in Neuroblastoma – Stage dependent expression of PI3K p110 isoforms. *(2011) Submitted*

IV. **Fransson S**, Uv A, Eriksson H, Andersson M K, Wettergren Y, Bergö M, & Ejeskär K. p37delta, a new isoform of PI3K p110delta that increases cell proliferation, is over expressed in human tumors. *(2011) Submitted*

OTHER RELEVANT PUBLICATIONS

These papers are relevant but not included in this thesis:

Carén H, Ejeskär K, **Fransson S**, Sjöberg R-M, Krona C, Hesson L, Latif F & Martinsson T.
A Cluster of 1p36 located genes are down-regulated in neuroblastomas with poor prognosis, but not due to CpG island methylation.
Molecular Cancer (*2005) Mar 1;4(1):10*

Ejeskär K, **Fransson S**, Zaibak F & Ioannou P.
Method for efficient transfection of *in vitro*-transcribed mRNA into SK-N-AS and 293 cells; Difference in the toxicity of nuclear EGFP compared to cytoplasmic EGFP.
Int J Mol Med. (2006) Jun;17(6):1011-6

CONTENT

ABBREVIATIONS

5´-aza-dC	5-Aza-2-deoxycytedine
BGS	bovine growth serum
bp	base pair
cDNA	complementary DNA
CNS	central nervous system
CNV	copy number variants
CpG	cytosine-guanine dinucleotide
da	daughterless
ddNTP	dideoxynucleotides
DNA	deoxyribonucleic acid
DOD	dead of disease
EGF	epidermal growth factor
ERK	extracellular receptor-stimulated kinase
GF	growth factor
GN	ganglioneuroma
GNB	ganglioneuroblastoma
GUSB	β-glucuronidase
HDAC	histone deacetylase
INRG	International neuroblastoma risk group
INSS	International neuroblastoma staging system
kb	kilobases (1000bp)
LOH	loss of heterozygogosity
MAPK	mitogen-activated protein kinase
MEF	mouse embryonal fibroblasts
mRNA	messenger RNA
MSP	methylation-specific PCR
NB	neuroblastoma
NED	no evidence of disease
NGF	nerve growth factor
NIH	national institute of health
ORF	open reading frame
PCR	polymerase chain reaction
PEST	penicillin/Streptomycin
PI3K	phosphoinositide 3-kinase
PIP_3	phosphatidylinositol 3,4,5-triphosphate
RA	retinoic acid
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription PCR
S473	serine 473
siRNA	short interfening RNA
SNP	single-nucleotide polymorphism
SNS	sympathetic nervous system/sympatiska nervsystemet
SRO	smallest region of overlap
T308	threonine 308
Trk	tropomyosin receptor kinase
TSA	trichostatin A
TSG	tumor suppressor gene
UAS	upstream activator sequence
WB	western blot
wt	wild type

INTRODUCTION

DNA and genes

The genome is the entire hereditary information of an organism and genetics is the study of it. The genome is built by DNA (deoxyribonucleic acid) or in the case of some viruses, by RNA (ribonucleic acid). In eukaryotes, DNA is found in the chromosomes of the nucleus, in the mitochondria and also in the chloroplasts of plants. The DNA serves as long term storage of information and is built by four different nucleotides; adenine (A), cytosine (C), guanine (G) and thymine (T). The usage of different combinations of these bases makes the genetic code. The chemical composition of RNA is similar to DNA but differ in the usage of ribose instead of deoxyribose and the usage of the nucleotide uracil (U) instead of thymine. The DNA is formed as a double helix held together by complementary hydrogen bonds between A-T and C-G base pairs and was first described by Watson and Crick in 1953 [1]. The human genome consists of more than 3 billion base pairs (bp), organized in 23 chromosome pairs (Figure 1).

A gene is a stretch of DNA that codes for a protein or a RNA-chain with biological function in the organism. In the classical view, the gene is composed of exons and introns where the exons code for the amino acid sequence and the introns are spliced off during RNA processing of the transcript. The promoter region constitutes the regulatory region in the 5'-region of the gene where transcription factors binds and directs the transcription. To this adds other regions that also affect transcription activity, these can be located far away from the gene and binds enhancers or silencers. However, the presence of non-coding RNA (ncRNA) and conserved regions outside the genes that perform functions, has challenged the concept of the gene. According to the official Guidelines for Human Gene Nomenclature a gene is currently defined as "a DNA segment that contributes to phenotype or function. In the absence of demonstrated function, a gene may be characterized by sequence, transcription or homology".

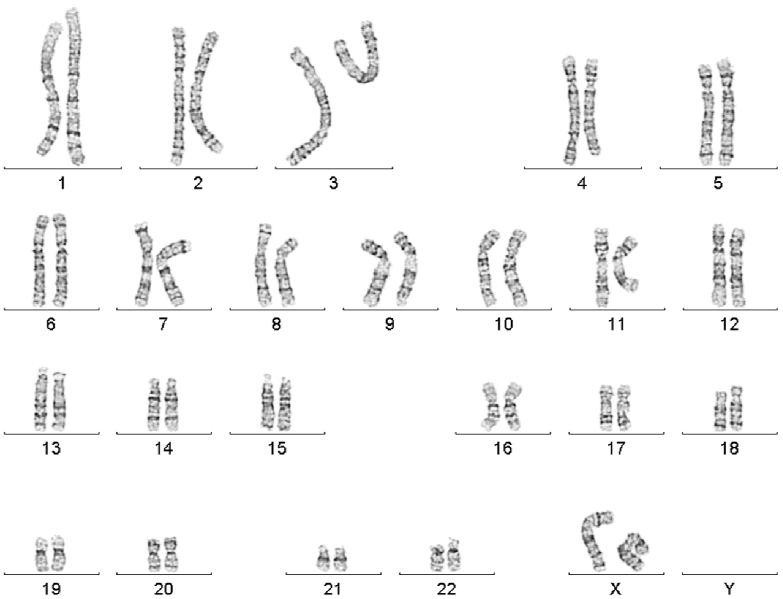

Figure 1. *A human female karyotype showing 23 chromosome pairs. Image kindly provided by Kirsten Schultz, Dept. Clinical genetics, SU/Sahlgrenska*

A first calculation of the number of genes in the human genome was presented in 1964 by Vogel who made an estimation of 6.7 million genes based on the molecular weight of an average gene and the weight of the chromosomes [2]. This was however before the knowledge that only 1-2 % of the genome is coding for proteins and the rest encodes non-translated RNA or constitutes regulatory sequences, introns, repetitive sequences, pseudogenes and DNA with (at the moment) unknown function. In 1990 National Institute of Health (NIH) presented a preliminary report on the Human Genome Project giving an estimate of 100 000 genes. Today the exact number of genes is still not known despite 99% coverage of the gene-coding parts of the genome, but approximately only a humble 23 000 protein coding genes are dispersed over our chromosomes. However, increased diversity of the proteome can be established by mechanisms such as post-translational modification or alternative splicing where one gene is the source for different transcripts. Another dimension of complexity is added through specific expression of the genes depending on cell type, tissue and also time of expression.

The central dogma

Proteins are the connection between genotype (genetic constitution) and phenotype (observed trait) in organisms. The route from DNA to protein is mediated by messenger RNA (mRNA) and has been described as the central dogma (Figure 2) [3]. The DNA serves as blueprint for the RNA-polymerase that transcribes the gene sequence to pre-mRNA. The pre-mRNA is processed to a mature mRNA through splicing, addition of a 5' protective cap and polyadenylation at the 3' end. Following post-transcriptional processing, the mRNA migrates from the nucleus to the cytoplasm where it engages ribosomes and other components in the translation of mRNA to a protein. The protein chain is composed of different amino acids and a group of three nucleotides, a codon in the RNA, is used to specify individual amino acids or the stop signals used for termination of translation (Table 1). To become a fully functional protein the amino acid chain folds up into a unique three-dimensional configuration and also possibly undergoes post-translational modification (e.g. covalent attatchment of chemical groups, protease cleavage or building of disulphide bridges).

Table 1. *The codons, corresponding amino acids and their one-letter code*

U			C			A			G			← First base / Second base ↓
UUU	Phenylalanine	F	CUU	Leucine	L	AUU	Isoleucine	I	GUU	Valine	V	**U**
UUC			CUC			AUC			GUC			
UUA	Leucine	L	CUA			AUA			GUA			
UUG			CUG			AUG	Methionine	M	GUG			
UCU	Serine	S	CCU	Proline	P	ACU	Threonine	T	GCU	Alanine	A	**C**
UCC			CCC			ACC			GCC			
UCA			CCA			ACA			GCA			
UCG			CCG			ACG			GCG			
UAU	Tyrosine	Y	CAU	Histidine	H	AAU	Aspargine	N	GAU	Aspartic acid	D	**A**
UAC			CAC			AAC			GAC			
UAA	Stop	X	CAA	Glutamine	Q	AAA	Lysine	K	GAA	Glutamic acid	E	
UAG			CAG			AAG			GAG			
UGU	Cysteine	C	CGU	Argenine	R	AGU	Serine	S	GGU	Glycine	G	**G**
UGC			CGC			AGC			GGC			
UGA	Stop	X	CGA			AGA	Argenine	R	GGA			
UGG	Tryptophan	W	CGG			AGG			GGG			

Figure 2. *A schematic description of the flow from gene to protein in the cell. The DNA is transcribed to pre-mRNA that undergoes RNA processing in form of splicing, 5' capping and 3' polyadenylation in order to stabilize the mRNA. The mature mRNA is transported out of the nucleus and translated in to proteins by ribosomes. The proteins can then undergo further post-translational modifications in form of building of disulphide bridges, or addition of functional groups (e.g. phosphorylation, myristoylation or glycosylation).*

The splicing process

The RNA transcript of eukaryotic genes undergoes processing that often involves removal of introns and rejoining of exons, a process called splicing. RNA splicing requires a specific nucleotide sequence at the exon/intron boundaries and in the vast majority of cases introns starts with GT and ends with AG. The splicing is mediated by the spliceosome complex, that consists of different small nuclear RNA (snRNA) and more than 50 proteins [4].

Alternative splicing is a process where the RNA could be reconnected in multiple ways and result in different mRNA (Figure 3). These may translate into different isoforms of a protein and thus could a single gene encode several different proteins. To the complexity of splicing adds the finding of chimeric transcripts resulting from splicing of adjacent genes [5]. This is however rare and the common modes of alternative splicing are;

Exon skipping

This is the most common mechanism of alternative splicing in mammalians and in this case the splicing apparatus uses another legitimate splice site with the consequence that an exon is discarded. One example is the Fas receptor that has two common isoforms in human. If exon 6 is retained this leads to production of a membrane bound protein with apoptosis promoting capacity. However, if exon 6 is skipped this will result in a soluble Fas receptor that does not promote apoptosis [6]. Another variant of exon skipping is the usage of mutually exclusive exons where one of two possible alternative exons can be included in the mRNA but never both.

Intron retention

In some cases the intron sequence is retained and this is most likely to occur with a small intron (< 200bp). Retention of introns could affect transportation of mRNA from the nucleus in order to avoid contact with the translational machinery [7]. If translated, it would presumably have major impact on the corresponding protein by addition of amino acids, or early truncation of the protein or a complete new amino acid sequence caused by frameshift in the open reading frame (ORF). However, retention of an intron could have biological functions as exemplified by one isoform from the mouse Tgi2 gene [8].

Alternative acceptor or donor sites

Different isoforms could also be created by using either an alternative 5' donor site or an alternative 3' acceptor site. One example is the tight junction protein Claudin-10 that besides two alternative first exons has an alternative splice donor site, causing a deletion of 57 nucleotides corresponding to an in-frame deletion of 19 amino acids. In addition, some Claudin-10 isoforms

lack exon 4. This complex splicing of Claudin-10 results in six different isoforms that exhibit distinct function, cellular localization and tissue specific expression [9].

Defects in mRNA splicing are important in several diseases as well as cancer and could be caused by mutation in splicing elements and also by changes in the nuclear environment that regulates the choice of splice site [10]. There are splice variants that are found solely in cancerous tissues and absent in normal tissues, such as the spleen tyrosine kinase that frequently is expressed in breast cancer but not in normal mammary tissues [11]. An example from neuroblastoma is the hypoxia-induced alternative splicing of the nerve growth factor TrkA. A double splicing change causes alteration in the extracellular N-terminal of the protein through excision of exon 6, 7 and 9, resulting in a constitutively activated receptor. This splice variant, called TrkAIII, does not respond to nerve growth factor (NGF) and antagonizes the function of the normal TrkA [12].

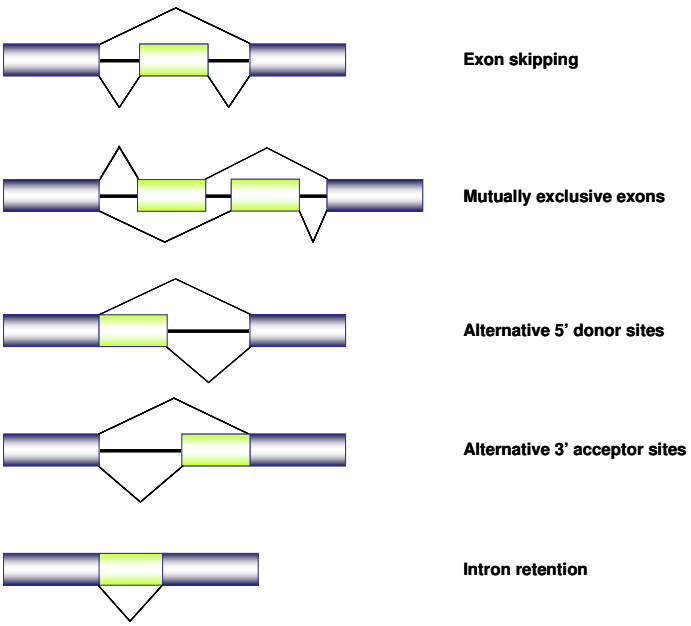

Figure 3. *Different modes of alternative splicing. The lower lines represent the common form of splicing and the upper lines alternative splicing. Image shows alternative 5´donor site and 3' acceptor sites positioned within exons but alternative splice sites could also be located within introns, resulting in partial intron retention.*

Variations in the genome

The DNA sequence is unique for each individual. Most of these differences occur naturally in the populations and are not disease causing, so called polymorphisms. Single-nucleotide polymorphism (SNP) is a common variation in the DNA sequences in which only one nucleotide differs between individuals at that precise position. Usually a SNP has two alleles although three- and four-allele SNP exist but are rare. Any change in the DNA is called a mutation and thus could polymorphisms also be referred to as mutations. However, the term "mutation" is more commonly used for pathogenic alterations and "polymorphism" for non-pathogenic alteration of the DNA. Mutations are often found in the coding region of the gene but could also be found in regulatory elements and other locations that could have implications of the biological function of

the gene. They could be induced by our environment through chemicals or radiation, by viruses or transposons or through spontaneous errors from our own DNA repair and replication machinery.

Mutations can, beside nucleotide substitution, also result from deletions or insertions. A single-base substitution results in a change of the codon and could cause an insertion of a stop codon (nonsense mutation), or a change of the usage of amino acid in corresponding protein (missense mutation). In some cases a single-base substitution has no effect since the genetic code is somewhat degenerated and there are several triplets encoding the same amino acid (silent mutation). A deletion or insertion in the DNA will cause a shift in the open reading frame if the number of involved nucleotides is not a multiple of three, and will most likely result in a truncated protein. Mutations could also affect splicing by either creating or destroying splice site signals. Cancer cells often exhibit large-scale chromosomal abnormalities such as translocations (when a part of a chromosome has joined with a non-homologous chromosome), loss or gain of whole chromosomes or larger chromosomal regions, or inversions (when a part is cut out and then inserted at "wrong" direction). There is also a form of large-scale polymorphisms that involves DNA copy number variants (CNV) caused by deletions or duplication in the genome. CNVs are variants where a stretch of more than 1 kb diverges from the normal diploid number of the autosomal chromosomes and could be found in the normal population [13]. The area displaying CNV could contain a single gene or a contiguous set of genes and thus affect expression dosage of affected genes. Changes of copy number is implicated to be important in many types of human diseases (e.g. monogenic disorders, infection, cancer or complex diseases) and some individual CNVs have been shown to associate with disease [14].

Organization of the genome

The genomic content of each cell corresponds to approximately 2 meters and needs to be highly ordered (Figure 4), especially during mitosis. The nucleosome is the most fundamental unit of packaging and consists of eight histones, upon which a stretch of 146 bp is coiled. The adjacent nucleosomes are separated by a short length of spacer-DNA and electron micrographs shows this as a "string of beads". This string is further coiled into a chromatin fiber that is organized into long loops that is heavily condensed during cell division, making it possible to visualize chromosomes in light microscope.

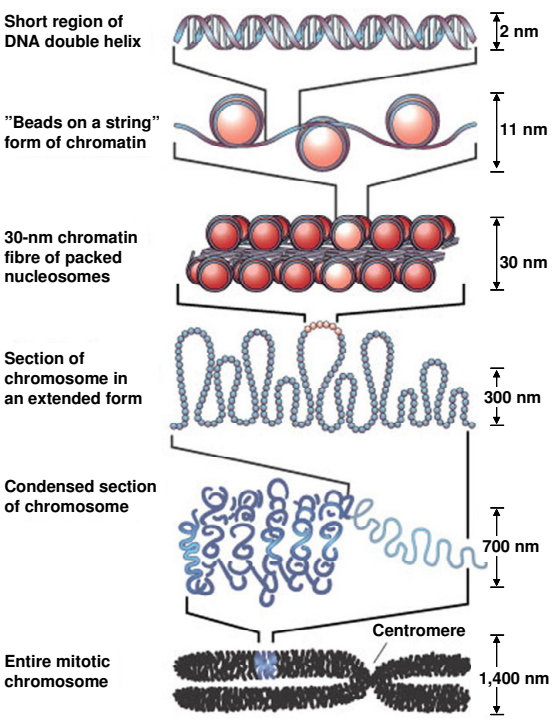

Figure 4. *The organization of DNA within the chromatin structure. Reprint by permission from Macmillan Publisher Ltd; Nature (Felsenfeld and Groudine) © 2003 [15]*

Epigenetics

Epigenetics is the study of changes in phenotype that is hereditable but does not involve changes in the underlaying DNA. Epigenetics can regulate gene expression and is behind cellular processes such as X-chromosome inactivation and chromosome imprinting. Regulation by epigenetical means could be extorted at different levels, from alternative usage of different modifications of histones to methylation in CpG-islands or expression of non-coding RNA. CpG-islands are areas with high presence of cytosine-guanine dinucleotides, most often located in repetitive elements and are also commonly present in the promoter and the first exon/exons of genes. The cytosine in the CpG nucleotide can be modified by methylation, a common modification of the DNA in the human genome. Methylation of the CpG-islands is associated with gene silencing and is normally found in repetitive elements, imprinted genes or genes on the inactivated X-chromosome in females. Cancer is characterized by genome-wide hypo-methylation of the chromatin together with gene-specific hypo- or hyper methylation and many tumor suppressor genes are inactivated through hypermethylation of promoter CpG islands.

The histones can also be modified in different ways, for example by acetylation, methylation, glycosylation or ubiquitination, and the combination of these modifications constitute a "histone code". The different modifications alter the interaction between histones, DNA and nuclear proteins. A transcriptional active gene is characterized by acetylated histones while deacetylation alters the charge and leads to a stronger interaction between the nucleosome and the negatively charged DNA with a more closed chromatin as a consequence.

Gene activity could also be affected by small ncRNAs that bind to target RNAs in a sequence-specific manner as they display complementary sequences. There are different classes; short interfering RNAs (siRNA), microRNA (miRNA) and PIWI-interacting RNA (piRNA) [16]. The siRNA and miRNA are generated in a Dicer-dependent way from double-stranded precursors. The siRNA has a double stranded structure and the miRNA a single stranded. miRNA commonly bind to the 3' untranslated regions of the mRNA and alters the stability or the translational efficiency. The piRNA is generated from single-stranded RNA and has a function in silencing of retrotransposons, particularly in germ cells. A single ncRNA can have hundreds of target mRNAs [17] and can thereby affect processes such as cell cycle progression, apoptosis or differentiation [18].

CANCER

Cancer is, in essence, a genetic disease and represents a heterogeneous group of diseases that shares certain biological properties such as uncontrolled growth and abnormal phenotype of cells. Untreated tumors will eventually affect surrounding tissues and organs and can cause inflammation or pain by adding pressure on nerves. The progression varies between types of cancer and between individuals and the course of events could be very slow or very rapid.
Sweden has about 50 000 cases each year and approximately 22 000 dies from cancer annually. The most common types of cancer are prostate (34 % of all cancers in men), breast cancer (29 % of all cancers in women) and colorectal cancer (12 % of all cancers in men and women). 250 children (0-14 years) are diagnosed with cancer each year and the most common forms of cancer is leukemia and lymphoma (40 %) and brain tumors (30 %). Neuroblastoma is the most common cancer in infants and is occasionally even found in fetuses. Cancer in children is generally more aggressive than in adults, and since the body is growing and developing, they are also more sensitive to therapy [19].

Cancer genetics

Cancer is caused by series of somatic mutations, with in some cases a hereditary predisposition, involving both activation of oncogenes and inactivation of tumor supressor genes. The tumorigenesis can be seen as an evolutionary process where genetic alterations with proliferating advantages are selected, ultimately disrupting the delicate balance between proliferation and differentiation (Figure 5). During tumor progression the genomes of tumor cells often become more and more unstable and thereby increasing the rate at which new mutations are aquired. However, due to the complexity of tumorigenesis most human cancers require long time to develop.

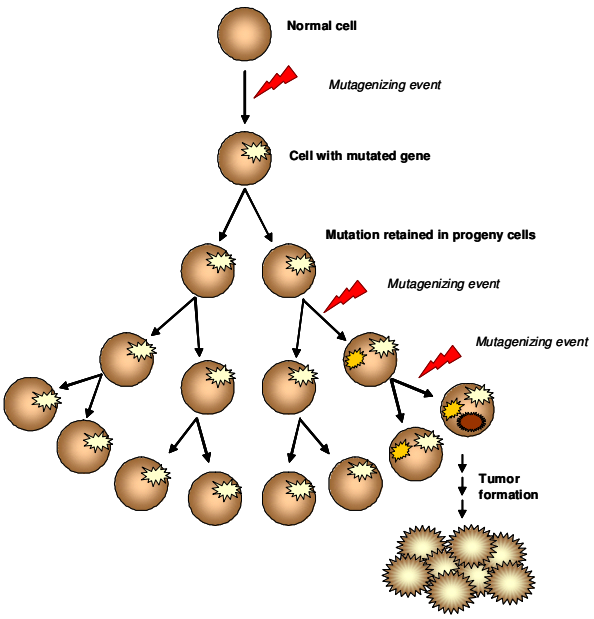

Figure 5. *Cellular transformation is a multistep process where several mutations that confer growth advantages are acquired for tumor formation.*

A classical review article by Hanahan and Weinberg [20] suggests six essential alterations as a part of the process that transforms normal cells to metastazing cancer;

Self-sufficiency in growth signals
Normal cells are dependent on growth-promoting signals in order to actively proliferate, but tumor cells have reduced their dependence on the normal microenvironment by generating their own growth signals. This can be done by autocrine signaling, deregulation of cell surface receptors or alterations of intracellular signaling pathways.

Insensitivity to anti-growth signals
Normal tissue homeostasis is also maintained by anti-growth signals that induce quiescence or differentiation, and tumor cells have rendered themselves insensitive to these types of signals.

Evasion of apoptosis
Programmed cell death, apoptosis, is a highly regulated process of attrition of unwanted cells. Apoptosis allows a normal cell to die in a controlled manner upon extra- or intracellular signals, however, tumor cells have gained resistance toward such signals.

Limitless replicative potential
Most mammalian cells have a limited potential of replication that cancer cells must overcome before a tumor can progress. The number of cell divisions for a normal cell is limited by telomere length since erosion of the telomeres leads to unprotected chromosomal ends, resulting in crisis and eventual cell death. Cancer cells avoids the problem of telomere shortening mainly by activating the telomerase enzyme and thereby get limitless replicative potential [21].

Sustained angiogenesis
All cells, including cancer cells, require nutrition and oxygen as well as waste disposal for proper function and survival. These functions are provided by the vasculature. Growth of new blood vessels, angiogenesis, is tightly regulated in normal cells and in order to develop macroscopically detectable tumors, cancer cells must obtain angiogenic ability.

Tissue invasion and metastasis
In order to transform solid tumors from benign to malignant tumors, acquisition of migration and invasion capacity is crucial. The process of metastasis is complex and cancer cells must achieve the ability to enter (and leave) blood and lymph vessels in order to establish metastases at distant sites.

Tumor suppressors and oncogenes
Two major groups of genes are involved in tumorigenesis; oncogenes and tumor suppressor genes (TSG). Proto-oncogenes are a heterogeneous group of genes with a positive effect on cell growth and the oncogene represent the mutated form. Activation of oncogenes is normally dominant and involves either an increase in the levels of the unaltered gene or a modified product resulting from a mutation or chimeric rearrangement. Oncogenes can act at different levels in the cell and function as growth factors, growth factor receptors (e.g. *ERBB*), signal transduction molecules (e.g. *RAS*), DNA-binding molecules (e.g. *MYCN*) or cell cycle regulators (e.g. *MDM2*).

While oncogenes drive proliferation, TSGs constrain cell growth and inactivation of TSGs is just as important as oncogene activation in cancer pathogenesis. TSGs normally inhibit events that lead to tumor formation and can be divided into gatekeepers and caretakers [22]. The gatekeepers

are involved in cellular processes that inhibit proliferation (e.g. pRB) or initiate cell cycle arrest or apoptosis of malfunctioning cells (e.g. p53). Caretakers are important in control of DNA integrity and while mutations in caretakers may not initiate cancer by itself, the resulting genomic instability would make the cell more likely to sustain other mutations.

Important insights of TSG function in tumor development came from the childhood eye tumor retinoblastoma. The observation that familiar retinoblastoma displayed earlier onset than sporadic made Alfred Knudsen to postulate the classical two-hit hypothesis [23]. This hypothesis suggests that TSGs are recessive and inactivation of both the maternal and paternal allele is required for tumor initiation. In familiar retinoblastoma one mutation of the *RB1* gene is inherited and found in all cells of the individual since they are descendants from germline. Subsequently, only one additional hit adressing the second allele is required for inactivation of the *RB1* gene in a cell. The second hit could be achieved by deletion, promoter hypermethylation or more rarely, through mutation (Figure 6). Children with sporadic retinoblastoma require two successive genetic alterations in a single retinal cell in order to inactivate both alleles of the *RB1* gene. Consequently, the risk of developing tumors is much higher when a TSG mutation is inherited and there is also higher risk of developing multiple tumors.

The two-hit hypothesis is however not universal and there is evidence that haploinsufficiency, inactivation of one allele, can contribute to the tumorigenic process. There are different potential mechanisms explaining the impact of loss-of-function in only one allele of a gene. It can be constitutionally hemizygous genes (located at the X- or Y-chromosome in males), genes that are monogenically expressed such as imprinted genes or genes at the inactivated X-chromosome in females. Other mechanisms can be that the single mutation acts dominant negative and blocks the function of the wild-type allele, as the case of some p53 mutants [24], or that the reduction in gene dosage is enough to facilitate tumor progression. There are also emerging evidence of post-transcriptional regulation of tumor suppressor genes by non-coding RNA, that binds to complementary sequences on target mRNA, resulting in translational repression and gene silencing [25].

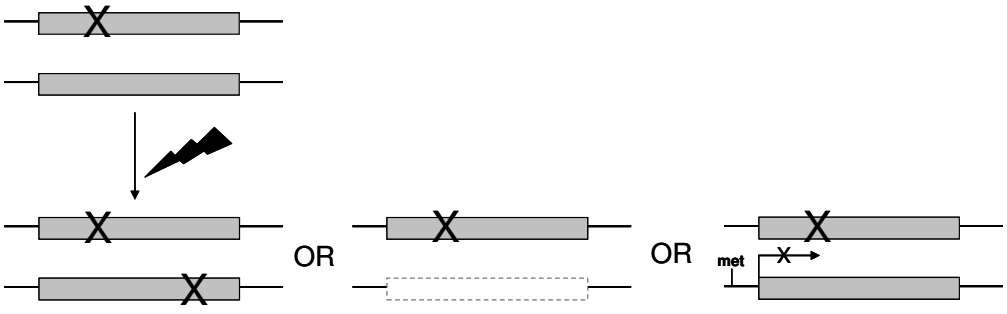

Figure 6. *According to Knudsen's two-hit model inactivation of both alleles of a tumor suppressor gene is required for tumor growth. The first hit can be a de novo mutation as in sporadic cancers or germline as in familiar cancer syndromes. The second hit can constitute of a second mutation, but more often a deletion of the second allele or promoter hypermethylation which silences this allele in this case.*

CELL SIGNALING

Normal tissue architechture and function depend on cooperation and communication among large groups of cells. Cell communication is based on production of signal molecules and their recognition by receptors that allow the cells to react to different stimuli. Activation of a receptor most often involve an enzyme cascade or generation of second messengers, however can also act through a single-step signal transduction such as steroid or thyroid hormones. Steroid and thyroid hormones, as well as retinoic acid, can diffuse through the membrane, bind intracellular receptors and directly act as transcription factors, modulating the expression of target genes. However, most common is signaling through transmembrane receptors where binding of a ligand causes conformational changes with consequent activation of intracellular signal transduction and ultimately, altered gene expression. Different types of cells use different combinations of signaling pathways to regulate growth and change of phenotype is the result of multiple converging signaling pathways. Activation and de-activation of different pathways is crucial in the transformation of normal cells to malignant counterparts and signaling through the phosphoinositide 3-kinase (PI3K)/Akt pathway has a key role in cancer development.

Activation of PI3K/Akt signaling

The PI3K signaling cascade is initiated by receptors with intrinsic tyrosine kinase activity that activates PI3K upon stimulation. The most extensively studied PI3K (Type IA) generates the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) that recruits downstream effectors such as PI-dependent kinase (PDK1), integrin-linked kinase (ILK-1) or Akt to the plasma membrane through their pleckstrin homology domain. After recruitment to the membrane Akt is activated through phosphorylation on two key residues: threonine 308 (T308) and serine 473 (S473), mediated by PDK1 and the rector/mTOR complex respectively. Several studies show that phosphorylation of T308 is sufficient to activate Akt, although phosphorylation at both T308 and S473 is required for full activation of Akt [26, 27]. Levels of phosphorylated Akt is affected by both levels of PIP_3 which depends on PI3K-activity, and the activity by phosphatases such as PTEN [28], SHIP [29] or PHLPP [30]. Activation of Akt is a key event in many important biological processes such as proliferation, apoptosis, differentiation, metabolism and migration (Figure 7) [31]. Deregulation of the PI3K/Akt pathway and increased levels of phosphorylated Akt has been reported in numerous human malignancies and is associated with aggressive phenotype and poor prognosis. Deregulation could be caused by loss of the tumor suppressor gene *PTEN* [32, 33] oncogenic activation of *PIK3CA* [34, 35] or increased stimulation by various growth factor receptors and its ligands (e.g. IGF-1, VEGF and EGF) [36-38].

Subunits of PI3K

PI3K is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit. There are five identified isoforms of p85 in mammals (p85α, p85β, p55γ, p55α or p50α) encoded by three different genes and three isoforms of catalytical subunits of the Type IA PI3K; p110α, p110β or p110δ, encoded by *PIK3CA*, *PIK3CB* and *PIK3CD* respectively. The regulatory subunit protects the catalytic subunit from degradation, inhibits its enzymatic activity in quiescent cells and mediates translocation to the plasma membrane and the PI3K substrates upon activation [39, 40]. The exact role of the distinct p85 isoforms are unknown but there are evidence of differential binding of p85 to receptors [41, 42]. The regulatory subunit p85, encoded by *PIK3R1*, have tumor suppressing capacity [43, 44], partly caused by its regulation of the p110 catalytic subunit but also by its association to Pten and its modulation of the phosphatase activity of Pten [45-47].

The distinct roles of the catalytic α-, β- and δ-isoforms are not fully understood, but differences in tissue distribution as well as the fact that knock-out in mice generates different phenotypes indicates isoform specific functions, reviewed by Vanhaesebroeck et al. 2005 [48]. p110α and p110β displays a broad tissue distribution in embryonic and adult tissue [49, 50], whereas p110δ, encoded by *PIK3CD,* is mainly expressed in leucocytes [50, 51] and also highly expressed in neurons [52]. However, any of the different Type IA isoforms can sustain cell proliferation and survival [53] and both the β- and δ-isoform induces oncogenic tranformation in chicken embryo fibroblasts [54]. The main focus in cancer research has primarily been on the catalytic activities of the α- and β-isoforms of p110 [55], but it has recently been shown that p110β also has distinct non-catalytic functions in cell proliferation and trafficking [56].

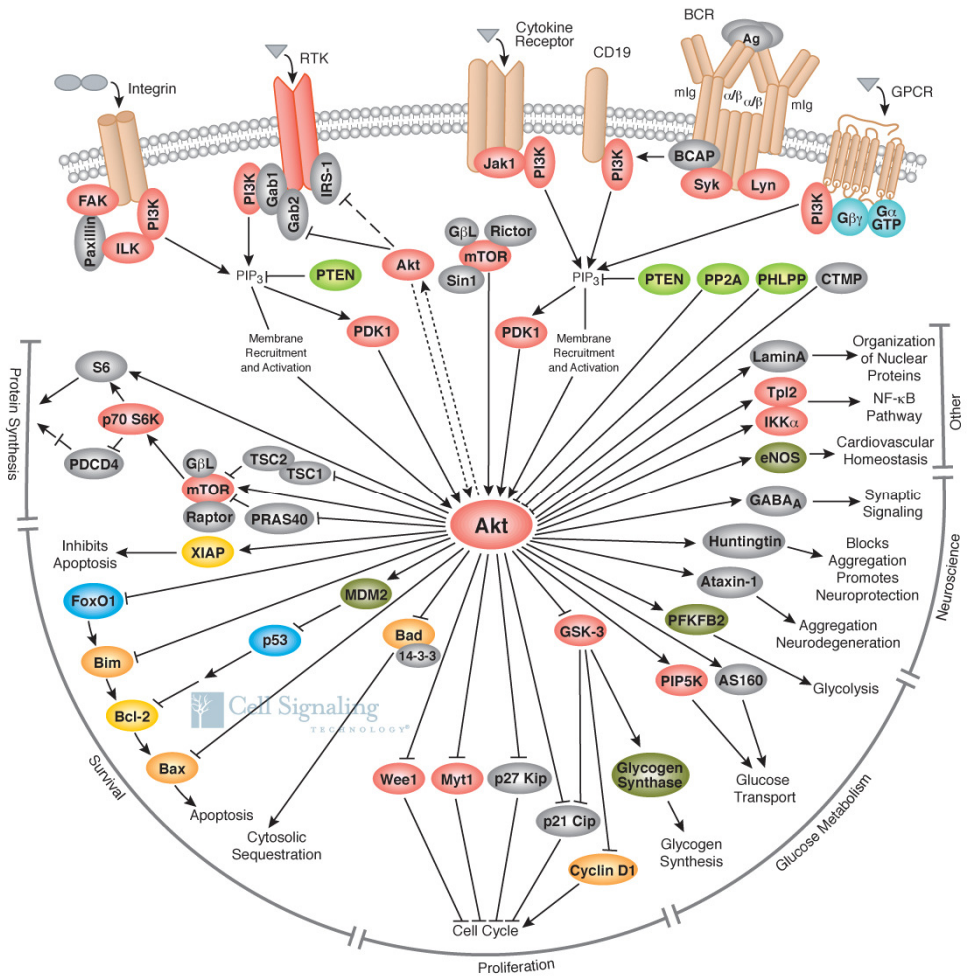

Figure 7. *Signaling through the PI3K/Akt pathway is complex. Activation through different tyrosine receptor kinases leads to a signaling cascade where phosphorylation of the effector Akt is central. Pathway diagram reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).*

PI3K and RAS crosstalk

The RAS signaling pathway is also frequently deregulated in cancer and RAS can be activated by the same receptor tyrosine kinases (RTKs) and growth factors as PI3K. The RAS subfamily consists of four proteins (H-RAS, N-RAS and two species of K-RAS) which are small GTPases that were discovered for their oncogenic capacity [57]. Upon stimulation of a RTK, RAS initiates a kinase cascade where phosphorylation of MEK and subsequent activation of ERK, a mitogen-activated protein kinase (MAPK), is central. Besides canonical signaling through MAPK/ERK, there is also crosstalk between RAS and the PI3K signaling pathway. Studies in *Drosophila* shows that RAS increases PI3Ks signaling response [58], possibly by enhancing the PI3K affinity for the receptors [59-61]. Activated RAS can directly bind to the RAS-binding domain of the different PI3K catalytic subunits [62] but interestingly, the greatest diversity in primary structure of the p110 isoforms is found within the RAS-binding domain, suggesting that RAS might affect PI3K in an isoform specific manner [63, 64].

PI3Ks are also implicated in activation or regulation of the RAS/ERK signaling pathways since pharmacological inhibitors of PI3K also affects ERK activation [65]. Furthermore, a constitutively activated mutant of the catalytical PI3K subunit caused activation of the RAS effector Raf-1 when expressed in *Xenopus laevis* oocytes [66]. This indicates a complex nature of the cross talk between the RAS and PI3K pathways.

The PI3K pathway as therapeutic target

Inhibition of PI3K has become an attractive target in cancer therapeutics and significant efforts have been made during the recent years to develop agents that inhibit the PI3K/Akt pathway. The first generation of PI3K inhibitors includes Wortmannin and LY294002, a derivative of Quercetin, the first compound discovered with inhibitory effect on PI3K [67]. Both have been widely used as pharmacological tools to study involvement of PI3K in various biological systems but have the drawback of also inhibiting kinases unrelated to PI3K [68, 69]. Based on these compounds, a new generation of drugs has been developed that are more stable and with better pharmacokinetic properties (e.g. SF1126 and PX-866). However, the compounds above are all global PI3K inhibitors and since PI3K-signaling is central in many cellular functions, global inhibition of PI3Ks is most likely to be deleterious. Consequently, development of drugs targeting specific PI3K isoforms is crucial. Despite the overall similarity of the ATP binding sites of the p110 isoforms, it has been possible to exploit minor differences in the binding pockets to produce selective kinase inhibitors. A range of new inhibiting compound with isoform specificity is being developed, one example is the δ-specific inhibitor CAL-101 that is in early clinical trial for hematological malignancies [70].

NEUROBLASTOMA

Occurrence

Neuroblastoma (NB) is the most common solid extra cranial tumor of childhood, accounting for 7% of all pediatric malignancies. NB is commonly diagnosed in the first years of life and is rarely seen after the age of 5. The prevalence is about 1 in 7 000 live births with approximately 15-20 new cases annually in Sweden and with no major differences in prevalence between genders. There have not been any epidemiological reports of difference in incidence rates depending on country and no environmental risk factors are known to contribute to development of NB.

NB is believed to arise from postganglionic sympathetic neuroblasts derived from the neural crest. Cells committed to the sympathetic nervous system (SNS) are characterized by synthesis of the neurotransmitter noradrenalin and controls autonomic functions such as regulation of heart rate and contraction of blood vessels. NB tumors are found in any site along the sympathetic nervous system chain, about 50% arise in the adrenal medulla and the rest in thoracic or abdominal paraspinal sympathetic ganglia or pelvic ganglia. Metastases can develop in lymph nodes, bone, bone marrow, liver and skin. Most NBs are undifferentiated tumors consisting of small round neuroblasts. Tumors characterized by partial histological differentiation are called ganglioneuroblastoma (GNB) while the most differentiated tumor type is called ganglioneuroma (GN).

The International Neuroblastoma Staging System

The International Neuroblastoma Staging System (INSS) is used for classification of NB based on clinical, radiographic and surgical evaluation of the children with the disease, (Table 2) [71, 72]. NB is classified in stages ranging from stage 1, the group with most favorable outcome consisting of well localized tumors to stage 4 NB with highly aggressive and fatal tumors. There is also a special form called stage 4S, limited to infants less than 1 year of age with localized primary tumors with dissemination to skin, liver and/or bone marrow involvement. This form of NB has the remarkable feature of cases with spontaneous regression, indicating the ability to respond to differentiation and/or apoptotic signals.

Table 2. *The International Neuroblastoma Staging system*

Stage	Description
1	Localized tumor with complete gross excision, with or without microscopic residual disease, representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached to and removed with the primary tumor may be positive)
2A	Localized tumor with incomplete gross excision; representative ipsilateral non-adherent lymph nodes negative for tumor microscopically
2B	Localized tumor with or without incomplete gross excision, with ipsilateral non-adherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically
3	Unresectable unilateral tumor infiltrating across the midline (vertebral column), with or without regional lymph node involvment; or localized unilateral tumor with contralateral regional lymph node involvment; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvment
4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or organs (except as defined by stage 4S)
4S	Localized primary tumor (as defined for stage 1, 2A or 2B), with dissemination limited to skin, liver, and/or bone marrow (only minimal involvment of the bone marrow). Limited to infants < 1 year of age

Recently the International Neuroblastoma Risk Group Staging System (INRGSS) was established in order to develop a uniform pretreatment risk stratification [73, 74]. This approach uses tumor imaging rather than surgical resection and it includes age, histology, grade of tumor differentiation, MYCN status, presence/absence of 11q deletion and tumor cell ploidy (Table 3).

Clinical manifestations

One feature of neuroblastoma is wide clinical variability, ranging from highly unfavorable tumors with adverse outcome to the patient to tumors displaying spontaneous regression. Although the percentage of surviving patients has increased during the last decades there is still a subset of tumors that are highly aggressive and resistant to all treatments. Consequently, NB is still fatal in more than 70% of high-risk cases [75]. The symptoms of NB vary depending on localization of tumor/tumors and their size. The most common symptoms of NB are caused by the tumor adding pressure on nearby tissues as it grows or by cancer spreading to the bone. Different symptoms include lumps in abdomen, neck or chest, bone pain, swollen stomach, diarrhea, fever, high blood pressure and fatigue. However, there are also patients with no symptoms at all. A large majority of NB patients has elevated levels catecholamines (adrenaline or noradrenalin) or its metabolites (e.g. dopamine). The tyrosine hydroxylase (TH) is the first enzyme in the synthesis of catecholamine and can be used for detection of NB cells in blood and bone marrow [76, 77]. NB is treated with surgery, chemotherapy, radiotherapy and biotherapy depending on stage of disease. In some cases of localized disease the tumor is only observed without further treatment.

Table 3. *INRG consensus pretreatment classification schema*

INRG stage	INRGSS description	Age (months)	Histological category	Grade of tumor differentiation	*MYCN*	11q-del.	Ploidy	Pretreatment risk group
L1/L2			GN maturing, GNB intermixed					A Very low
L1	Localized tumor not involving vital structures as defined by a list of image-defined risk factors* and confined to one body compartment				NA			B Very low
					amp			K High
L2	Locoregional tumor with presence of one or more image-defined risk factors	<18	Any, except GN maturing or GNB intermixed		NA	No		D low
						Yes		G Intermediate
		>18	GNB nodular; neuroblastoma	Differentiating	NA			E Low
				Poorly differentiated or undifferentiated	NA			H Intermediate
					Amp			N High
M	Distant metastatic disease (except stage MS)	<18			NA		Hyperdiploid	F Low
		<12			NA		Diploid	I Intermediate
		12-18			NA		Diploid	J Intermediate
		<18			Amp			Q High
		>18						P High
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bonemarrow				NA	No		C Very low
						Yes		Q High
					Amp			R High

GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified

* For image-defined risk factors see Monclair et al (2009)

Adapted from Cohn et al (2009) [74] and Monclair et al (2009) [73]

Genetical alterations

The genetics behind NB is fairly well studied, however the exact process of the tumorigenesis is not fully understood. There are several cytogenetic abnormalities and other alterations with clinical relevance in NB.

1p-deletions
Deletion of 1p is reported in approximately 35% of all NB and with even higher frequency considering only advanced tumors [78-80]. Deletion of 1p is not restricted to NB but is also found in other neoplasms of the nervous system or of embryonic origin such as meningioma [81], phaechromocytomas [82] and germ cell tumors [83] as well as different carcinomas [84]. The recurrent deletions of this region in NB together with the finding that transfer of 1p chromosomal material reduced tumorigenicity in a NB cell line [85], indicates that this region harbors one or several tumor suppressor genes. A number of chromosomal changes involving 1p has been reported but does not describe a single region [86] indicating that there might be several distinct regions at chromosome 1 contributing to progression of NB. Studies of our Scandinavian material at 1p36 have revealed a shortest region of overlapping deletions (SRO) of 25 cM, defined by markers D1S244 and D1S80 [87]. By including germ cell tumors that share embryonic origin with NB and also displays frequent deletions of 1p, this region was delimitated to a more foreseeable 5 cM [78].

Chromosome arm 2p: MYCN and ALK
The oncogene *MYCN* is located at 2p24 and encodes a transcription factor that activates targets such as *ODC* and *MCM7*, leading to cell cycle progression [88, 89]. *MYCN* is amplified in a subset of neuroblastomas and highly associated with advanced stage of disease and poor prognosis. The amplicons of *MYCN* can be arranged sequential in homogenously stained regions (HSR) or in double minutes (Figure 8 A-B).

A small subset of NB is hereditary and differs from the sporadic NB in that they are diagnosed at earlier age and that patients are more prone to develop multiple tumors. There is gentic linkage of hereditary NB predisposition to chromosomes 16p12-13 [90], 4p16 [91] and 2p23-24 [92]. Located in the latter region is the oncogene *ALK*, which now is believed to be the major familiar predisposition gene. *ALK* is also frequently co-occurring in the *MYCN* amplicon at 2p in sporadic NB and has activating mutations in 8.4% of high risk tumors [92]. Mutations and amplification of *ALK* are mainly found in large primary tumors or metastatic tumors [93].

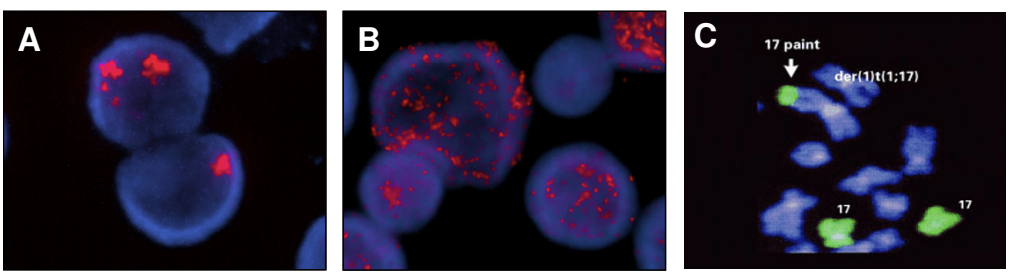

Figure 8. *Genetic alterations in neuroblastoma. Amplification of the oncogene MYCN in NB visualized using FISH showing A) sequential amplification of MYCN in HSR and B) in double minutes. FISH-images kindly provided by the department of clinical genetics, SU/Sahlgrenska. C) Translocation of 17q to 1p causing gain of 17q and loss of 1p chromosomal material. Image reprinted by permission of Massachusetts Medical Society; New England Journal of Medicine (Nick Bown et al.) © 1999 [94]*

17q gain
Gain of 17q is probably the most prevalent cytogenetic alteration in NB, detected in about 50% of tumors and correlated to adverse outcome [94-97]. Gains of 17q always include the terminal part and it has been postulated that genes in this region gives selective advantages. There are also frequent cases of unbalanced translocation between chromosome 1 and chromosome 17, leading to loss of 1p and gain of 17q (Figure 8C) [94]. However, the translocation breakpoints varies, making it difficult to identify important genes [98, 99].

11q-deletions
Deletion of 11q is found in 20-40% of all NB [96, 100]. It is associated with deletion of 14q but shows inverse correlation to *MYCN* amplification and 1p-deletion. It is also a marker of unfavorable tumors with features distinct from *MYCN* amplified tumors, such as the number of segmental rearrangement and lower expression levels of the DNA damage response histone *H2AFX* [101].

Ploidy
Besides chromosomal rearrangements, tumor cell ploidy is also predictive of patient outcome in infants. A hyper-diploid or near triploid karyotype is mostly found in low stage NB, while diploid tumors associates with poor prognosis. However , the prognostic significance of ploidy is lost for patients older than 1-2 years [102].

Neurotrophin receptors
Signals from neurotrophic factors are mediated by the Trk family of tyrosine kinases and have an important role in normal neural development as reviewed by Barbacid [103]. The expression of the different neurotrophin receptors (Trk A, B and C) is correlated to biological and clinical features of NB [104]. Expression of the high-affinity nerve growth factor receptor TrkA is high in NB with favorable prognosis [105]. Upon activation through NGF, TrkA signals survival or differentiation while inhibition of TrkA can lead to apoptosis [106, 107]. Just as TrkA, TrkC is highly expressed in NB with good prognosis [108, 109]. The opposite is seen for the brain-derived neurotrophic factor receptor TrkB where high expression is associated with poor prognosis [110]

PI3K/Akt signaling in neuroblastoma
As in many other malignancies, signaling through the PI3K/Akt pathway is alleged to be important in NB, however the contribution of PI3K/Akt deregulation in NB is not fully understood. High levels of phosphorylated Akt is detected in all primary NB tumors compared to non-malignant adrenal medulla [111] and the levels of phosphorylated Akt is higher in aggressive neuroblastomas compared to neuroblastomas with favorable biology [111-113]. Elevated levels of phosphorylated Akt can be caused by either increased PI3K activity or decreased phosphatase activity. However, mutations in the key components of the PI3K signaling cascade (e.g. *PIK3CA* and *PTEN)* are rare in NB [114, 115]. RNA-expression of *PTEN* is possibly epigenetically regulated in primary NB tumors [116, 117]. A few mutations have been reported in *PIK3CD* [118] and it is also shown that *PIK3CD* is expressed at lower levels in aggressive NB compared to NB with more favorable biology [119-121]. *PIK3R1,* encoding the regulatory PI3K subunit p85α, is also lower expressed in aggressive neuroblastoma compared to the favorable ones [113, 119]. There is also cooperation between MYCN and PI3K/Akt signaling in NB since inhibition of PI3K destabilized N-myc at the protein level and prevented tumor progression in a murine model of NB [122].

Even though signaling through PI3K is associated with proliferation, PI3K is also important in neural differentiation. Differentiation of NB cell lines by different means (e.g. retinoic acid) results in phosphorylation of Akt and inhibition of PI3K diminishes differentiation and neurite outgrowth [97, 123-125].

Epigenetic deregulation

There are several genes that are deregulated by epigenetical means in NB and include *CASP8*, *RASSF1A* and *ZMYND10*. *CASP8* encodes a key enzyme in the apoptotic cascade and is one of the first genes to be reported methylated in NB [126]. Methylation of *CASP8* correlates with amplification of *MYCN* and also with poor event-free survival [116]. The chromosomal region 3p21 is also a subject of deletion in NB [127] and both *RASSF1A* and *ZMYND10* (*BLU*) is located in this region. Promoter methylation of *RASSF1A* is reported in nearly 100% of all NB [116, 128, 129] while *ZMYND10* is methylated in 15-41% of all NB [116, 130].

OBJECTIVES

The objective of this thesis was to explore genes and gene products that might contribute to initiation and progression of neuroblastoma and possibly also of other malignancies.

Specific aims

Paper I

- To explore mRNA expression of genes located within our specific region of interest at 1p36.2-3 using primary NB tumors.
- To investigate differences at the mRNA level between favorable and unfavorable NB tumors or between tumors with or without 1p-deletion.

Paper II

- To perform an epigenetic screening of 30 genes at 1p36.2-3 in NB cell lines
- To further analyze genes with possible epigenetical regulation in NB cell lines in detail in primary NB tumors by bisulfite sequencing, expression analysis and mutation screening.

Paper III

- To explore the expression of genes that participates in PI3K/Akt signaling in NB.
- To investigate protein levels of the different catalytic isoforms of Type IA PI3K as well as the levels of phosphorylated Akt in correlation to protein levels of Pten.

Paper IV

- To explore the presence of a novel splice variant of *PIK3CD* at mRNA and protein levels in normal tissues and human tumors (i.e. colorectal and ovarian cancer)
- To analyze the function of p110δ and the splice variant p37δ and their possible involvement in tumorigenesis.

MATERIALS

Tumors, cell lines and control material

In this thesis have we used primary tumors mainly from Sweden. We have also used several cell lines derived from neuroblastoma and other cancers, as well as a transformed kidney cell line and mouse embryonal fibroblasts (MEF). In addition, we used protein and mRNA from various normal tissues including adrenal gland, murine spleen and lung.

The majority of NBs were diagnostic pre-treatment samples, biopsies or surgically removed tumor samples. Genomic DNA was extracted from fresh or fresh frozen (-70°C) tumors with DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to suppliers protocol, or phenol extracted using phase lock gel (Eppendorf AG, Hamburg, Germany) according standard procedure. Total RNA was extracted using RNeasy RNA extraction kit (Qiagen) or using Totally RNA (Ambion, Austin, Texas) and treated with DNA-free (Ambion). Purity and integrity of the RNA were assessed by spectrophotometer and RNA 6000 Nano Bioanalyzer (Agilent, Palo Alto, CA) before reverse transcription to cDNA using superscript II (Amersham, Buckinghamshire, UK) according standard procedure. Proteins were extracted from tumors and cell lines using RIPA buffer (Pierce, Rockford, IL) supplemented with HALT™ phosphatase inhibitor cocktail (Pierce) and a protease inhibitor cocktail (Roche, Basel, Switzerland) according standard protocol.

Paper I

- Tumor cDNA from 55 NB patients

Paper II

- A total of 66 NB tumors were used:
  - Tumor DNA from 46 NB patients
  - cDNA from 35 NB patients (15 tumors overlapping)
- Cultures of nine NB cell lines (IMR32, SKNAS, SKNBE(2), SKNDZ, SKNFI, SKNSH, Kelly, NB69 and SH-SY5Y)
- Blood lymphocytes from 120 healthy individuals

Paper III

- A total of 64 NB tumors were used:
  - cDNA from 16 NB patients for cDNA micro array analysis
  - cDNA from 52 NB patients for TaqMan
  - protein from 24 NB patients for western blot
- Normal adrenal gland; cDNA (40 pooled donors) and protein (20 pooled donors)
- Protein from cell lines derived from NB (SKNAS, SKNBE(2), SKNDZ, SHSY5Y, Kelly and NB69), medulloblastoma (DAYO) and cervix (HeLa)

Paper IV

- 27 cDNA and 24 protein from colorectal tumors and corresponding adjacent mucosa from a total of 27 patients
- Eight cDNA and two protein from a total of eight ovarian cancer patients
- cDNA pooled from a minimum of three donors for 34 normal human tissues
- Protein for 10 normal human tissues and two normal mouse tissues
- Cell lines; HEK-293 and mouse embryonal fibroblasts (MEF)

METHODS

Polymerase Chain Reaction (PCR)

This is nowadays a fundamental technique in most genetic analyses and has revolutionized molecular genetics since its introduction in the mid-1980s [131]. PCR is used for multiplying shorter stretches of a specific DNA sequence within a heterogeneous collection of DNA sequences (e.g. total genomic DNA). It is a chemical process that uses a thermo stable DNA polymerase developed from the archea bacteria *Thermus aquaticus*. Short complementary oligonucleotides (primers) specify which sequence that will be amplified and also serves as a starting point in the replication process for the *Taq* DNA-polymerase. The principle is based on consecutive rounds of (I) denaturation of the double-stranded molecules, (II) annealing of the primers to single-stranded template and (III) extension/DNA synthesis (Figure 9). The newly synthesized DNA strands will serve as template in following cycles and the PCR products are doubled every cycle, until the reaction components is consumed.

The PCR-products are often evaluated on agarose gel after staining with ethidium bromide to estimate size, specificity and concentration. In this thesis, we have used the PCR technique for different applications; i.e. gene expression (*Paper I-IV),* molecular cloning (*Paper IV*), DNA sequencing (*Paper II*) and bisulfite sequencing (*Paper II*).

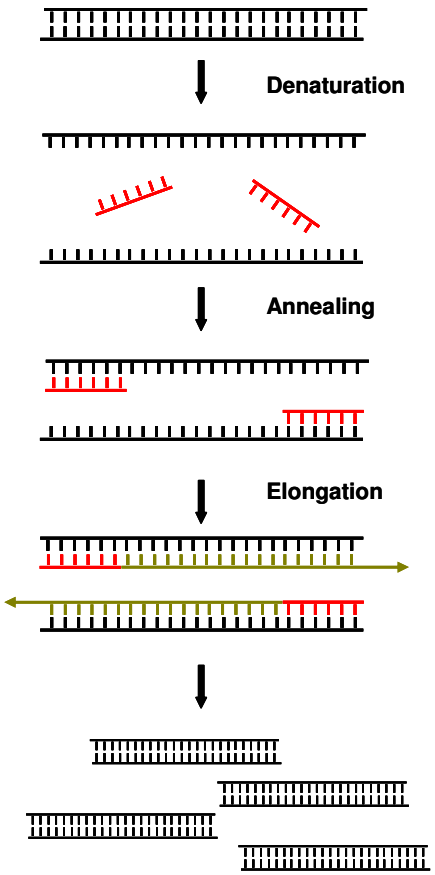

Figure 9. *PCR amplification. The double-stranded DNA molecules are denatured under high temperature (95°C). The temperature is then decreased (50-60°C) allowing adherents of the primers to the single-stranded template under the annealing phase. This is followed by DNA replication under slightly higher temperatures (72°C). These three steps are repeated in consecutive rounds (20-26 cycles). Given temperatures and cycles representative of a standard PCR.*

Real-time reverse transcriptase PCR (real-time RT-PCR)

The real-time RT-PCR is a sensible and reproducible method for determine mRNA levels in cells and tissues [132]. In contrast to conventional PCR, that is measured at end-point, real-time RT-PCR is monitored as the amplification process proceeds (i.e. in real-time). Detection of PCR-products is possible either by usage of fluorescent probes (e.g. TaqMan) or by DNA-binding dyes (e.g. SYBR Green), whereof TaqMan technology (Applied Biosystems, Foster City, CA) was used for analysis of mRNA expression in this thesis. The detection has highest precision in the exponential phase of the amplification curve where a threshold is set and from the cycle number (C_T) is read. A low C_T-value indicates high number of transcripts while high C_T-values correspond to low number of transcripts.

The TaqMan system uses the 5'-3' exonuclease activity [133] of *Taq* DNA polymerase and dual-labeled probes with a 5' fluorescent reporter and 3' quencher dye. The TaqMan probe is complementary and anneals to the target sequence between forward and reverse primer. When the TaqMan-probe is intact, fluorescent emission of the reporter dye will be absorbed by the quencher dye while degradation induces a fluorescent signal (Figure 10). During the elongation step the probe will be cleaved by the exonuclease activity of the *Taq* DNA polymerase, and thus increase in reporter fluorescence emission. The fluorescence generated is directly proportional to the amount of generated PCR product.

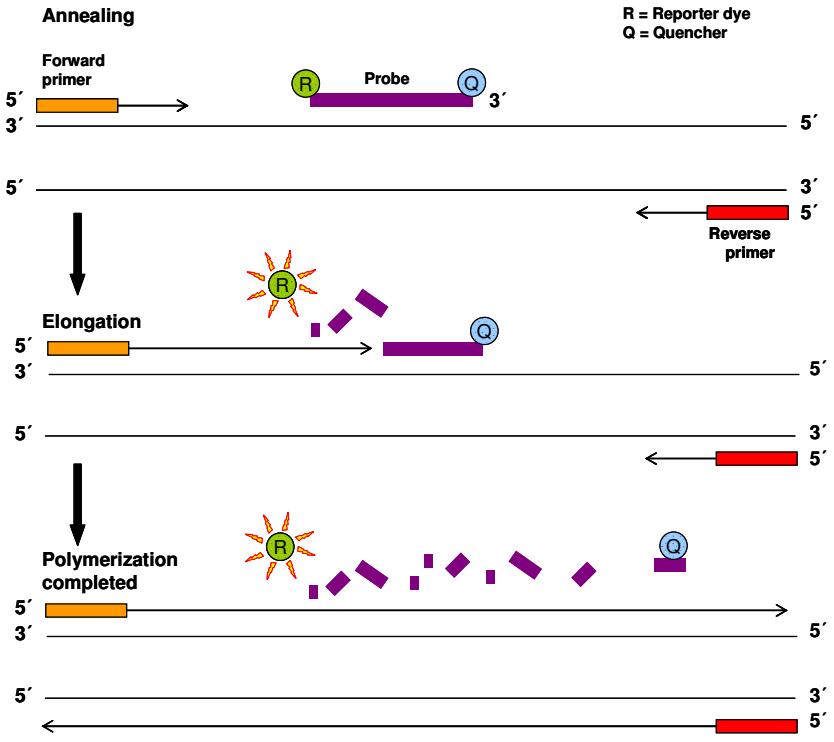

Figure 10. *Principle of TaqMan. Primers and probe anneals to the target gene and under the elongation process the probe is cleaved by the 5'-3' exonuclease activity of the Taq polymerase, allowing fluorescence emission.*

Quantification of results from real-time RT-PCR can be obtained using different methods; most common is the standard curve method (used in this thesis) and the comparative C_T-method. The standard curve method is based on interpolation of the sample against a standard curve generated by amplification of serial dilutions of a standard sample. In the comparative C_T-method there is no need to construct a standard, however the PCR efficiencies of target and internal control must be identical. Correction for experimental variations such as differences in starting amount of RNA or differences in cDNA conversion efficiency is normally done by normalization to a house keeping gene (e.g. *GAPDH, β-ACTIN* or *GUSB*). In this thesis *GUSB* (encoding β-glucoronidase) was used for normalization since it previously was shown to have constant expression in neuroblastoma tumors of various stages [134].

Primers and probes were ordered from Applied Biosystems and reactions were carried out in duplicate or triplicate in 385-well plates using an ABI PRISM® 7900HT sequence Detection System (Applied Biosystems). Replicates were considered to be reliably reproduced if ΔC_T was less then 1, which corresponds to a two-fold change in expression at maximum PCR efficiency. Two non-template controls were included in each assay in order to detect contamination.

Protein detection with antibodies

Analysis of proteins commonly includes antibodies with high specificity to a specific antigen on the protein. There are several different techniques that utilizes protein-antibody interaction; Western blot (WB), immunoprecipitation (IP), flow cytometry or immunohistochemistry, where of WB (*Paper III and IV*) and IP (*Paper IV)* was used in this thesis.

Western blot

WB (immunoblotting) is a semi-quantitative method introduced by Towbin et al in 1979 [135]. Proteins are separated in a gel based on length (denatured proteins) or 3D-structure (native proteins) using gel electrophoresis before transferring of proteins to a membrane. Primary antibodies against the protein of interest are added to the membrane after incubation with a blocking buffer (e.g. skimmed milk) that decreases non-specific binding of the antibody. Detection of the proteins is made by using a labeled secondary antibody that binds to the primary antibody. There are different detection systems, allowing usage of either fluorescence-labeled secondary antibodies or the more commonly used horseradich-peroxidase conjugated secondary antibodies. Horseradish peroxidase cleaves a chemiluminiscent substrate leading to emission of light that can be detected. As with real-time RT-PCR endogenous controls (e.g. Gapdh and β-actin) are used to normalize samples and estimate relative protein levels.

Immunoprecipitation

Immunoprecipitation of proteins is performed to separate and enrich the protein of interest or to study protein-protein interactions (Co-IP). The method used in *Paper IV* is based on conjugation of primary antibodies to magnetic beads and subsequent binding of proteins to the antibodies. The bound protein is eluted and depending on the stringency of the washing steps, possible also other interacting proteins. The collected protein sample can then be analyzed by immunoblotting, with antibodies against known proteins or proteins thought to interact with the immunoprecipitated protein.

Cell based assays

Primary cell cultures established from fresh tissues normally has a limited number of replications before entering senescence (the Hayflick limit) [136], in contrast to immortalized cells with unlimited replicative capacity. One example of an immortalized cell line is HeLa, established in

1951, that still is widely used in scientific research. Transformation can occur spontaneously or be induced by viral infections [137, 138]. Cell lines are used as model systems for study of biological issues and are relatively easy to manipulate through pharmacological or physiological treatments. The effect of a manipulation can be assessed in numerous ways by studying effect on mRNA- and/or protein levels, morphology, proliferation, apoptosis, cell cycle arrest, migration, colony forming capacity etc.

Cells derived from mammals are commonly cultured on solid surfaces in culturing media supplemented with nutrients and antibiotics. Cell lines used in this thesis was cultured under standard conditions (5% CO_2 , 37°C) in DMEM supplemented with 10% bovine growth serum (BGS) and 1% penicillin/streptomycin (PEST) [117]. MEFs were cultured in DMEM with 1µM β-Mercaptoethanol supplemented with 10% BGS, 1% PEST, 1% non-essential amino acids and 1% glutamine.

Pharmacological treatments
Treatment with 5´-Aza-2-deoxycytidine (5´-Aza-dC) and Trichostatin A (TSA) is commonly used for epigenetical studies (Figure 11). 5´-Aza-dC is a modified cytosine analogue that is incorporated during DNA synthesis and binds all available DNA-methyltransferases in the cell, which prevents methylation of CpG-sites and leads to increased gene transcription. TSA inhibits histone deacetylases (HDAC) and enables removal of acetyl groups from the histone tails and thereby increases transcription. Up-regulation in mRNA expression after treatment with 5´-Aza-dC or TSA indicates possible epigenetical regulation of the target genes or their effectors. These agents were used in *Paper II* and outcome was analyzed by quantitative RT-PCR.

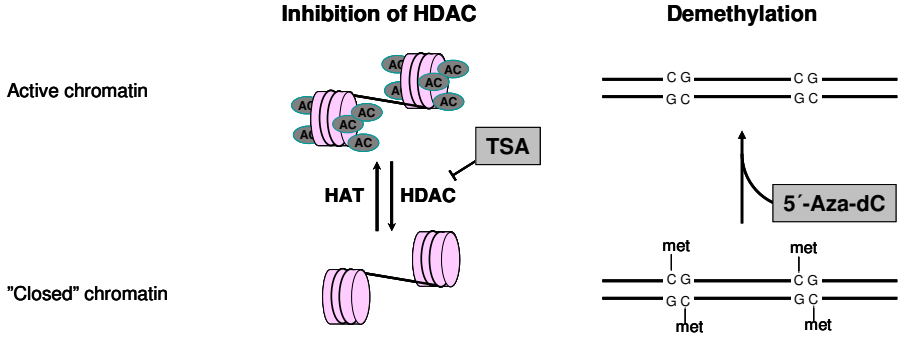

Figure 11. *Functions of TSA and 5'-Aza-dC. TSA inhibits HDAC that removes acetyl groups from the histone tails. This leads to decreased interaction between the nuleosome and the DNA and hence, an active chromatin. Treatment with 5'-Aza-dC leads to a demethylated DNA, associated with transcriptionally active chromatin. HAT = histone acetyltransferase*

Transfections
Cells can be manipulated in order to add or change mRNA expression of a specific transcript and corresponding protein levels. This can be achieved by transfection of expression vectors, *in vitro*-transcribed mRNA [139] or RNA that interferes with transcripts in the cell. In this thesis we have performed both stable and transient transfections of HEK-293 cells in order to explore the localization and function of ectotopically expressed p37δ as well transfections of siRNA targeting

transcripts derived from *PIK3CD* (*Paper IV*). Fluorescent fusion-constructs were generated using human cDNA or MGC image clones cloned into pDsRed-Monomer-N1 or pAc-GFP1-N2 vectors (Clontech, Mountain View, CA), creating constructs both with and without dsRed/GFP1-fusion tags. All constructs were verified by BigDye Cycle Sequencing (Applied Biosystems).

Assays for analyzing tumorigenic capacity
Transfection of a gene with oncogenic potential into a cell is expected to increase properties associated with malignant cells such as increased proliferation, invasive capacity or colony forming capacity. The viability assay CellTiter96 Aqueous One Solution (Promega, Madison, WI), used in *Paper IV*, is a colormetric method based on an enzymatic reaction in metabolic active cells, resulting in measurable shift in absorbance where high absorbance indicates high number of living cells. The viability assay measures the absolute number of cells and can not distinguish if increase in viability is caused by increased proliferation or augmented cell death.

In *Paper IV* we also used BD BioCoat™ Matrigel™ Invasion Chamber (BD Bioscience, Bedford MA) in order to analyze the invasive capacity of cells. This assay evaluates metastatic potential based on the cells capacity to migrate towards a chemoattractant (i.e. from serumfree media to complete media) through a membrane coated with a layer of matrigel matrix. This layer reconstitutes an *in vitro* basement membrane and blocks non-invasive cells while invasive cells can migrate through the matrix and the pores of the membrane.

A common method to study transformation is in soft agar assays (colony formation assay) where cells are seeded in semi-solid media. This reduces the cells movement and allows individual cells to grow into colonies. A majority of normal cells need to attach to a solid surface to grow, and fail to divide in viscous media. However, when cells are transformed they become anchorage-independent and are able to grow in semi-solid media. This change in phenotype is assumed to be related to the process of *in vivo* carcinogenesis.

DNA sequencing
DNA sequencing is used to determine the exact sequence of nucleotides in a region of interest. The method commonly used was originally developed by Sanger *et al.* 1997 [140] and is based on fluorescent labeled nucleotides lacking the hydroxyl-group at the 3' carbon atom, dideoxynucleotides (ddNTP). The ddNTPs are added to the sequence reaction together with normal nucleotides, DNA polymerase and one primer. The ddNTPs will be incorporated randomly and because of the lacking hydroxyl-group at the 3' carbon atom, further elongation is not possible. The sequence reaction will result in nucleotide chains of different lengths, from chains ending directly after the forward primer to chains covering the whole amplicon. The nucleotide chains are then separated and analyzed with laser excitation of terminal fluorescent dye and the DNA sequence is evaluated. Big Dye Terminator ddNTP (Applied biosystems) was used to sequence genomic DNA, bisulfit treated genomic DNA (*Paper II*) and cloned constructs (*Paper IV*).

Bisulfite modification
Conventional sequencing can not be used to detect methylated cytosines unless the DNA is treated with bisulfite [141]. Treatment with bisulfite leads to deamination of cytosines into uracil unless they are methylated. The modified template is amplified by PCR where methylated cytosines remains as cytosines while deaminated cytosines are converted to uracil, and recognized as thymines in the sequencing reaction.

Methylation status can be analyzed by methylation-specific PCR (MSP) where two set of primers with preference for either methylated or unmethylated template are used. PCR-products are then assessed on agarose gel. However, the golden standard in methylation analysis is bisulfite sequencing, used in *Paper II*. With this method, amplifications are performed with primers without preference for methylated or unmethylated DNA and followed by sequencing. Bisulfite sequencing has the advantage of analyzing a large number of CpG-sites and could also provide information on the efficiency of the bisulfite conversion.

Drosophila Melanogaster and GAL4/UAS

Drosophila Melanogaster is commonly known as the fruit fly and has been used as model organism for studies in genetics and developmental biology since 1906 [142]. The fruit fly has the advantage of a short generation time, being easily reared in laboratory and production of high number of offspring. Another advantage is that transgenic flies are easily made using transposons (P elements) for insertion of a gene or construct. A method to study gene expression in *Drosophila* was developed in 1993 by Brand and Perrimon based on the GAL4 gene and an Upstream Activator Sequence (UAS) [143]. In this system GAL4, encoding Gal4, is controlled by a native promoter. Gal4 binds to the UAS region and drive the expression of genes located downstream of the UAS element. Thousands of different *Drosophila* lines have been developed where GAL4 are set under control of different promoters with various transcriptional patterns, thus allowing study of gene expression in specific tissues and developmental time. In *Paper IV* we used a construct of p37δ in a line of *Drosophila* where GAL4 was placed under control of the *Daughterless* (*da*) promoter (Figure 12). *Da* is normally found in the embryonic fly with maximum levels during stage 9-11 and is present in ectodermal cells as well as in neuronal precursors, which shows slightly higher levels of Da, whereas *da* mainly is expressed in the ovary under egg chamber morphogenesis in the adult fly.

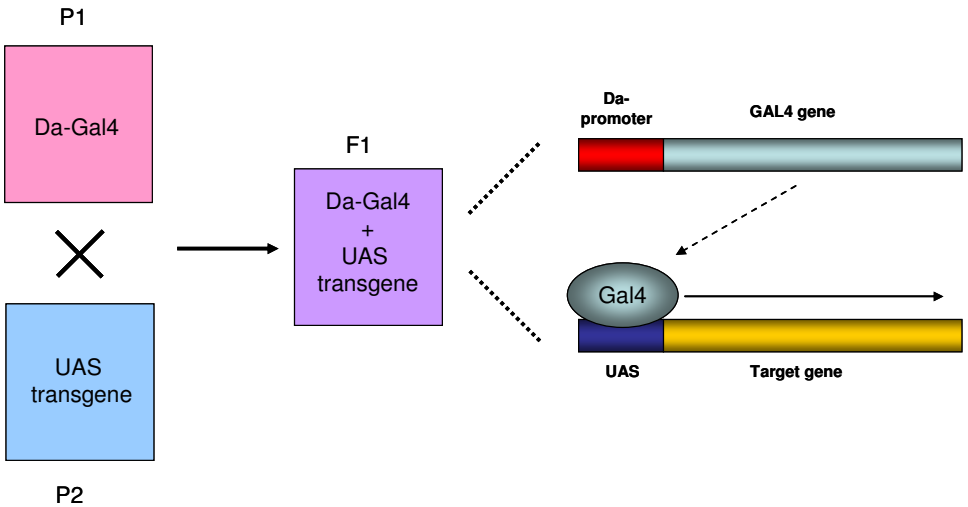

Figure 12. *The GAL4/UAS system in drosophila. Transgenic flies expressing Gal4 under the da promoter were crossed with transgenic strains expressing different constructs under control of UAS. Progeny carrying both the GAL4-element and the UAS transgene are selected for further studies. Gal4 binds to the UAS element, enabling transcription of the target gene only when and where Da is expressed.*

Statistical methods

Two-tailed t-test
Two-tailed t-test is used to compare two populations and was used in all papers. In *Paper I-III* to compare mRNA- and protein levels between two groups of NB; 1p-deleted vs. non-deleted and unfavorable vs. favorable; no evidence of disease (NED) vs. dead of disease (DOD); stage 1-2 vs. stage 4 and *MYCN*-amplified vs. non-amplified respectively. Two-tailed t-test was also used to assess protein levels (*Paper III and IV*), proliferation and migration (*Paper IV*). The relative gene expression and protein levels were first log transformed to achieve a more normal distribution.

One-tailed t-test
One-tailed t-test can be used if assuming only one direction of the difference between two groups. One-tailed t-test was used in *Paper III* comparing stage 1-2 vs. stage 4 NB in the TaqMan validation of cDNA microarray data and in analyze of mRNA expression with other clinical markers of aggressive disease besides stage.

Bonferroni correction
Conducting a large number of tests on the same data-set increases the risk of getting false positive results (Type I error). The Bonferroni correction deals with this problem by adjusting the p-values by multiplying the achieved p-value by the total number of tests that have been conducted. Bonferroni correction is reasonable for a small number of comparisons but for a large number of comparisons there is a high risk of getting false negative results (Type II error).
Stepdown Bonferroni is a less conservative method with smaller risk of Type II errors compared to regular Bonferroni [144] and was used in *Paper I and II* to correct for multiple testing because of the large number of compared genes.

Spearman's rank correlation coefficient
Spearman's rank correlation coefficient is a non-parametric test of relationship between two variables. This test was used to analyze correlation in mRNA expression (*Paper III*) and protein levels (*Paper IV*).

Boxplots
Boxplots have been used to graphically describe statistics of groups (*Paper I-III*). Explanation of boxplots; upper hinge of the box, 75^{th} percentile; lower hinge of the box, 25^{th} percentile; thick horizontal line within box, median. The whiskers are indicating range, open circles represent outliers while asterisks represent extremes.

RESULTS AND DISCUSSION

Analysis of genes located at 1p36.2-3 in NB (*Paper I-III*)

The distal part of chromosome one is frequently deleted in aggressive neuroblastomas and it is believed that one or several putative tumor suppressor genes are located within this region. Several attempts have been made to narrow the critical region and previously a shortest region of overlap (SRO) of deletions between markers D1S80 and D1S244 was defined [87, 145]. This region of 25 cM was further delimited by including germ line tumors, another embryonic cancer with frequent deletions of 1p [146], to a combined SRO of deletion of 5 cM defined by markers D1S508 and D1S244 [78]. Since only a few rare mutations have been found in the genes located within this region [147-154], we wanted to explore their mRNA expression and possible epigenetical regulation.

mRNA expression (Paper I-III)

Expression of 30 known and putative genes located between markers D1S508 and D1S244 were analyzed by real-time RT-PCR (TaqMan) in order to gain further insight of the biological differences between subgroups of neuroblastoma. A decrease of expression levels in stage 4 compared to favorable tumors (i.e. stage 1-2 and stage 4S) was seen for *RERE*, *PIK3CD*, *LZIC*, *PGD* and *PEX14* while the opposite was seen for *SLC2A5* that showed lower expression in favorable tumors. Comparing 1p-deleted with NB tumors with intact 1p, *TNFRSF9*, *RERE*, *PIK3CD*, *CLSTN1*, *CTNNBIP1* and *CASZ1* showed lower mRNA expression in 1p-deleted tumors. However, after correction for multiple testing by step-down Bonferroni only *PIK3CD* and *TNFRSF9* was found to be significantly lower expressed in 1p-deleted NB compared to non-deleted. In *Paper II* we found that *CASZ1* and *PIK3CD* had lower expression in unfavorable NB tumors (i.e. dead of disease) compared to favorable (i.e. no evidence of disease). The expression pattern of *PIK3CD* was also confirmed in *Paper III* using both cDNA micro arrays and real-time RT-PCR. Besides lower expression in stage 4, expression also correlated to other markers of aggressive disease such as INRG-staging and *MYCN*-amplification.

Decreased expression of genes located at 1p in aggressive NB could partly be due to higher frequency of 1p-deletions in these tumors. Albeit lower expression was seen for some of the genes located at 1p36.2-3, no single gene showed complete loss of expression in advanced neuroblastoma tumors. It is possible that haploinsufficiency of one or several genes located within this deleted region can contribute to tumor development or that decreased expression of a specific combination of genes at 1p36 is required.

Epigenetic regulation and mutation screening (Paper II)

In this study we treated NB cell lines with the demethylation agent 5´-Aza-dC or the histone deacetylase inhibitor TSA in order to explore epigenetic mechanisms in regulation of the genes in the 1p36 chromosomal region. The treatment was optimized by analyzing a panel of known methylated genes in NB and a panel of reference genes not affected by the treatment. Three of the 30 genes analyzed (RBP7, *CASZ1* and *PIK3CD*) was found to be up regulated more than two-fold after treatment with TSA or 5´-Aza-dC, and *ERRFI1* after treatment with TSA, indicating possible epigenetical inactivation. A few sequence variants were found in *ERRFI1* and *PIK3CD* in NB tumors and bisulfite sequencing revealed methylation in the promoters of *PIK3CD* and *CASZ1*.

ERRFI1 (also known as *MIG-6* or *RALT*) negatively regulates signaling through the epidermal growth factor (EGF) receptor by inhibiting its catalytic function and stimulate degradation [155] and thus has tumor suppressive activity [156]. We did not detect any difference in expression when comparing favorable with unfavorable neuroblastomas, and treatment with 5'-Aza-dC did

not affect expression levels. Also, *ERRFI1* does not harbor a CpG-island, consequently we concluded that this gene is not regulated by DNA methylation. However, expression of *ERRFI1* was up-regulated after treatment with TSA, indicating that histone deacteylation can have a role in regulation of *ERRFI1* or its effectors. Sequencing revealed two different mutations in two tumors, whereof one resulted in an amino-acid change in a conserved region.

RBP7 is a retinol-binding protein found to be epigenetically silenced in high frequency by DNA-methylation in nasopharyngeal carcinomas [157]. No difference in mRNA expression was seen comparing favorable and unfavorable NB but *RBP7* was up-regulated after treatment with either TSA or 5'-Aza-dC. However, DNA methylation was not detected around the transcription start, indicating that up-regulation of *RBP7* after treatment with TSA or 5'-Aza-dC could be due to increased levels of its effectors or by an unspecific drug effect.

The gene *CASZ1* encodes a homolog to the transcription factor Castor. Castor is involved in neuronal cell linage specification, required for CNS neuronal development in *Drosophila Melanogaster* [158, 159]. Expression of *CASZ1* was up-regulated after treatment with either TSA or 5'-Aza-dC and DNA methylation was seen in cell lines and primary NB tumors. Four fragments covering three CpG-islands were analyzed; one in the CpG-island 1 at the transcription start, two fragments covering the CpG-island 2 at the start of translation and one fragment in CpG-island 3 located at the second translated exon of *CASZ1*. CpG-island 1, as well as the second fragment in CpG-island 2, was unmethylated while the first fragment of CpG-island 2 were heavily methylated in NB cell lines and partially methylated in primary NB tumors and blood from healthy donors. CpG-island 3 was methylated in cell lines and healthy blood controls but not in neuroblastoma tumors. *CASZ1* is almost completely methylated in testes, although the pattern of methylation in other tissues is highly variable [160, 161], indicating that methylation could be important in regulating tissue specific expression. Since it is involved in neuronal development and shows decreased expression in fatal NB, one might speculate that aberrant methylation of *CASZ1* could have implications in the development of neuroblastoma.

Signaling through PI3K/Akt is central in numerous biological functions and deregulation of this pathway has also been implicated in neuroblastoma. *PIK3CD* encodes one of the catalytical subunits of the Type IA PI3K. *PIK3CD* is mainly expressed in leukocytes and is also found in neurons. Resequencing of *PIK3CD* showed two patients harboring missense mutations leading to changes of five amino-acids, all located within evolutionary conserved regions. The tumors with mutations also had low gene expression levels. Expression of *PIK3CD* was lower in unfavorable neuroblastoma compared to favorable and treatment with either TSA or 5'-Aza-dC led to increased mRNA expression in NB cell lines, indicating involvement of epigenetic regulation. As for *CASZ1*, *PIK3CD* has a tissue specific pattern of methylation [161]. In this study we found partial methylation of CpG-island 1, located at the transcription start of *PIK3CD,* in NB cell lines and also methylation within CpG-island 3 in cell lines and leukocytes from healthy donors. However, no methylation was detected in primary NB tumors, indicating that low expression of *PIK3CD* in unfavorable neuroblastoma probably is due to the frequent deletions of 1p in aggressive NB.

Expression analysis of PI3K/Akt genes (*Paper III*)

In previous studies we found *PIK3CD,* encoding one isoform of the catalytic subunit of PI3K, to be lower expressed in NBs with 1p-deletion compared to non-deleted tumors or in tumors with fatal outcome compared to tumors with favorable outcome. PI3K plays an important role in many human cancers with much attention paid on two central players; *PIK3CA* and *PTEN*. Constitutively activating mutations in *PIK3CA* are frequently seen in common cancers of the

breast, prostate, colon and endometrium while the tumor suppressor gene *PTEN* is mutated or deregulated in several other advanced cancers [105]. In neuroblastoma mutations in these two genes are rare [114, 115] and expression of *PIK3CD* is lower in aggressive NB compared to NB with more favorable biology. Despite this, levels of phosphorylated Akt is high in NB primary tumors compared to adrenal gland and predicts outcome [112]. Increased levels of phosphorylated Akt can be achieved in different ways, by increased RTK stimulation, enhanced PI3K activity or decreased phosphatase activity. Given the importance of PI3K in cancer, we investigated genes in this pathway at mRNA and/or protein level to gain further insight of this pathway in neuroblastoma.

The gene *PDGFRA,* encoding a RTK of the platelet-derived growth factor receptor-family (Prkcα), showed higher expression in stage 4 neuroblastomas compared to stage 1-2. This gene is important in the development of neural crest-derived cells [162], and differentiation of SHSY5Y by TPA leads to down-regulation of Prkcα [163]. Favorable NB tumors express more markers of neural differentiation [164] and consequently should lower levels of *PRKCA* be anticipated in low stage NB. However, the PDGF-recptors can also become powerful oncogenes when over expressed [165, 166] and it is possible that *PDGFRA* is generally over-expressed in neuroblastoma tumors but to higher extent in stage 1-2.

The genes *PRKCZ*, *PRKCB1, EIF4EBP1*, *PIK3RI* and *PIK3CD* showed lower expression in stage 4 compared to stage 1-2 tumors. *PIK3CD* and *PRKCZ* are both located in the chromosomal region 1p36 and decreased expression in stage 4 tumors can partly be due to higher frequency of 1p-deletions in this subgroup. However, expression of *PRKCZ*, *PRKCB1*, *PIK3CD* and *PIK3RI* also associated with *MYCN*-amplification, 1p-deletion, outcome and staging according to INRG, indicating that lower expression of these genes is a general marker of aggressive disease.

Two members of the PKC family (*PRKCZ* and *PRKCB1)* were expressed at higher levels in stage 1-2 NB tumors compared to stage 4. The different members of this family has unique and even opposite effects in the cell and although a degree of redundancy has been suggested, increasing evidence supports individual, non-redundant, albeit subtle roles for members of the PKC family [167]. Silencing of PKCζ, encoded by *PRKCZ,* leads to impaired migration in glioblastoma, indicating a role in metastasis [168] that stands in contrast to a report of pro-apoptotic function in ovarian cancer [169]. PKCβI, encoded by *PRKCBI*, stimulated growth and proliferation in neuroblastoma [170] but activation of PKCβ induced apoptosis in HL60-cells [171]. This suggests different roles of the PKC isoforms depending on stimuli and that further effort is needed to elucidate the function of the various members of the PKC family in neuroblastoma.

On mRNA level, the only catalytical subunit of type IA PI3K showing differential expression between stage 1-2 and stage 4 was *PIK3CD*, which was confirmed at the protein level by western blot. Albeit no difference at the mRNA level, protein levels of p110α were significantly higher in stage 4 NB compared to stage 1-2 NB. It is possible that there is a redundancy between the different catalytic subunits of PI3K where p110δ is more important in favorable neuroblastomas and p110α in aggressive NB. Since PI3K also is required in neural differentiation one could hypothesize that the expression pattern of the different catalytic subunits reflects the degree of differentiation and that p110δ is required for axonal growth and regeneration of neurons [52]. However, the contribution of the different catalytic isoforms in neural differentiation needs further attention.

PIK3R1 encoding p85α, a regulatory subunit of PI3K, showed lower mRNA expression in stage 4 tumors compared to stage1-2 NB. It has been suggested that p85 functions as a tumor suppressor gene through its regulation of PI3K activity, and decreased expression is indeed seen

in cancer (e.g. ovarian, prostate and liver). p85 has dual function in the negative regulation of PI3K-signaling, partly by regulation of PI3K, but also by stabilizing Pten and enhancing its phosphatase activity. Mutations and silencing of Pten are reported in several malignancies but we could not detect any significant difference in mRNA and protein levels of Pten comparing stage 1-2 and stage 4 NB. However, levels of Pten correlated negatively with levels of phosphorylated Akt T308 and S473, which were found in higher levels in stage 4 NB compared to stage 1-2, concordant with other studies [111, 112].

Localization and function of p37δ, a novel splice variant of *PIK3CD* (*Paper IV*)

PIK3CD was found to be differentially expressed in our previous studies (*Paper I-III*) where a lower mRNA level was associated to different markers of aggressive disease. During screening of *PIK3CD* mRNA, a novel isoform of p110δ was discovered. Alternative splicing by usage of an alternative donor site leads to a previously unknown protein, p37δ. This protein lacks the catalytic domain but retains the p85-binding domain, a part of the RAS-binding domain and has a unique carboxy-terminal region (Figure 13).

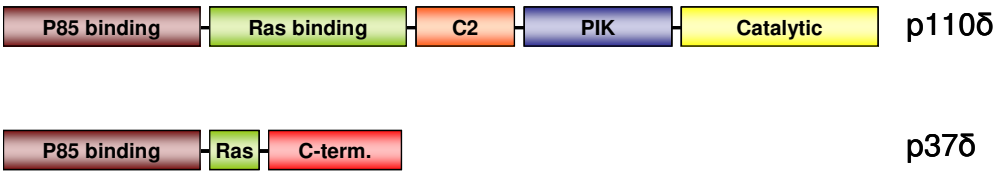

Figure 13. *Domains of p110δ and p37δ. Alternative splicing leads to an early stop and a truncated protein corresponding to 37kDa. Besides a partial RAS binding domain and a complete p85 binding domain it also has a unique C-terminal region.*

Co-immunoprecipitation revealed association between p37δ and p85α as well as RAS, despite the truncation in the RAS-binding domain and independent of RAS' GTP status. Interestingly, in colorectal mucosa and tumors, protein levels of p37δ correlated with RAS which in turn, correlated to levels of phosphorylated ERK. The stabilizing function of p37δ on RAS was abrogated by transfection of siRNA targeting both full-length and alternatively spliced *PIK3CD*, which suppressed p37δ and led to decreased levels of RAS and phosphorylated ERK. Transgenic expression of human p37δ in *Drosophila* led to flies with 7% increased body weight and increased levels of phosphorylated ERK.

The levels of alternatively spliced *PIK3CD* mRNA and p37δ were low in most normal tissues analyzed, besides in lymphocytes, thymus, spleen and lung, whereas levels of p37δ were moderate in colorectal tumors and high in ovarian cancer. In colorectal cancer levels of p37δ were higher in both tumors and adjacent mucosa compared to normal colon, with significantly higher levels of p37δ in the adjacent mucosa compared to the tumors themselves. Genetic alterations in histologically normal mucosa adjacent to tumors are not uncommon and are important for prognosis [172-174]. This indicates that expression of p37δ could be an early event in colorectal tumorigenesis.

Over-expression of p37δ in HEK-293 cells and MEFs significantly increased proliferation compared to both control and cells over-expressing p110δ. Furthermore, p37δ also increased invasiveness of MEFs. This indicates that p37δ has tumor promoting capacity, but not enough for full transformation since p37δ-expressing cells had no colony forming capability.
Co-transfected p85α fused with GFP and p37δ fused with dsRed aggregated in cytoplasmic foci consistent with aggregates seen for ectopically and endogenous expressed p85α [175]. This pattern was not seen when p37δ was transfected solely or for co-transfection of p85α and p110δ. One could postulate that p85 function as a tumor suppressor gene through controlling the catalytic activity of PI3K and increasing stability and phosphatase activity of Pten. Since we found that over-expression of p37δ leads to decreased levels of p85α, is it possible that besides stabilization of RAS, p37δ also contributes to tumorigenesis through binding and inactivation of p85.

GENERAL DISCUSSION

This thesis has increased the knowledge about the biology of the childhood cancer neuroblastoma. Comprehensive studies of the chromosomal region 1p36.2-3 have gained insight of expression, epigenetical regulation and mutations of the genes located within this region.

PIK3CD, located at 1p36, was found to be expressed at lower levels in unfavorable compared to favorable NB. *PIK3CD* encodes p110δ, a catalytical isoform of PI3K. Deregulation of the PI3K/Akt pathway is implicated in the pathogenesis of several malignancies. Increased levels of phosphorylated Akt was detected in stage 4 compared to stage 1-2 NB, indicating that the PI3K/Akt pathway is deregulated in NB as well. We also found that low stage tumors (stage 1-2) showed high levels of p110δ while stage 4 tumors showed high levels of p110α. The PI3K-pathway has become an attractive target in cancer therapeutics, since it is commonly up-regulated in cancer. PI3K is involved in many cellular processes and probably would a global PI3K-inhibition have severe side-effects. However, several drugs targeting specific PI3K isoform are under development and some are in early clinical trials. Because of their specific inhibition they are believed to be more useful in cancer treatment, as lesser toxicity is expected compared to general PI3K inhibitors. NB tumors express different p110-isoforms depending on stage, indicating that the patients with NB tumors of different stages would benefit from receiving different PI3K-inhibitors (when available), as part of the treatment.

The different levels of the α- and δ-isoforms in different stages of NB could indicate that there is redundancy in the usage of p110-isoforms in PI3K/Akt signaling or that they have different roles depending on NB subtype. It is known that signaling through PI3K is essential for differentiation of NB cell lines and that p110δ is required for neurite outgrowth. In low stage NB, which usually express more markers of neural differentiation compared to stage 4 tumors, high levels of p110δ were detected. This indicates that the levels of the different PI3K p110-isoforms reflect different degree of differentiation of NB tumors, however this hypothesis requires further attention. Planned studies addressing this question includes analyzes of the impact of PI3K-isoform specific inhibition, either through siRNA or pharmacological treatment, on differentiation of NB cell lines.

We also found a novel splice variant, p37δ, encoded by *PIK3CD*, that is highly expressed in ovarian- and colorectal cancer and that also is expressed in neuroblastoma tumors (unpublished data). p37δ confer growth advantages in cell culture and increased body size in *Drosophila*, possibly through the interaction between p37δ and RAS, and/or between p37δ and p85. Current p110-isoform specific inhibitors are all targeted against the ATP-binding site within the catalytical domain. These compounds are promising in inactivation of PI3K-activity but the emerging kinase-independent functions of these kinases can be more challenging to target. Example of kinases with kinase-independent function is Aurora A that stabilizes Myc [176], or p110β that have a role in cell proliferation and trafficking [56]. If p110δ also have functions not related to its catalytical activity it is possible that the functions of p37δ are related to kinase-independent functions of p110δ. It is also possible that the unique C-terminal portion of p37δ have properties that contribute to the growth promoting effects of p37δ in cell lines and *Drosophila*. Further studies addressing this question include functional studies of truncated or mutated constructs of *PIK3CD*/p110δ, in cell lines and *Drosophila*. As cell lines and fruit flies have limitations in *in vivo* mammalian biological relevance, development of a mouse model expressing p37δ is crucial in order to elucidate the function of p37δ.

Cancer is an enigmatic disease, and despite recent progress in treatment, mortality is still high in many forms of cancer. This is also true for children with neuroblastoma, where prognosis has

improved in recent decades, although the outcome for patients with high stage NB is still poor. Because of this it is essential to learn more about the genes and pathways involved in the pathogenesis of NB. Better understanding of the underlying mechanisms can lead to better treatment, either by identifying new therapeutic targets or by patient stratification, thus improving efficiency of already available treatments. Translational research and constructive dialogues between biologists and clinicians are also important to increase survival for children with NB.

SAMMANFATTNING PÅ SVENSKA

Neuroblastom (NB) är en allvarlig barncancerform som drabbar 1 av 7000 födda barn vilket motsvarar 15-20 nya fall per år i Sverige. Hälften av alla fall av NB upptäcks innan 1 års ålder och sällan efter 5 års ålder. Tumörer uppkommer från omogna celler (neuroblaster) i det sympatiska nervsystemet (SNS) och hittas således på alla de platser där SNS är lokaliserat men vanligen i binjurarna. NB är klinisk heterogen, från tumörer som försvinner av sig själva till tumörer som är resistenta mot alla typer av behandling. Även om överlevnaden har ökat totalt sett under de senaste åren så är överlevnaden för högriskpatienterna fortfarande mindre än 30 %. De som överlever drabbas tyvärr också av allvarliga sidoeffekter från kemo- och strålningsterapi (t.ex. hörselnedsättning eller ökad risk för andra cancerformer). På grund av patienternas låga ålder och den i många gånger fatala utgången är det viktigt att förstå mer om varför NB uppkommer och hur man kan hitta bättre behandlingsmetoder.

Det finns flera genetiska förändringar som är vanligt förekommande i aggressiva NB, däribland amplifiering av onkogenen *MYCN* eller deletion av den yttersta delen av den korta armen på kromosom 1 (1p). Deletion av 1p förekommer i cirka 30 % av all NB och är associerat till dålig prognos. På grund av detta, samt att återförsel av 1p till en 1p-deleterad cell linje ledde till minskade tumöregenskaper, så tros en eller flera möjliga gener med tumörsuppressande funktion vara lokaliserade här. Sedan tidigare har vi i vårt svenska material definierat ett intressesant område lokaliserat till den kromosomala regionen 1p36.2-3. Några gener i området med intressant funktion (ur cancerperspektiv) har sekvenserats, men bara några fåtal mutationer har hittats och ingen klar ”neuroblastomgen” är identifierad. Därför har vi också analyserat generna i området med avseende på expression och epigenetisk reglering. I samband med dessa studier fann vi att genen *PIK3CD* hade lägre uttryck i elakartade NB jämfört med godartade och dessutom kunde genuttrycket uppregleras i cell linjer efter farmakologisk behandling. *PIK3CD* kodar för en katalytisk subenhet av PI3-Kinas (PI3K) som har en central roll i många cellulära funktioner såsom programmerad celldöd, celltillväxt eller differentiering. Regleringen av PI3K-signalering är ofta felaktig i många cancerformer och även i NB såg vi en ökad aktivering av Akt, ett protein nedströms om PI3K. För full aktivering av Akt krävs fosforylering på två positioner, S473 och T308, och vi fann att båda positioner var fosforylerade i större utsträckning i stadium 4 NB jämfört med stadium 1-2. Dessutom fanns det en omvänd korrelation med fosforylering på T308 och proteinnivåer av tumörssuppressorgenen Pten. Expression av *PIK3CD* och motsvarande protein p110δ är lägre i aggressiva tumörer jämfört med tumörer med mer gynnsam biologi. *PIK3CA* och *PIK3CB* (p110α respektive p110β) kodar för andra katalytiska subenheter av PI3K och har setts bidra till ökad Akt-signalering i andra cancerformer. På mRNA-nivå kunde vi inte se någon skillnad i uttryck av *PIK3CA* eller *PIK3CB* mellan stadium 4 och stadium 1-2 NB men på proteinnivå detekterade vi däremot högre nivåer av p110α i stadium 4 än i stadium 1-2. Detta kan tolkas som att det finns en viss redundans i användningen av de olika katalytiska isoformerna, där p110δ är vanligare i lågstadietumörer och p110α har större betydelse i aggressiva tumörer. p110δ och PI3K är också viktiga i den embryonala utvecklingen av neuron och det är därför också möjligt att uttrycket av *PIK3CD*/p110δ speglar tumörernas differentieringsgrad med högt uttryck av *PIK3CD* i stadium 1-2 NB, som också brukar uttrycka fler markörer för neural differentiering än stadium 4 tumörer. Den exakta rollen för de olika katalytiska isoformerna i neural differentiering kräver dock vidare undersökning. Uttrycket av de olika katalytiska isoformerna i NB är också intressant ur terapeutiskt syfte eftersom flera cancermediciner som riktar sig mot specifika p110-isoformer är under utveckling

Vi har också sett att det förekommer alternativ splicing av *PIK3CD* som leder till ett kortare protein på 302 aminosyror istället för 1032 som normalt. Den nya splicevarianten, kallad 37δ,

saknar den katalytiska domänen och kan därför inte fosforylera protein (t.ex. Akt) nedströms i signalkedjan. Proteinnivåerna av p37δ är låga i de flesta normala vävnader som undersökts förutom i lymfocyter (vita blodkroppar), lunga och thymus (brässen). Däremot fanns proteinet uttryckt i högre nivåer i ovarie- och kolorektalcancer jämfört med motsvarande normalvävnad. I kolorektal cancer såg vi ett intressant mönster där p37δ var högre uttryckt i mucosan precis intill tumören än i själva tumören, vilket tyder på att proteinet kan vara viktigt för själva uppkomsten av cancern. Proteinnivåerna av p37δ korrelerar starkt med proteinnivåerna av RAS och försök tyder på att p37δ stabiliserar RAS. Däremot verkar p37δ destabilisera p85α, den regulatoriska subenheten av PI3K, och skulle därmed också kunna påverka PI3K/Akt signalering, även om vi inte har kunnat se att p37δ påverkar nivåerna av fosforylerat Akt. Överuttryck av p37δ i olika cellinjer leder till ökad viabilitet och ökad migration vilket kan tolkas som att denna splicevariant kan bidra till uppkomst och utveckling av cancer. Vidare så såg vi också att transgena bananflugor (*Drosophila Melanogaster*) som uttryckte humant p37δ i hela kroppen blev 7 % större än kontrollflugor.

Eftersom felaktig reglering av PI3K-signalering är vanligt i många cancerformer arbetar vi vidare med detta, dels med fortsatt kartläggning om de olika isoformernas roll i differentiering av neuron och dels med ytterligare studier av funktion hos p37δ. Vi vill undersöka mer exakt hur p37δ stabiliserar RAS och om p37δ reflekterar funktioner hos p110δ, som inte är beroende av den katalytiska domänen.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to all those who helped me complete this thesis, with special thanks to;

Dr. **Katarina Ejeskär**, my supervisor, for always being encouraging, enthusiastic and inspiring. I admire your positive spirit and am thankful for opportunity to work with this exciting project. It has been a privilege and pleasure to work together with you.

All other collegues with common interest in neuroblastoma; especially **Rose-Marie Sjöberg** for your great sense of humor, sharing of your expertise in the lab and for our mutual interest in "Bergslagssten" as well as the Westma catalogue. **Helena Eriksson,** for support in the lab and all superior western blots. **Helena Carén,** my super supervisor in epigenetics. Thanks for help and support in the lab and for being a good friend**. Cecilia Krona,** my hero in the field of planning and organizing. **Hanna Kryh** for all amusing conversations. **Frida Abel**, **Anna Djos**, **Jennie Erichsen** and **Nilofar Javanmardi,** for all discussions (scientific or non-scientific), laughs and contributing to the cheerful atmosphere in the NB-lab. Professor **Tommy Martinsson**, co-supervisor, for introducing me to the field of neuroblastoma and for always laughing at my jokes.

Maria Yhr, for being a friend and co-worker with a big heart, positive spirit and always showing great hospitality.

All fellow graduate students, former and present, for nice company and sharing of knowledge. Especially thanks to **Stina Petterson** and **Emman Shubbar**, for sharing time, space and antibodies. **Hanna Carlsson** for all conversations about small and big things in life or lab. I know it is selfish, but I wished that you had stayed.

Friends and co-workers at the Department of Clinical Genetics for creating a pleasant working environment and sharing of knowledge, time and space. I have felt supported from the first day of my Master of Science project and all the way through.

Camilla Stiller and **Catrine Forssén** at Genomics core facility for technical assistance.

Therese Börjesson, for being a good friend and reminding me that there is life outside work.

A special thank to **Mira Melander** for your patience when learning me karyotyping, your big heart and being like a grandmother to my children. You mean a lot to me and my family!

My wonderful crowd of boys at home; **Peter**, my fantastic husband, for loving and supporting me, whether or not he is in the country**.** My lovely sons**, Ivar** and **Alvar** for their unconditional love and for always making me smile. I love you all endlessly.

Finally, my deepest gratitude to all families with children with neuroblastoma for their willingness to contribute to this project.

The work in this thesis was supported by grants from the Swedish Cancer Society, the Children's Cancer Foundation, the Nilsson-Ehle Foundation, the Wilhelm and Martina Lundgren's Research Foundation, the Sahlgrenska University Hospitel Foundation and the Foundation in Memory of Lars Hierta, Gunvor and Ivan Svensson's Foundation, Åke Wiberg's Foundation, the Assar Gabrielsson Foundation, Mary Béves Stiftelse för Barncancerforskning and Frimurare Barnhusdirektionen

REFERENCES

1. Watson JD, Crick FH: **The structure of DNA.** *Cold Spring Harb Symp Quant Biol* 1953, **18:**123-131.
2. Vogel F: **A Preliminary Estimate Of The Number Of Human Genes.** *Nature* 1964, **201:**847.
3. Crick FH: **On protein synthesis.** *Symp Soc Exp Biol* 1958, **12:**138-163.
4. Hastings ML, Krainer AR: **Pre-mRNA splicing in the new millennium.** *Curr Opin Cell Biol* 2001, **13:**302-309.
5. Maher CA, Palanisamy N, Brenner JC, Cao X, Kalyana-Sundaram S, Luo S, Khrebtukova I, Barrette TR, Grasso C, Yu J, et al: **Chimeric transcript discovery by paired-end transcriptome sequencing.** *Proc Natl Acad Sci U S A* 2009, **106:**12353-12358.
6. Izquierdo JM, Majos N, Bonnal S, Martinez C, Castelo R, Guigo R, Bilbao D, Valcarcel J: **Regulation of Fas alternative splicing by antagonistic effects of TIA-1 and PTB on exon definition.** *Mol Cell* 2005, **19:**475-484.
7. Luo MJ, Reed R: **Splicing is required for rapid and efficient mRNA export in metazoans.** *Proc Natl Acad Sci U S A* 1999, **96:**14937-14942.
8. Melhuish TA, Wotton D: **The Tgif2 gene contains a retained intron within the coding sequence.** *BMC Mol Biol* 2006, **7:**2.
9. Gunzel D, Stuiver M, Kausalya PJ, Haisch L, Krug SM, Rosenthal R, Meij IC, Hunziker W, Fromm M, Muller D: **Claudin-10 exists in six alternatively spliced isoforms that exhibit distinct localization and function.** *J Cell Sci* 2009, **122:**1507-1517.
10. Grosso AR, Gomes AQ, Barbosa-Morais NL, Caldeira S, Thorne NP, Grech G, von Lindern M, Carmo-Fonseca M: **Tissue-specific splicing factor gene expression signatures.** *Nucleic Acids Res* 2008, **36:**4823-4832.
11. Wang L, Duke L, Zhang PS, Arlinghaus RB, Symmans WF, Sahin A, Mendez R, Dai JL: **Alternative splicing disrupts a nuclear localization signal in spleen tyrosine kinase that is required for invasion suppression in breast cancer.** *Cancer Res* 2003, **63:**4724-4730.
12. Tacconelli A, Farina AR, Cappabianca L, Desantis G, Tessitore A, Vetuschi A, Sferra R, Rucci N, Argenti B, Screpanti I, et al: **TrkA alternative splicing: a regulated tumor-promoting switch in human neuroblastoma.** *Cancer Cell* 2004, **6:**347-360.
13. Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, Fiegler H, Shapero MH, Carson AR, Chen W, et al: **Global variation in copy number in the human genome.** *Nature* 2006, **444:**444-454.
14. de Smith AJ, Walters RG, Froguel P, Blakemore AI: **Human genes involved in copy number variation: mechanisms of origin, functional effects and implications for disease.** *Cytogenet Genome Res* 2008, **123:**17-26.
15. Felsenfeld G, Groudine M: **Controlling the double helix.** *Nature* 2003, **421:**448-453.
16. Jinek M, Doudna JA: **A three-dimensional view of the molecular machinery of RNA interference.** *Nature* 2009, **457:**405-412.
17. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM: **Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs.** *Nature* 2005, **433:**769-773.
18. Davalos V, Esteller M: **MicroRNAs and cancer epigenetics: a macrorevolution.** *Curr Opin Oncol*, **22:**35-45.
19. **Cancerfonden och Socialsyrelsen i samarbete; Cancer i siffror 2009.** 2009.
20. Hanahan D, Weinberg RA: **The hallmarks of cancer.** *Cell* 2000, **100:**57-70.
21. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW: **Specific association of human telomerase activity with immortal cells and cancer.** *Science* 1994, **266:**2011-2015.
22. Kinzler KW, Vogelstein B: **Cancer-susceptibility genes. Gatekeepers and caretakers.** *Nature* 1997, **386:**761, 763.
23. Knudson AG, Jr.: **Mutation and cancer: statistical study of retinoblastoma.** *Proc Natl Acad Sci U S A* 1971, **68:**820-823.
24. Marutani M, Tonoki H, Tada M, Takahashi M, Kashiwazaki H, Hida Y, Hamada J, Asaka M, Moriuchi T: **Dominant-negative mutations of the tumor suppressor p53 relating to early onset of glioblastoma multiforme.** *Cancer Res* 1999, **59:**4765-4769.
25. He L: **Posttranscriptional Regulation of PTEN Dosage by Noncoding RNAs.** *Sci Signal*, **3:**pe39.
26. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM: **Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex.** *Science* 2005, **307:**1098-1101.
27. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA: **Mechanism of activation of protein kinase B by insulin and IGF-1.** *Embo J* 1996, **15:**6541-6551.

28. Cantley LC, Neel BG: **New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway.** *Proc Natl Acad Sci U S A* 1999, **96:**4240-4245.
29. Rohrschneider LR, Fuller JF, Wolf I, Liu Y, Lucas DM: **Structure, function, and biology of SHIP proteins.** *Genes Dev* 2000, **14:**505-520.
30. Gao T, Furnari F, Newton AC: **PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth.** *Mol Cell* 2005, **18:**13-24.
31. Engelman JA, Luo J, Cantley LC: **The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism.** *Nat Rev Genet* 2006, **7:**606-619.
32. Saal LH, Johansson P, Holm K, Gruvberger-Saal SK, She QB, Maurer M, Koujak S, Ferrando AA, Malmstrom P, Memeo L, et al: **Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity.** *Proc Natl Acad Sci U S A* 2007, **104:**7564-7569.
33. Tang JM, He QY, Guo RX, Chang XJ: **Phosphorylated Akt overexpression and loss of PTEN expression in non-small cell lung cancer confers poor prognosis.** *Lung Cancer* 2006, **51:**181-191.
34. Aleskandarany MA, Rakha EA, Ahmed MA, Powe DG, Paish EC, Macmillan RD, Ellis IO, Green AR: **PIK3CA expression in invasive breast cancer: a biomarker of poor prognosis.** *Breast Cancer Res Treat* 2009.
35. Kato S, Iida S, Higuchi T, Ishikawa T, Takagi Y, Yasuno M, Enomoto M, Uetake H, Sugihara K: **PIK3CA mutation is predictive of poor survival in patients with colorectal cancer.** *Int J Cancer* 2007, **121:**1771-1778.
36. Singleton JR, Randolph AE, Feldman EL: **Insulin-like growth factor I receptor prevents apoptosis and enhances neuroblastoma tumorigenesis.** *Cancer Res* 1996, **56:**4522-4529.
37. Ho R, Minturn JE, Hishiki T, Zhao H, Wang Q, Cnaan A, Maris J, Evans AE, Brodeur GM: **Proliferation of human neuroblastomas mediated by the epidermal growth factor receptor.** *Cancer Res* 2005, **65:**9868-9875.
38. Muders MH, Zhang H, Wang E, Tindall DJ, Datta K: **Vascular endothelial growth factor-C protects prostate cancer cells from oxidative stress by the activation of mammalian target of rapamycin complex-2 and AKT-1.** *Cancer Res* 2009, **69:**6042-6048.
39. Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer JM: **Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit.** *Mol Cell Biol* 1998, **18:**1379-1387.
40. Skolnik EY, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, Ullrich A, Schlessinger J: **Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases.** *Cell* 1991, **65:**83-90.
41. Baltensperger K, Kozma LM, Jaspers SR, Czech MP: **Regulation by insulin of phosphatidylinositol 3'-kinase bound to alpha- and beta-isoforms of p85 regulatory subunit.** *J Biol Chem* 1994, **269:**28937-28946.
42. Inukai K, Funaki M, Ogihara T, Katagiri H, Kanda A, Anai M, Fukushima Y, Hosaka T, Suzuki M, Shin BC, et al: **p85alpha gene generates three isoforms of regulatory subunit for phosphatidylinositol 3-kinase (PI 3-Kinase), p50alpha, p55alpha, and p85alpha, with different PI 3-kinase activity elevating responses to insulin.** *J Biol Chem* 1997, **272:**7873-7882.
43. Taniguchi CM, Winnay J, Kondo T, Bronson RT, Guimaraes AR, Aleman JO, Luo J, Stephanopoulos G, Weissleder R, Cantley LC, Kahn CR: **The phosphoinositide 3-kinase regulatory subunit p85alpha can exert tumor suppressor properties through negative regulation of growth factor signaling.** *Cancer Res*, **70:**5305-5315.
44. Luo J, Sobkiw CL, Logsdon NM, Watt JM, Signoretti S, O'Connell F, Shin E, Shim Y, Pao L, Neel BG, et al: **Modulation of epithelial neoplasia and lymphoid hyperplasia in PTEN+/- mice by the p85 regulatory subunits of phosphoinositide 3-kinase.** *Proc Natl Acad Sci U S A* 2005, **102:**10238-10243.
45. Barber DF, Alvarado-Kristensson M, Gonzalez-Garcia A, Pulido R, Carrera AC: **PTEN regulation, a novel function for the p85 subunit of phosphoinositide 3-kinase.** *Sci STKE* 2006, **2006:**pe49.
46. Rabinovsky R, Pochanard P, McNear C, Brachmann SM, Duke-Cohan JS, Garraway LA, Sellers WR: **p85 Associates with unphosphorylated PTEN and the PTEN-associated complex.** *Mol Cell Biol* 2009, **29:**5377-5388.
47. Chagpar RB, Links PH, Pastor MC, Furber LA, Hawrysh AD, Chamberlain MD, Anderson DH: **Direct positive regulation of PTEN by the p85 subunit of phosphatidylinositol 3-kinase.** *Proc Natl Acad Sci U S A*, **107:**5471-5476.
48. Vanhaesebroeck B, Ali K, Bilancio A, Geering B, Foukas LC: **Signalling by PI3K isoforms: insights from gene-targeted mice.** *Trends Biochem Sci* 2005, **30:**194-204.
49. Hu P, Mondino A, Skolnik EY, Schlessinger J: **Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85.** *Mol Cell Biol* 1993, **13:**7677-7688.

50. Vanhaesebroeck B, Welham MJ, Kotani K, Stein R, Warne PH, Zvelebil MJ, Higashi K, Volinia S, Downward J, Waterfield MD: **P110delta, a novel phosphoinositide 3-kinase in leukocytes.** *Proc Natl Acad Sci U S A* 1997, **94:**4330-4335.
51. Chantry D, Vojtek A, Kashishian A, Holtzman DA, Wood C, Gray PW, Cooper JA, Hoekstra MF: **p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes.** *J Biol Chem* 1997, **272:**19236-19241.
52. Eickholt BJ, Ahmed AI, Davies M, Papakonstanti EA, Pearce W, Starkey ML, Bilancio A, Need AC, Smith AJ, Hall SM, et al: **Control of axonal growth and regeneration of sensory neurons by the p110delta PI 3-kinase.** *PLoS One* 2007, **2:**e869.
53. Foukas LC, Berenjeno IM, Gray A, Khwaja A, Vanhaesebroeck B: **Activity of any class IA PI3K isoform can sustain cell proliferation and survival.** *Proc Natl Acad Sci U S A*, **107:**11381-11386.
54. Kang S, Denley A, Vanhaesebroeck B, Vogt PK: **Oncogenic transformation induced by the p110beta, -gamma, and -delta isoforms of class I phosphoinositide 3-kinase.** *Proc Natl Acad Sci U S A* 2006, **103:**1289-1294.
55. Vogt PK, Gymnopoulos M, Hart JR: **PI 3-kinase and cancer: changing accents.** *Curr Opin Genet Dev* 2009, **19:**12-17.
56. Jia S, Liu Z, Zhang S, Liu P, Zhang L, Lee SH, Zhang J, Signoretti S, Loda M, Roberts TM, Zhao JJ: **Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis.** *Nature* 2008, **454:**776-779.
57. Malumbres M, Barbacid M: **RAS oncogenes: the first 30 years.** *Nat Rev Cancer* 2003, **3:**459-465.
58. Orme MH, Alrubaie S, Bradley GL, Walker CD, Leevers SJ: **Input from Ras is required for maximal PI(3)K signalling in Drosophila.** *Nat Cell Biol* 2006, **8:**1298-1302.
59. Rodriguez-Viciana P, Marte BM, Warne PH, Downward J: **Phosphatidylinositol 3' kinase: one of the effectors of Ras.** *Philos Trans R Soc Lond B Biol Sci* 1996, **351:**225-231; discussion 231-222.
60. Rodriguez-Viciana P, Warne PH, Vanhaesebroeck B, Waterfield MD, Downward J: **Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation.** *Embo J* 1996, **15:**2442-2451.
61. Xue L, Murray JH, Tolkovsky AM: **The Ras/phosphatidylinositol 3-kinase and Ras/ERK pathways function as independent survival modules each of which inhibits a distinct apoptotic signaling pathway in sympathetic neurons.** *J Biol Chem* 2000, **275:**8817-8824.
62. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J: **Phosphatidylinositol-3-OH kinase as a direct target of Ras.** *Nature* 1994, **370:**527-532.
63. Deora AA, Win T, Vanhaesebroeck B, Lander HM: **A redox-triggered ras-effector interaction. Recruitment of phosphatidylinositol 3'-kinase to Ras by redox stress.** *J Biol Chem* 1998, **273:**29923-29928.
64. Kinashi T, Katagiri K, Watanabe S, Vanhaesebroeck B, Downward J, Takatsu K: **Distinct mechanisms of alpha 5beta 1 integrin activation by Ha-Ras and R-Ras.** *J Biol Chem* 2000, **275:**22590-22596.
65. Karnitz LM, Burns LA, Sutor SL, Blenis J, Abraham RT: **Interleukin-2 triggers a novel phosphatidylinositol 3-kinase-dependent MEK activation pathway.** *Mol Cell Biol* 1995, **15:**3049-3057.
66. Hu Q, Klippel A, Muslin AJ, Fantl WJ, Williams LT: **Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase.** *Science* 1995, **268:**100-102.
67. Matter WF, Brown RF, Vlahos CJ: **The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs.** *Biochem Biophys Res Commun* 1992, **186:**624-631.
68. Gharbi SI, Zvelebil MJ, Shuttleworth SJ, Hancox T, Saghir N, Timms JF, Waterfield MD: **Exploring the specificity of the PI3K family inhibitor LY294002.** *Biochem J* 2007, **404:**15-21.
69. Wipf P, Halter RJ: **Chemistry and biology of wortmannin.** *Org Biomol Chem* 2005, **3:**2053-2061.
70. Billottet C, Grandage VL, Gale RE, Quattropani A, Rommel C, Vanhaesebroeck B, Khwaja A: **A selective inhibitor of the p110delta isoform of PI 3-kinase inhibits AML cell proliferation and survival and increases the cytotoxic effects of VP16.** *Oncogene* 2006, **25:**6648-6659.
71. Brodeur GM, Seeger RC, Barrett A, Castleberry RP, D'Angio G, De Bernardi B, Evans AE, Favrot M, Freeman AI, Haase G, et al.: **International criteria for diagnosis, staging and response to treatment in patients with neuroblastoma.** *Prog Clin Biol Res* 1988, **271:**509-524.
72. 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* 1993, **11:**1466-1477.
73. Monclair T, Brodeur GM, Ambros PF, Brisse HJ, Cecchetto G, Holmes K, Kaneko M, London WB, Matthay KK, Nuchtern JG, et al: **The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report.** *J Clin Oncol* 2009, **27:**298-303.
74. Cohn SL, Pearson AD, London WB, Monclair T, Ambros PF, Brodeur GM, Faldum A, Hero B, Iehara T, Machin D, et al: **The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report.** *J Clin Oncol* 2009, **27:**289-297.
75. Maris JM, Hogarty MD, Bagatell R, Cohn SL: **Neuroblastoma.** *Lancet* 2007, **369:**2106-2120.

76. Trager C, Kogner P, Lindskog M, Ponthan F, Kullman A, Kagedal B: **Quantitative analysis of tyrosine hydroxylase mRNA for sensitive detection of neuroblastoma cells in blood and bone marrow.** *Clin Chem* 2003, **49:**104-112.
77. Lambooy LH, Gidding CE, van den Heuvel LP, Hulsbergen-van de Kaa CA, Ligtenberg M, Bokkerink JP, De Abreu RA: **Real-time analysis of tyrosine hydroxylase gene expression: a sensitive and semiquantitative marker for minimal residual disease detection of neuroblastoma.** *Clin Cancer Res* 2003, **9:**812-819.
78. Ejeskar K, Sjoberg RM, Abel F, Kogner P, Ambros PF, Martinsson T: **Fine mapping of a tumour suppressor candidate gene region in 1p36.2-3, commonly deleted in neuroblastomas and germ cell tumours.** *Med Pediatr Oncol* 2001, **36:**61-66.
79. Fong CT, Dracopoli NC, White PS, Merrill PT, Griffith RC, Housman DE, Brodeur GM: **Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification.** *Proc Natl Acad Sci U S A* 1989, **86:**3753-3757.
80. Mora J, Cheung NK, Kushner BH, LaQuaglia MP, Kramer K, Fazzari M, Heller G, Chen L, Gerald WL: **Clinical categories of neuroblastoma are associated with different patterns of loss of heterozygosity on chromosome arm 1p.** *J Mol Diagn* 2000, **2:**37-46.
81. Bello MJ, Leone PE, Nebreda P, de Campos JM, Kusak ME, Vaquero J, Sarasa JL, Garcia-Miguel P, Queizan A, Hernandez-Moneo JL, et al.: **Allelic status of chromosome 1 in neoplasms of the nervous system.** *Cancer Genet Cytogenet* 1995, **83:**160-164.
82. Benn DE, Dwight T, Richardson AL, Delbridge L, Bambach CP, Stowasser M, Gordon RD, Marsh DJ, Robinson BG: **Sporadic and familial pheochromocytomas are associated with loss of at least two discrete intervals on chromosome 1p.** *Cancer Res* 2000, **60:**7048-7051.
83. Stock C, Ambros IM, Strehl S, Zoubek A, Fink FM, Gadner H, Ambros PF: **[Cytogenetic aspects of pediatric germ cell tumors].** *Klin Padiatr* 1995, **207:**235-241.
84. Schwab M, Praml C, Amler LC: **Genomic instability in 1p and human malignancies.** *Genes Chromosomes Cancer* 1996, **16:**211-229.
85. Bader SA, Fasching C, Brodeur GM, Stanbridge EJ: **Dissociation of suppression of tumorigenicity and differentiation in vitro effected by transfer of single human chromosomes into human neuroblastoma cells.** *Cell Growth Differ* 1991, **2:**245-255.
86. Brodeur GM: **Neuroblastoma: biological insights into a clinical enigma.** *Nat Rev Cancer* 2003, **3:**203-216.
87. Martinsson T, Sjoberg 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* 1997, **33:**1997-2001.
88. Hogarty MD, Norris MD, Davis K, Liu X, Evageliou NF, Hayes CS, Pawel B, Guo R, Zhao H, Sekyere E, et al: **ODC1 is a critical determinant of MYCN oncogenesis and a therapeutic target in neuroblastoma.** *Cancer Res* 2008, **68:**9735-9745.
89. Shohet JM, Hicks MJ, Plon SE, Burlingame SM, Stuart S, Chen SY, Brenner MK, Nuchtern JG: **Minichromosome maintenance protein MCM7 is a direct target of the MYCN transcription factor in neuroblastoma.** *Cancer Res* 2002, **62:**1123-1128.
90. Maris JM, Weiss MJ, Mosse Y, Hii G, Guo C, White PS, Hogarty MD, Mirensky T, Brodeur GM, Rebbeck TR, et al: **Evidence for a hereditary neuroblastoma predisposition locus at chromosome 16p12-13.** *Cancer Res* 2002, **62:**6651-6658.
91. Perri P, Longo L, Cusano R, McConville CM, Rees SA, Devoto M, Conte M, Ferrara GB, Seri M, Romeo G, Tonini GP: **Weak linkage at 4p16 to predisposition for human neuroblastoma.** *Oncogene* 2002, **21:**8356-8360.
92. Mosse YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, Laquaglia MJ, Sennett R, Lynch JE, Perri P, et al: **Identification of ALK as a major familial neuroblastoma predisposition gene.** *Nature* 2008, **455:**930-935.
93. Caren H, Abel F, Kogner P, Martinsson T: **High incidence of DNA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours.** *Biochem J* 2008, **416:**153-159.
94. Bown N, Cotterill S, Lastowska M, O'Neill S, Pearson AD, Plantaz D, Meddeb M, Danglot G, Brinkschmidt C, Christiansen H, et al: **Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma.** *N Engl J Med* 1999, **340:**1954-1961.
95. Caron H: **Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavourable prognostic markers in neuroblastoma.** *Med Pediatr Oncol* 1995, **24:**215-221.
96. Caren H, Erichsen J, Olsson L, Enerback C, Sjoberg RM, Abrahamsson J, Kogner P, Martinsson T: **High-resolution array copy number analyses for detection of deletion, gain, amplification and copy-neutral LOH in primary neuroblastoma tumors: four cases of homozygous deletions of the CDKN2A gene.** *BMC Genomics* 2008, **9:**353.
97. Abel F, Ejeskar K, Kogner P, Martinsson T: **Gain of chromosome arm 17q is associated with unfavourable prognosis in neuroblastoma, but does not involve mutations in the somatostatin receptor 2(SSTR2) gene at 17q24.** *Br J Cancer* 1999, **81:**1402-1409.

98. Van Roy N, Laureys G, Van Gele M, Opdenakker G, Miura R, van der Drift P, Chan A, Versteeg R, Speleman F: **Analysis of 1;17 translocation breakpoints in neuroblastoma: implications for mapping of neuroblastoma genes.** *Eur J Cancer* 1997, **33:**1974-1978.
99. Laureys G, Speleman F, Versteeg R, van der Drift P, Chan A, Leroy J, Francke U, Opdenakker G, Van Roy N: **Constitutional translocation t(1;17)(p36.31-p36.13;q11.2-q12.1) in a neuroblastoma patient. Establishment of somatic cell hybrids and identification of PND/A12M2 on chromosome 1 and NF1/SCYA7 on chromosome 17 as breakpoint flanking single copy markers.** *Oncogene* 1995, **10:**1087-1093.
100. Guo C, White PS, Weiss MJ, Hogarty MD, Thompson PM, Stram DO, Gerbing R, Matthay KK, Seeger RC, Brodeur GM, Maris JM: **Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas.** *Oncogene* 1999, **18:**4948-4957.
101. Caren H, Kryh H, Nethander M, Sjoberg RM, Trager C, Nilsson S, Abrahamsson J, Kogner P, Martinsson T: **High-risk neuroblastoma tumors with 11q-deletion display a poor prognostic, chromosome instability phenotype with later onset.** *Proc Natl Acad Sci U S A*, **107:**4323-4328.
102. Look AT, Hayes FA, Shuster JJ, Douglass EC, Castleberry RP, Bowman LC, Smith EI, Brodeur GM: **Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study.** *J Clin Oncol* 1991, **9:**581-591.
103. Barbacid M: **Neurotrophic factors and their receptors.** *Curr Opin Cell Biol* 1995, **7:**148-155.
104. Brodeur GM, Nakagawara A, Yamashiro DJ, Ikegaki N, Liu XG, Azar CG, Lee CP, Evans AE: **Expression of TrkA, TrkB and TrkC in human neuroblastomas.** *J Neurooncol* 1997, **31:**49-55.
105. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, et al: **Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers.** *Nat Genet* 1997, **15:**356-362.
106. Kogner P, Barbany G, Dominici C, Castello MA, Raschella G, Persson H: **Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis.** *Cancer Res* 1993, **53:**2044-2050.
107. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM: **Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma.** *N Engl J Med* 1993, **328:**847-854.
108. Svensson T, Ryden M, Schilling FH, Dominici C, Sehgal R, Ibanez CF, Kogner P: **Coexpression of mRNA for the full-length neurotrophin receptor trk-C and trk-A in favourable neuroblastoma.** *Eur J Cancer* 1997, **33:**2058-2063.
109. Yamashiro DJ, Liu XG, Lee CP, Nakagawara A, Ikegaki N, McGregor LM, Baylin SB, Brodeur GM: **Expression and function of Trk-C in favourable human neuroblastomas.** *Eur J Cancer* 1997, **33:**2054-2057.
110. Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM: **Expression and function of TRK-B and BDNF in human neuroblastomas.** *Mol Cell Biol* 1994, **14:**759-767.
111. Johnsen JI, Segerstrom L, Orrego A, Elfman L, Henriksson M, Kagedal B, Eksborg S, Sveinbjornsson B, Kogner P: **Inhibitors of mammalian target of rapamycin downregulate MYCN protein expression and inhibit neuroblastoma growth in vitro and in vivo.** *Oncogene* 2008, **27:**2910-2922.
112. Opel D, Poremba C, Simon T, Debatin KM, Fulda S: **Activation of Akt predicts poor outcome in neuroblastoma.** *Cancer Res* 2007, **67:**735-745.
113. Fransson S, Abel F, Eriksson H, Kogner P, Martinsson T, Ejeskar K: **Analysis of the PI3K/Akt signaling pathway in Neuroblastoma – Stage dependent usage of PI3K p110 isoforms.** *Submitted* 2011.
114. Dam V, Morgan BT, Mazanek P, Hogarty MD: **Mutations in PIK3CA are infrequent in neuroblastoma.** *BMC Cancer* 2006, **6:**177.
115. Moritake H, Horii Y, Kuroda H, Sugimoto T: **Analysis of PTEN/MMAC1 alteration in neuroblastoma.** *Cancer Genet Cytogenet* 2001, **125:**151-155.
116. 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* 2009, **273:**336-346.
117. Lazcoz P, Munoz J, Nistal M, Pestana A, Encio IJ, Castresana JS: **Loss of heterozygosity and microsatellite instability on chromosome arm 10q in neuroblastoma.** *Cancer Genet Cytogenet* 2007, **174:**1-8.
118. Caren H, Fransson S, Ejeskar K, Kogner P, Martinsson T: **Genetic and epigenetic changes in the common 1p36 deletion in neuroblastoma tumours.** *Br J Cancer* 2007, **97:**1416-1424.
119. Boller D, Schramm A, Doepfner KT, Shalaby T, von Bueren AO, Eggert A, Grotzer MA, Arcaro A: **Targeting the phosphoinositide 3-kinase isoform p110delta impairs growth and survival in neuroblastoma cells.** *Clin Cancer Res* 2008, **14:**1172-1181.
120. Fransson S, Martinsson T, Ejeskar K: **Neuroblastoma tumors with favorable and unfavorable outcomes: Significant differences in mRNA expression of genes mapped at 1p36.2.** *Genes Chromosomes Cancer* 2007, **46:**45-52.

121. Wang Q, Diskin S, Rappaport E, Attiyeh E, Mosse Y, Shue D, Seiser E, Jagannathan J, Shusterman S, Bansal M, et al: **Integrative genomics identifies distinct molecular classes of neuroblastoma and shows that multiple genes are targeted by regional alterations in DNA copy number.** *Cancer Res* 2006, **66:**6050-6062.
122. Chesler L, Schlieve C, Goldenberg DD, Kenney A, Kim G, McMillan A, Matthay KK, Rowitch D, Weiss WA: **Inhibition of phosphatidylinositol 3-kinase destabilizes Mycn protein and blocks malignant progression in neuroblastoma.** *Cancer Res* 2006, **66:**8139-8146.
123. Evangelopoulos ME, Weis J, Kruttgen A: **Signalling pathways leading to neuroblastoma differentiation after serum withdrawal: HDL blocks neuroblastoma differentiation by inhibition of EGFR.** *Oncogene* 2005, **24:**3309-3318.
124. Evangelopoulos ME, Weis J, Kruttgen A: **Mevastatin-induced neurite outgrowth of neuroblastoma cells via activation of EGFR.** *J Neurosci Res* 2009, **87:**2138-2144.
125. Lopez-Carballo G, Moreno L, Masia S, Perez P, Barettino D: **Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells.** *J Biol Chem* 2002, **277:**25297-25304.
126. 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* 2000, **6:**529-535.
127. Ejeskar K, Aburatani H, Abrahamsson J, Kogner P, Martinsson T: **Loss of heterozygosity of 3p markers in neuroblastoma tumours implicate a tumour-suppressor locus distal to the FHIT gene.** *Br J Cancer* 1998, **77:**1787-1791.
128. Astuti D, Agathanggelou A, Honorio S, Dallol A, Martinsson T, Kogner P, Cummins C, Neumann HP, Voutilainen R, Dahia P, et al: **RASSF1A promoter region CpG island hypermethylation in phaeochromocytomas and neuroblastoma tumours.** *Oncogene* 2001, **20:**7573-7577.
129. 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* 2008, **50:**29-32.
130. Agathanggelou A, Dallol A, Zochbauer-Muller S, Morrissey C, Honorio S, Hesson L, Martinsson T, Fong KM, Kuo MJ, Yuen PW, et al: **Epigenetic inactivation of the candidate 3p21.3 suppressor gene BLU in human cancers.** *Oncogene* 2003, **22:**1580-1588.
131. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H: **Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction.** *Cold Spring Harb Symp Quant Biol* 1986, **51 Pt 1:**263-273.
132. Heid CA, Stevens J, Livak KJ, Williams PM: **Real time quantitative PCR.** *Genome Res* 1996, **6:**986-994.
133. Holland PM, Abramson RD, Watson R, Gelfand DH: **Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase.** *Proc Natl Acad Sci U S A* 1991, **88:**7276-7280.
134. Abel F, Sjoberg RM, Nilsson S, Kogner P, Martinsson T: **Imbalance of the mitochondrial pro- and anti-apoptotic mediators in neuroblastoma tumours with unfavourable biology.** *Eur J Cancer* 2005, **41:**635-646.
135. Towbin H, Staehelin T, Gordon J: **Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.** *Proc Natl Acad Sci U S A* 1979, **76:**4350-4354.
136. Hayflick L: **The cell biology of aging.** *J Invest Dermatol* 1979, **73:**8-14.
137. Rassoulzadegan M, Naghashfar Z, Cowie A, Carr A, Grisoni M, Kamen R, Cuzin F: **Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblast cell lines.** *Proc Natl Acad Sci U S A* 1983, **80:**4354-4358.
138. Wall FE, Henkel RD, Stern MP, Jenson HB, Moyer MP: **An efficient method for routine Epstein-Barr virus immortalization of human B lymphocytes.** *In Vitro Cell Dev Biol Anim* 1995, **31:**156-159.
139. Ejeskar K, Fransson S, Zaibak F, Ioannou PA: **Method for efficient transfection of in vitro-transcribed mRNA into SK-N-AS and HEK293 cells: difference in the toxicity of nuclear EGFP compared to cytoplasmic EGFP.** *Int J Mol Med* 2006, **17:**1011-1016.
140. Sanger F, Nicklen S, Coulson AR: **DNA sequencing with chain-terminating inhibitors.** *Proc Natl Acad Sci U S A* 1977, **74:**5463-5467.
141. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL: **A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands.** *Proc Natl Acad Sci U S A* 1992, **89:**1827-1831.
142. Morgan TH: **Sex Limited Inheritance In Drosophila.** *Science* 1910, **32:**120-122.
143. Brand AH, Perrimon N: **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.** *Development* 1993, **118:**401-415.
144. Holm S: **A simple sequentially rejective multiple test procedure.** *Scand J statist* 1979**:**65-70.
145. Martinsson T, Sjoberg RM, Hedborg F, Kogner P: **Deletion of chromosome 1p loci and microsatellite instability in neuroblastomas analyzed with short-tandem repeat polymorphisms.** *Cancer Res* 1995, **55:**5681-5686.

146. Zahn S, Sievers S, Alemazkour K, Orb S, Harms D, Schulz WA, Calaminus G, Gobel U, Schneider DT: **Imbalances of chromosome arm 1p in pediatric and adult germ cell tumors are caused by true allelic loss: a combined comparative genomic hybridization and microsatellite analysis.** *Genes Chromosomes Cancer* 2006, **45:**995-1006.
147. Abel F, Sjoberg RM, Ejeskar K, Krona C, Martinsson T: **Analyses of apoptotic regulators CASP9 and DFFA at 1P36.2, reveal rare allele variants in human neuroblastoma tumours.** *Br J Cancer* 2002, **86:**596-604.
148. Abel F, Sjoberg RM, Krona C, Nilsson S, Martinsson T: **Mutations in the N-terminal domain of DFF45 in a primary germ cell tumor and in neuroblastoma tumors.** *Int J Oncol* 2004, **25:**1297-1302.
149. Krona C, Ejeskar K, Caren H, Abel F, Sjoberg RM, Martinsson T: **A novel 1p36.2 located gene, APITD1, with tumour-suppressive properties and a putative p53-binding domain, shows low expression in neuroblastoma tumours.** *Br J Cancer* 2004, **91:**1119-1130.
150. Ejeskar K, Abel F, Sjoberg R, Backstrom J, Kogner P, Martinsson T: **Fine mapping of the human preprocortistatin gene (CORT) to neuroblastoma consensus deletion region 1p36.3-->p36.2, but absence of mutations in primary tumors.** *Cytogenet Cell Genet* 2000, **89:**62-66.
151. Krona C, Ejeskar K, Abel F, Kogner P, Bjelke J, Bjork E, Sjoberg RM, Martinsson T: **Screening for gene mutations in a 500 kb neuroblastoma tumor suppressor candidate region in chromosome 1p; mutation and stage-specific expression in UBE4B/UFD2.** *Oncogene* 2003, **22:**2343-2351.
152. Judson H, van Roy N, Strain L, Vandesompele J, Van Gele M, Speleman F, Bonthron DT: **Structure and mutation analysis of the gene encoding DNA fragmentation factor 40 (caspase-activated nuclease), a candidate neuroblastoma tumour suppressor gene.** *Hum Genet* 2000, **106:**406-413.
153. Yang HW, Chen YZ, Piao HY, Takita J, Soeda E, Hayashi Y: **DNA fragmentation factor 45 (DFF45) gene at 1p36.2 is homozygously deleted and encodes variant transcripts in neuroblastoma cell line.** *Neoplasia* 2001, **3:**165-169.
154. Chen YY, Takita J, Chen YZ, Yang HW, Hanada R, Yamamoto K, Hayashi Y: **Genomic structure and mutational analysis of the human KIF1Balpha gene located at 1p36.2 in neuroblastoma.** *Int J Oncol* 2003, **23:**737-744.
155. Frosi Y, Anastasi S, Ballaro C, Varsano G, Castellani L, Maspero E, Polo S, Alema S, Segatto O: **A two-tiered mechanism of EGFR inhibition by RALT/MIG6 via kinase suppression and receptor degradation.** *J Cell Biol*, **189:**557-571.
156. Zhang X, Pickin KA, Bose R, Jura N, Cole PA, Kuriyan J: **Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface.** *Nature* 2007, **450:**741-744.
157. Kwong J, Lo KW, Chow LS, To KF, Choy KW, Chan FL, Mok SC, Huang DP: **Epigenetic silencing of cellular retinol-binding proteins in nasopharyngeal carcinoma.** *Neoplasia* 2005, **7:**67-74.
158. Mellerick DM, Kassis JA, Zhang SD, Odenwald WF: **castor encodes a novel zinc finger protein required for the development of a subset of CNS neurons in Drosophila.** *Neuron* 1992, **9:**789-803.
159. Edenfeld G, Pielage J, Klambt C: **Cell lineage specification in the nervous system.** *Curr Opin Genet Dev* 2002, **12:**473-477.
160. Igarashi J, Muroi S, Kawashima H, Wang X, Shinojima Y, Kitamura E, Oinuma T, Nemoto N, Song F, Ghosh S, et al: **Quantitative analysis of human tissue-specific differences in methylation.** *Biochem Biophys Res Commun* 2008, **376:**658-664.
161. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, et al: **The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores.** *Nat Genet* 2009, **41:**178-186.
162. Matsui T, Sano K, Tsukamoto T, Ito M, Takaishi T, Nakata H, Nakamura H, Chihara K: **Human neuroblastoma cells express alpha and beta platelet-derived growth factor receptors coupling with neurotrophic and chemotactic signaling.** *J Clin Invest* 1993, **92:**1153-1160.
163. Pahlman S, Johansson I, Westermark B, Nister M: **Platelet-derived growth factor potentiates phorbol ester-induced neuronal differentiation of human neuroblastoma cells.** *Cell Growth Differ* 1992, **3:**783-790.
164. Fredlund E, Ringner M, Maris JM, Pahlman S: **High Myc pathway activity and low stage of neuronal differentiation associate with poor outcome in neuroblastoma.** *Proc Natl Acad Sci U S A* 2008, **105:**14094-14099.
165. Blom T, Roselli A, Hayry V, Tynninen O, Wartiovaara K, Korja M, Nordfors K, Haapasalo H, Nupponen NN: **Amplification and overexpression of KIT, PDGFRA, and VEGFR2 in medulloblastomas and primitive neuroectodermal tumors.** *J Neurooncol*, **97:**217-224.
166. Carvalho I, Milanezi F, Martins A, Reis RM, Schmitt F: **Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression.** *Breast Cancer Res* 2005, **7:**R788-795.
167. Rosse C, Linch M, Kermorgant S, Cameron AJ, Boeckeler K, Parker PJ: **PKC and the control of localized signal dynamics.** *Nat Rev Mol Cell Biol*, **11:**103-112.
168. Guo H, Gu F, Li W, Zhang B, Niu R, Fu L, Zhang N, Ma Y: **Reduction of protein kinase C zeta inhibits migration and invasion of human glioblastoma cells.** *J Neurochem* 2009, **109:**203-213.

169. Nazarenko I, Jenny M, Keil J, Gieseler C, Weisshaupt K, Sehouli J, Legewie S, Herbst L, Weichert W, Darb-Esfahani S, et al: **Atypical protein kinase C zeta exhibits a proapoptotic function in ovarian cancer.** *Mol Cancer Res*, **8:**919-934.
170. Svensson K, Zeidman R, Troller U, Schultz A, Larsson C: **Protein kinase C beta1 is implicated in the regulation of neuroblastoma cell growth and proliferation.** *Cell Growth Differ* 2000, **11:**641-648.
171. Macfarlane DE, Manzel L: **Activation of beta-isozyme of protein kinase C (PKC beta) is necessary and sufficient for phorbol ester-induced differentiation of HL-60 promyelocytes. Studies with PKC beta-defective PET mutant.** *J Biol Chem* 1994, **269:**4327-4331.
172. Chen LC, Hao CY, Chiu YS, Wong P, Melnick JS, Brotman M, Moretto J, Mendes F, Smith AP, Bennington JL, et al: **Alteration of gene expression in normal-appearing colon mucosa of APC(min) mice and human cancer patients.** *Cancer Res* 2004, **64:**3694-3700.
173. Odin E, Wettergren Y, Nilsson S, Willen R, Carlsson G, Spears CP, Larsson L, Gustavsson B: **Altered gene expression of folate enzymes in adjacent mucosa is associated with outcome of colorectal cancer patients.** *Clin Cancer Res* 2003, **9:**6012-6019.
174. Wettergren Y, Odin E, Nilsson S, Carlsson G, Gustavsson B: **p16INK4a gene promoter hypermethylation in mucosa as a prognostic factor for patients with colorectal cancer.** *Mol Med* 2008, **14:**412-421.
175. Luo J, Field SJ, Lee JY, Engelman JA, Cantley LC: **The p85 regulatory subunit of phosphoinositide 3-kinase down-regulates IRS-1 signaling via the formation of a sequestration complex.** *J Cell Biol* 2005, **170:**455-464.
176. Otto T, Horn S, Brockmann M, Eilers U, Schuttrumpf L, Popov N, Kenney AM, Schulte JH, Beijersbergen R, Christiansen H, et al: **Stabilization of N-Myc is a critical function of Aurora A in human neuroblastoma.** *Cancer Cell* 2009, **15:**67-78.

The range of what we think
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is limited by what we fail
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And because we fail to notice
what we fail to notice
there is little we can do
to change
Until we notice
how failing to notice
shapes our thoughts and deeds

R.D. Laing