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Notch Signaling in Human Neuroblastoma Cells

Marie-Thérése Stockhausen

Academic Dissertation

By due permission of the Faculty of Medicine, Lund University, Sweden, to be defended at the main lecture hall, Pathology building, Malmö University Hospital, MAS, on Friday 16th of September, at 9.15 for the degree of Doctor of Philosophy, Faculty of Medicine

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Abstract
Neuroblastoma is a childhood tumor derived from the sympathetic nervous system (SNS). It is believed that the tumors arise from cells halted in their differentiation and due to their immature phenotype; they express proteins normally only detected during embryogenesis. One such protein is Hash-1, which is required for formation of the SNS. Hash-1 is a component of the Notch signaling cascade, which is involved in many cell fate decisions. In general, Notch activity maintains a pool of undifferentiated cells and dysregulated Notch signaling has been linked to development of several cancers. It has been shown that the Notch cascade is transiently induced during neuroblastoma cell differentiation *in vitro* and that persistent Notch expression inhibits this differentiation. These observations imply a role for Notch signaling in the blocked differentiation of neuroblastoma cells. In addition, neuroblastoma cells exposed to hypoxia, a common event of solid tumors, dedifferentiate. During this process, components of the Notch signaling cascade are up regulated. In this thesis we show that Hash-1 interacts with ubiquilin-1, a protein involved in protecting proteins from degradation. In addition, we show that valproic acid (VPA) induces differentiation of neuroblastoma cells by modulation of the Notch signaling cascade. Aberrant signaling through the EGF receptor is involved in the genesis of some human cancers. Several reports have shown cross talk between EGFR signaling and the Notch cascade. We show here that the Notch target Hes-1 can be directly regulated by Ras/MAPK signaling at both normoxia and hypoxia, without the activation of Notch receptors.

Key words: Notch, Hes-1, Hash-1, neuroblastoma, ubiquilin-1, valproic acid, differentiation, Ras/MAPK, ERK1/2, EGFR, TGF-alpha, hypoxia

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Signature Marie Stockhausen Date 28th of July, 2005

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List of papers

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

I Ubiquilin-1 is a novel HASH-1-complexing protein that regulates levels of neuronal bHLH transcription factors in human neuroblastoma cells
Paula Persson, Marie-Thérése Stockhausen, Sven Påhlman, and Håkan Axelson
Int J Oncology 25: 1213-1221, 2004

II Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells
Marie-Thérése Stockhausen, Jonas Sjölund, Christina Manetopoulos, and Håkan Axelson
Br J Cancer 92: 751-759, 2005

III Regulation of the Notch target gene Hes-1 by TGFα induced Ras/MAPK signaling in human neuroblastoma cells
Marie-Thérése Stockhausen, Jonas Sjölund, and Håkan Axelson
Accepted for publication in Exp Cell Research

IV Regulation of the Notch target gene Hes-1 by hypoxia in human neuroblastoma cells
Marie-Thérése Stockhausen, Jonas Sjölund, Christina Manetopoulos, and Håkan Axelson
Manuscript

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Abbreviations

ADAM	a disintegrin and metalloprotease domain
ANK	ankyrin-like
AP	acute pancreatitis
APL	acute promyelocytic leukemia
APP	amyloid precursor protein
bHLH	basic helix-loop-helix
BMP2	bone morphogenic protein 2
CNS	central nervous system
CSL	CBF, Su(H), Lag-1
DBH	dopamine beta-hydroxylase
Dlk	Delta-like ligand
DLL	Delta-like
DSL	Delta/Serrate/Lag-2
ECN	extracellular Notch
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPO	erythropoietin
ER	endoplasmatic reticulum
ERK	extracellular regulated kinase
FGF	fibroblast growth factor
FIH	factor inhibiting HIF
GAP-43	growth associated protein-43
GSK3β	glycogen synthase kinase-3β
Hash-1	human *achaete-scute* homologue-1
HAT	histone acetylase
HDAC	histone deacetylase
Hes	Hairy/enhancer of split
HIF	hypoxia inducible factor
HRE	hypoxia response element
ICN	intracellular Notch
Id	inhibitor of DNA binding/ inhibitor of differentiation
IFN-α	interferon-α
INSS	international neuroblastoma staging system
JAG	Jagged
LNR	Lin12/Notch repeats
LOH	loss of heterozygosity
MAML	mastermind-like
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase

MAPKKK	mitogen-activated protein kinase kinase kinase
Mash-1	mammalian *achaete-scute* homologue-1
MMTV	mouse mammary tumor virus
NGF	nerve growth factor
NLS	nuclear localization signal
NPY	neuropeptide tyrosine Y
PHD	prolyl hydroxylase
PI3-K	phosphatidyl inositol 3-kinase
PKC	protein kinase C
PLZF	promyelocytic zinc finger
PML	promyelocytic leukemia protein
PNS	peripheral nervous system
RA	retinoic acid
RARE	retinoic acid response element
RAR	retinoic acid receptor
RTK	receptor tyrosine kinase
SCLC	small cell lung cancer
SIF	small intensely fluorescent
SKIP	ski interacting protein
SNS	sympathetic nervous system
Su(dx)	suppressor of deltex
Su(H)	suppressor of hairless
TACE	tumor-necrosis-factor-α converting enzyme
TAD	transcriptional activation domain
T-ALL	T-cell acute lymphoblastic leukemia
TCRβ	T-cell receptor β
TGFα	transforming growth factor α
TH	tyrosine hydroxylase
TKI	tyrosine-kinase inhibitor
TMN	transmembrane Notch
TSA	trichostatin A
UBL	ubiquitin-like protein
UDP	ubiquitin-like domain protein
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau
VPA	valproic acid

Background

The Notch receptor was originally discovered in the early nineteen hundreds based on an observation made in the fruit fly *Drosophila melanogaster*, in which partial loss of function resulted in characteristic notches at the wing margin [1]. Further studies revealed that mutations in Notch generated a neurogenic phenotype at the expense of epidermis [2], indicating a role for the receptor in restricting cell fate choices during development. Today we know that the highly regulated Notch signaling pathway is involved in a wide array of developmental processes and also in the genesis of several human cancers. The general view is that an active Notch signaling cascade prevents progenitor cells from adapting a primary cell fate by default, maintaining a pool of undifferentiated proliferative cells, a process referred to as lateral inhibition. Many of the key components of the cascade were originally recognized by loss of function studies generating a neurogenic phenotype resembling that of the Notch mutant. The Notch signaling pathway is highly conserved from *Drosophila* to humans and consists of Notch receptors, ligands, positive and negative modulators and down stream transcription factors.

The core axis of Notch signaling

The Notch receptors

The Notch receptors (Notch-1-4 in vertebrates) [3-6] are single-pass transmembrane receptors composed of an extracellular part non-covalently liked to the transmembrane subunit extending into the cytoplasm (Fig. 1). The extracellular part of Notch (ECN) consists largely of a ligand-binding domain composed of tandem epidermal growth factor (EGF)-like repeats (typically 36 in Notch-1 and Notch-2, 34 in Notch-3 and 29 in Notch-4), followed by three cysteine-rich Lin12/Notch repeats (LNR) that appear to restrict improper, ligand-independent receptor activation. The transmembrane Notch subunit (TMN) contains a short extracellular part, a membrane-spanning region and a large intracellular domain. This intracellular Notch (ICN) domain comprises a RAM sequence, six cdc10/ankyrin-like repeats (ANK), both involved in binding the transcriptional repressor/activator CSL as described later, two nuclear localization signals (NLS), and a C-terminal PEST region rich in proline (P), glutamine (E), serine (S) and threonine (T) important for protein stability. In addition to these subunits, Notch-1 to –3 contain cytokine response sequences (NCR) C-terminal to the ANK repeats and Notch-1 and –2 harbor a transcriptional activation domain (TAD) (reviewed in [7-9]).

The Notch ligands

There are two classes of related ligands in the Notch signaling pathway, called Delta-like (DLL-1, -3 and -4) and Jagged (JAG-1 and -2) in mammals (Delta and Serrate in *Drosophila* and Apx-1 and Lag-2 in *Caenorhabditis elegans* (*C. elegans*) respectively) (Fig. 1) [10, 11]. The ligands are transmembrane proteins and contain multiple EGF repeats in their extracellular domains similar to the Notch receptors. In addition, both types of ligands contain an N-terminal modified EGF repeat referred to as the DSL (Delta/Serrate/Lag-2) domain which is involved in receptor interaction. The intracellular domains of the

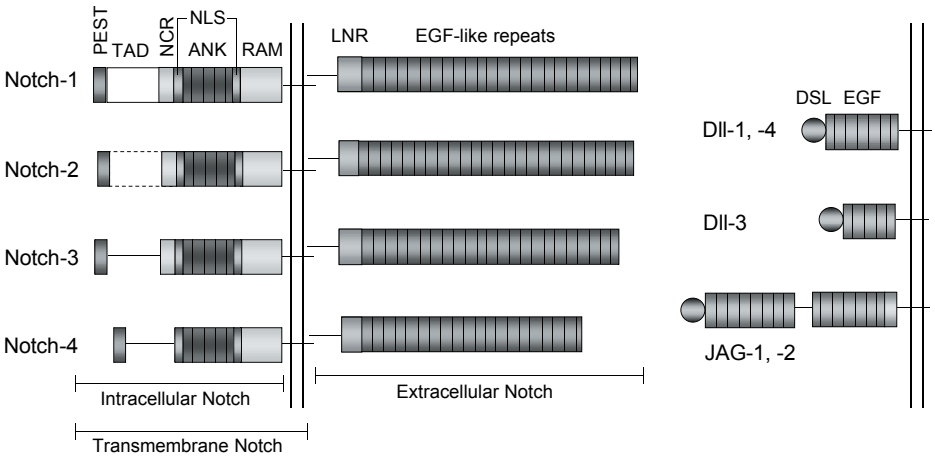

Figure 1. Schematic illustration of the vertebrate Notch receptors and ligands as described in the text. Modified from Radtke et al. [9].

ligands are short and poorly conserved. There are data showing that some of the ligands are sequentially cleaved to generate extracellular and intracellular fragments (reviewed in [12]). The intracellular fragments have been proposed to transmit a signal into the ligand bearing cell and it is also possible that the released extracellular fragments might bind the Notch receptors as soluble ligands. If this results in receptor activation or is antagonistic for Notch activation is not clear. Data also suggest that cleavage of at least Delta in *Drosophila* mediates its degradation, favoring a scenario in which the ligands are active only when membrane bound. (reviewed in [12]).

The mammalian Delta-like ligand (Dlk, also referred to as pG2) is a transmembrane protein containing multiple extracellular EGF repeats, a transmembrane region and a short cytoplasmic tail. The structure of the Dlk EGF repeats show close homology to those present in the Delta proteins, though one important difference is that Dlk lacks the N-terminal DSL domain needed for interaction with the Notch receptor. Still there are data showing that Dlk is able to interact with Notch-1 both *in vitro* and *in vivo* in mice [13]. Dlk has been proposed to play a role in several differentiation processes including adipogenesis, hematopoiesis and adrenal gland and neuroendocrine cell differentiation. In addition, Dlk is expressed in tumors with neuroendocrine features and might correlate with maturation along the chromaffin lineages [14, 15]. Interestingly it seems as if signaling through Dlk-Notch interaction is inhibitory to the Notch cascade in some cell types [13].

Proteolytic processing of Notch

The Notch receptors are synthesized as large 300 kDa proteins and modified by several proteolytic cleavages before activation (Fig. 2). Vertebrate Notch is first cleaved while the protein is in the Golgi apparatus in the secretory pathway. This S1 cleavage is thought to result from the action of a furin-like convertase, and occurs in the extracellular part of the receptor [16] between the Notch EGF repeats and the transmembrane domain at a site matching the furin consensus sequence (RXR/KR) present in Notch-1 and –3 [17]. Cleavage at the S1 site generates a 180 kDa fragment containing

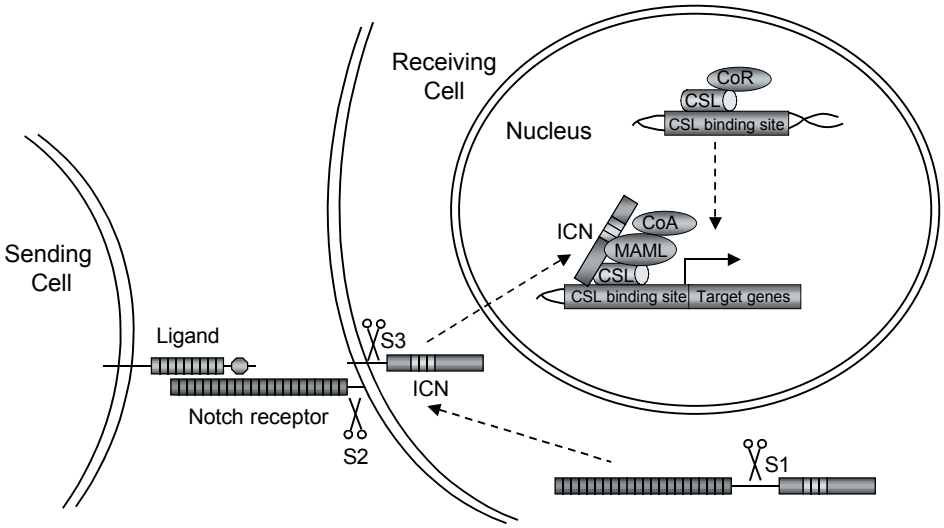

Figure 2. The core axis of Notch signaling. The Notch receptor is cleaved in the Golgi apparatus and resides as a heterodimer in the cell membrane. Upon ligand binding the receptor goes through two concequtive cleavages and the intracellular part of the receptor (ICN) translocates into the nucleus. Notch target genes are repressed by CSL and co-repressors (CoR), which upon binding of ICN are replaced by a co-activator complex (CoA) that initiates transcription. See text for further details. Modified from Sjölund et al. 2005.

the majority of the extracellular domain and a 120 kDa fragment corresponding to the transmembrane Notch subunit. The two fragments remain associated non-covalently and are positioned in the membrane as a heterodimeric receptor [16, 18]. The second cleavage occurs at a site called S2 in the extracellular domain in close proximity to the transmembrane region. The S2 cleavage is induced by receptor-ligand interaction on adjacent cells and is performed by a member of the ADAM (a disintegrin and metalloprotease domain) family of metalloproteases called TACE (tumor-necrosis-factor-α (TNF-α) converting enzyme, also known as ADAM17) in vertebrates [19, 20]. It has been suggested that ligand binding leads to endocytosis of the extracellular part of the Notch receptor into the ligand expressing cell, resulting in a stress-induced conformational change of Notch, exposing the S2 site for proteolysis [21, 22]. This is conflicting with data showing that it is possible to induce Notch signaling with soluble ligands. In this experimental setting, no endocytosis can occur and further studies are required to fully clarify the effect of ligand binding on processing of the receptor. Regulation of ligand and/or receptor endocytosis and the result of such an action will be discussed in further detail below. The S2 cleavage is thought to remove inhibiting components of the extracellular part of the receptor and is required for exposure of the S3 cleavage site (Val1744 in Notch-1) present in the transmembrane region. After S2 cleavage and removal of the extracellular domain the S3 cleavage takes place resulting in release of the intracellular (ICN) part of the receptor into the cytoplasm [23]. Several studies have shown that the S3 cleavage of Notch is dependent on the catalytic activity of presenilin-1 and –2 [24, 25]. In mammals, presenilin is associated

with nicastrin, Aph-1 and Pen-2, together composing the membrane bound γ-secretase complex (reviewed in [26, 27]). This complex has been implicated in the cleavage of both the Notch receptors and the amyloid precursor protein (APP) involved in Alzheimer's disease ([28] and reviewed in [26]) and inhibitors of γ-secretase have proven effective in inhibiting cleavage of both proteins. After cleavage by the γ-secretase complex, the ICN translocates into the nucleus and associates with the DNA binding protein CSL (CBF, Suppressor of Hairless (Su(H)), LAG-1; also referred to as RBP-Jκ in mammals) [29-31]. Binding of ICN to CSL converts CSL from a transcriptional repressor to an activator, initiating transcription of Notch target genes (Fig. 2).

The multimeric CSL-transcriptional complex

In the absence of ICN, CSL is bound to specific DNA sequences, GTGGGAA, in Notch target genes and acts as a transcriptional repressor (Fig. 2). At least two co-repressor complexes bind to CSL namely the SMRT/NCoR/histone deacetylase 1 (HDAC1) [32] and the CIR/HDAC2/SAP30 [33] complexes. The function of HDACs and their counterpart histone acetylases (HATs) will be discussed elsewhere in this thesis. In addition a protein called SKIP (Ski interacting protein) has been shown to interact with CSL and mediate repression possibly through interaction with the SMRT/HDAC complex [34]. Interestingly, SKIP also binds the fourth ANK repeat of ICN and even seems to promote the ICN-CSL interaction. The binding of SMRT or ICN to SKIP is mutually exclusive, though the affinity for ICN to SKIP is much higher, thus allowing low levels of ICN to convert CSL into a transcriptional activator [34]. Binding of ICN to CSL displaces the co-repressor complex and instead a transcriptional activating complex is formed (Fig. 2). This complex has been shown to contain factors with a general role in transcriptional regulation such as CBP/p300 [35], pCAF and GCN5 [36], all with histone acetylase (HAT) activity, associated with active transcription. In addition, the mastermind-like (MAML) proteins were recently identified as key transcriptional co-activators of Notch in mammals [37-40]. The MAML proteins contain at least one TAD capable of recruiting CBP/p300 [39, 41]. There are currently three MAML genes (denoted *MAML1-3*) described in mammals [40] all of which are capable of binding to CSL and the ANK repeats of ICN1-4 [40, 42]. It seems as if the interaction between ICN and CSL creates a site for MAML binding to CSL, thus generating a regulatory step in which ICN is required for MAML binding, an interaction strongly enhanced by the presence of CSL [39, 43]. An additional function of MAML might be in the control of signal termination as it has been shown that MAML is involved in ICN turnover [41, 43].

Observations in *Drosophila* that the *Notch* mutant phenotype is more severe than that of the *Su(H)* mutants indicate that other pathways for Notch signaling than those mediated through Su(H) are present (reviewed in [44]). Indeed, Deltex, a putative E3 ubiquitin ligase that binds to the ANK repeats of Notch, has been implicated in Su(H) independent Notch signaling [45-47].

Notch-interacting and –modifying proteins

Several proteins have been implicated in the regulation and termination of Notch signaling (reviewed in [48]). In the Golgi apparatus, before transport to the cell membrane, the Notch receptor is glycosylated by Fringe

proteins (Lunatic-, Manic- and Radical Fringe in mammals). The glycosylation occurs on *O*-fucose adducts on the extracellular EGF repeats of the Notch receptor and determines the output of receptor-ligand interaction leading to inhibition of Serrate/Jagged and activation of Delta signaling [49-52]. Glycosylation by Fringe is crucial for proper Notch signaling and has shown to be important in restricting Notch activation along the dorsal-ventral border in the development of the *Drosophila* wing.

The negative Notch regulator Numb localizes asymmetrically during cell division, ultimately repressing Notch signaling in one cell and permitting it in another. Numb interacts with the cytoplasmic domain of Notch, but exactly how it regulates Notch negatively is not clear [53-55]. It has however been shown that Numb can interact with E3 ubiquitin ligases such as Itch and/or Suppressor of deltex, Su(dx) in *Drosophila* resulting in polyubiquitination of ICN and subsequent degradation by the proteasomal machinery [56-58]. Another possibility is that Numb links ICN to components involved in endocytosis and targets the endocytosed Notch for degradation thereby preventing it from translocating to the nucleus.

As mentioned above, receptor-ligand interaction might cause internalization of the ligand together with the extracellular part of the receptor into the adjacent ligand-bearing cell. This step might be necessary for exposure of the S2 site for TACE, alternatively it is important for removal of excess extracellular parts of Notch that will remain after S2 cleavage and might act in an inhibitory fashion [21]. The E3 ubiqiutin ligase Neuralized has been implicated in the receptor-ligand endocytosis. An additional role for Neuralized is in promoting internalization and degradation of Delta [59]. Removal of excess ligand expressed on the signal-receiving cell might be important since it has been shown that signaling in *cis*, i.e. receptor-ligand interaction on the same cell is antagonistic.

Additionally, overexpression of a protein called Hairless mimics the phenotype of Notch mutants. It has been shown that Hairless binds to the DNA binding region of CSL and prevents CSL from binding DNA thus antagonizing CSL dependent Notch signaling [60].

Turning off the Notch signal is important and it has been shown that the E3 ubiquitin ligase Sel-10 regulates the stability of the ICN-CSL-MAML complex [61-63]. And as mentioned above MAML itself promotes ICN phosphorylation and degradation [41], abrogating Notch signaling.

Signaling downstream the Notch receptor

To date there are only a few known well-characterized transcriptional targets of Notch signaling in mammals. Among these is one family of basic helix-loop-helix transcription (bHLH) factors called Hes (Hairy/enhancer of split). The Hes proteins belong to a subgroup of bHLH proteins functioning as transcriptional repressors (see below). Other known Notch targets include yet another family of bHLH transcriptional repressors related to the Hes proteins called Herp (also known as Hey, Hesr, HRT, CHF and gridlock) as well as p21, pre-Tα, ErbB2 and NF-κB2 (reviewed in [64]). In addition, the cyclin D1 promoter has been shown to contain a binding site for RBP-Jκ, and *cyclin D1* mRNA was up regulated by overexpression of ICN [65]. Additionally, it was shown that Deltex, a putative inhibitor of Notch, was able to suppress the expression of cyclin D1 in mouse mammary epithelium,

further indicating that Notch is involved in cyclin D1 regulation [66].

The basic helix-loop-helix family of transcription factors

Members of the bHLH family of transcriptional regulators play crucial roles during the development of various tissues and organs, including the nervous system, the heart, the pancreas and the vasculature. The proteins are composed of a basic domain responsible for binding to specific DNA sequences called E-boxes (CANNTG) and a HLH domain involved in protein-protein interactions. The bHLH proteins can be classified into two major groups, namely the ubiquitously expressed class A proteins, also known as the E proteins, and the class B proteins which show a more tissue-specific expression (for a more detailed classification see [67]). The class B proteins can form heterodimers with the class A proteins, and some are also capable of forming homodimers. Importantly, binding to DNA requires the presence of two basic domains and thus Id (inhibitors of DNA binding, inhibitors of differentiation) proteins, belonging to a subgroup of HLH proteins that lack the basic domain, function as dominant negative inhibitors of other bHLHs.

The Hes family

There are currently seven Hes (Hes-1 to -7) members identified in mammals, all of which show high similarity to their *Drosophila* counterparts. The Hes proteins differ structurally from other bHLH proteins in that they contain an Orange domain, a C-terminal WRPW motif and a proline residue in the basic domain. The Orange domain contains two extra helices (helix 3 and 4) and has been shown to be involved in transcriptional repression, possibly in concert with WRPW. The WRPW motif is involved in transcriptional repression of target genes. It has been shown that the WRPW motif interacts with co-repressors such as TLE (Groucho in *Drosophila*) and possible suppress transcription by recruiting HDAC. In addition to this active form of repression, Hes proteins can also form non-functional heterodimers with E proteins thereby disrupting the formation of functional heterodimers. The presence of a proline in the basic domain of the Hes proteins alters their binding specificity for the E-box and instead the preferred binding site is a so-called N-box, CACNAG.

Hes-1 is the most extensively studied member of the Hes family and it is also the protein to which most target genes have been established. One target is Hes-1 itself and it has been shown that Hes-1 negatively regulates its own activity by binding to N-boxes present in the promoter [68]. Binding of Hes-1 to its own promoter creates a negative feedback loop resulting in an oscillating expression pattern of Hes-1 mRNA and protein. This oscillation has been shown to be important for proper formation of somites during development, a process referred to as the segmentation clock. Other known Hes-1 targets include the cell cycle regulators p27 [69] and p21, and the bHLH protein mammalian achaete-scute homologue-1 (Mash-1) which will be discussed in further detail in the section covering Notch signaling in the nervous system

Hes proteins as effectors of Notch signaling

Several lines of evidence have suggested that Hes-1 and Hes-5 are downstream targets of the Notch receptor [70]. For example overexpression of ICN in neural precursor cells from mice caused up regulation of both

Hes-1 and Hes-5 and led to inhibition of neuronal differentiation [70]. In another study it was shown that ICN was able to associate with RBP-Jκ and induce transcription of the Hes-1 promoter [31]. In addition, co-culture with Notch ligand expressing cells to obtain a more physiological Notch signaling activity in combination with inhibitors of *de novo* protein synthesis to avoid secondary effects (by induction of other proteins) have provided strong evidence that Hes-1 is a direct target of Notch activity [71, 72]. Further, there are data showing that mutation of the RBP-Jκ binding sites in the Hes-1 promoter abolish activation by Notch [31, 71, 73] (and reviewed in [64]). Though, in contrast, de la Pompa et al showed that knocking out either Notch-1 or RBP-Jκ did not reduce the expression of Hes-1 *in vivo* [74], indicating Notch independent pathways in the regulation of Hes-1. Indeed, a rat homolog of *Drosophila* hairy, was shown to respond as an immediately early gene upon stimulation with growth factors [75]. In addition, serum treatment of cultured cells induces Hes-1 mRNA and protein [76]. Under present investigation I will discuss the finding that Hes-1 is regulated through the activity of phosphorylated ERK1/2 in human neuroblastoma cells.

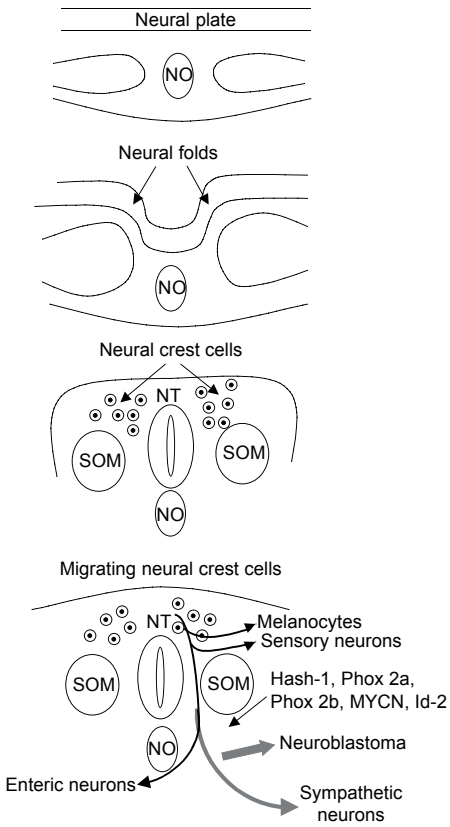

Figure 3. Formation of the neural crest and its derivates. Neuroblastoma originate from precursors of the sympathetic nervous system. NO=notochord, NT=neural tube, SOM=somites. Modified from Nakagawara 2004.

Development of the sympathetic nervous system

Formation of the neural crest and its derived cell types

During the third week of embryonal development in humans a structure called the neural plate is formed from the outer germinal layer, the ectoderm. Invagination of the borders of the neural plate creates the neural tube in anterior posterior orientation (Fig. 3). Formation of the neural tube is called neurulation and will eventually give rise to the central nervous system (CNS) comprising the brain and the spinal cord. From the lateral margins of the folding neural plate the neural crest cells will evolve. The neural crest cells are multipotent progenitor cells that, subjected to extrinsic factors, will give rise to a remarkable diversity of cell types including melanocytes, cartilage, smooth muscle cells, supportive glial cells and most importantly the peripheral nervous system (PNS) (Fig. 3) [77, 78]. The PNS is composed of the somatic and autonomic nervous systems, where the

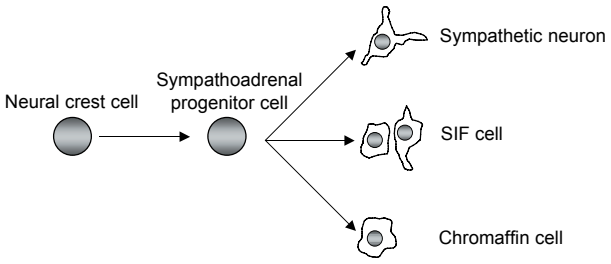

Figure 4. The sympathoadrenal progenitor cell gives rise to the three main cell types of the sympathetic nervous system; sympathetic neurons, SIF cells and chromaffin cells.

somatic system controls skeletal muscles and the autonomic system innervates inner organs. A further sub-division of the autonomic nervous system into the parasympathetic and sympathetic branches can be made.

The sympathetic nervous system

The sympathetic nervous system (SNS) is responsible for the so-called "fight and flight" response induced by stress and includes sympathetic neurons, chromaffin cells and small intensely fluorescent (SIF) cells (Fig. 4). The sympathetic neurons are located along the spinal cord in sympathetic chain ganglia and in the truncus region forming truncus ganglia. Sympathetic neurons are also present in the adrenal glands [79]. In adults, most of the chromaffin cells are located in the adrenal medulla, but can also exist outside the adrenal glands forming the paraganglia. SIF cells can be found interspersed between the sympathetic neurons in ganglia proper. The cells of the SNS produce transmittor substances such as acetylcholine and the catecholamine noradrenaline. Both chromaffin cells and the SIF cells produce catecholamines (adrenaline and noradrenaline) that are secreted directly into the blood stream, thus these cells are referred to as neuroendocrine. During prenatal and early postnatal life, the SNS also include cells that are though to regress either by apoptosis or by differentiation into mature neurons or chromaffin cells during the first years after birth [80, 81]. The organ of Zuckerkandl, located close to the abdominal aorta, is the largest paraganglia found in the human fetus and is thought to be the main producer of catecholamines before the adrenal glands are properly functional [82]. Clusters of immature neuroblastic cells can also be seen in the developing adrenal glands and the amount of SIF cells is higher in the embryonic than in adult SNS.

The three lineages of the SNS originate from a common sympathoadrenal progenitor derived from the neural crest cells of the trunc region (Fig. 4) [83]. Depending on the extrinsic signals this progenitor encounter, it can develop into either one of the structures composing the SNS. In response to fibroblast growth factor (FGF) or nerve growth factor (NGF), the sympathoadrenal precursor cell will become sympathetic neurons, while glucocorticoids promote differentiation into chromaffin cells, also including the SIF cells [84, 85]. During the differentiation from neural crest cells into terminally differentiated cells of the SNS, the cells express distinct sets of genes, enabling identification of cell type and differentiation stage. These marker genes have been extensively used to determine the origin and maturation grade of the SNS derived neuroblastoma.

Notch signaling in the nervous system

Proper development of multicellular organisms requires a coordinated spatial and temporal communication between neighboring cells. During embryonic development the specification of cell fate is often governed by the Notch signaling pathway. The Notch cascade has been implicated in a wide array of developmental processes such as hematopoiesis, T-cell development, vasculogenesis and neurogenesis. Underlining the importance of Notch signaling in neurogenesis; ablation of Notch-1, Delta-1, Hes-1 or RBP-Jκ in mice results in premature neurogenesis and embryonal lethality.

Lateral inhibition in neurogenesis

In the developing mammalian nervous system, neurons and glial cells differentiate from common precursor cells. In this setting, Notch signaling is important for maintaining a pool of undifferentiated cells, i.e inhibiting differentiation into neuronal cells. Recent data have shown that these undifferentiated cells are instructed to adopt another cell fate than the default one, for instance they can become glial cells [86, 87]. The process by which Notch acts to specify cell fates is called lateral inhibition and is highly conserved from *Drosophila* to vertebrates. Lateral inhibition, best described in *Drosophila*, occurs between cells that have similar developmental potential. In a simplified model using two cells, Notch and its receptor are expressed equally on both cells. For reasons currently unknown, one cell will become the signal-receiving cell, i.e. its Notch receptor will bind a ligand on the adjacent cell and start the intracellular Notch cascade. This results in repression of pro-neuronal genes such as *achaete-scute* that promotes transcription of the Notch ligand, thus creating a negative feedback loop. Ultimately, the cell that transmits the signal will produce less ligand than the neighboring cell. The outcome of this process is that the cell that express the pro-neuronal genes will become neuronal whereas the signal-receiving cell will stay undifferentiated.

Notch signaling in neurogenesis

Notch expression has been associated with undifferentiated cells of the developing CNS, whereas expression is reduced in the adult. During embryonal development, Notch signaling is involved in the dorsal ventral patterning of the neural tube [88] and formation of the CNS. Within the neural tube, Notch is expressed in proliferating cells whereas the ligand Delta can be detected in cells eventually becoming neurons by the process of lateral inhibition [74, 89]. Notch has been shown to inhibit neurogenesis in the brain through the downstream target Hes and promote stem cell survival by up regulation of anti-apoptotic genes [90]. Importantly, Notch plays a role in neuronal stem cell maintenance but not for establishing it [91].

Notch family genes are expressed in the neural crest [4, 88] and seem to be important for its formation [74]. During development of the ganglia of the PNS, Notch-1 is expressed in neural crest cells migrating ventrally from the neural tube and remains expressed in the immature cells before ganglion formation [88]. The neuroblasts in the ganglia, i.e. the cells that will become neurons, express Notch ligands [10, 11, 92]. Furthermore, Notch signaling promotes a switch from neurogenesis to glia in the PNS [87]. This is probably mediated through lateral inhibition in a two-step manner. First, some of the neural crest cells differentiate into neurons. These cells will then inhibit the surrounding

undifferentiated cells to adapt the default neuronal fate, and instead Notch signaling will promote the differentiation into glia.

Hes-1 expression in the nervous system

Studies in mice have revealed that Hes-1 is expressed in neuronal precursor cells but not in mature neurons [93, 94]. Thus, as neuronal differentiation proceeds, Hes-1 expression decreases in the nervous system suggesting that down regulation of the protein is required for proper differentiation of neuronal precursors. Indeed, overexpression studies demonstrate that persistent expression of Hes-1 inhibits neuronal differentiation both *in vivo* and *in vitro* [93, 95]. In contrast, Hes-1 null mice fail to close the neural tube resulting in open brain, anencephaly [96]. In addition, they exhibit premature neuronal differentiation, possibly a consequence of increased expression of *Mash-1*. These observations imply that Hes-1 plays an important role in timing the development and differentiation of the nervous system and thus proper regulation of the protein is potentially important, an issue discussed in further detail under present investigation.

Mash and neuronal development

The mammalian bHLH gene *Mash-1* was originally cloned from sympathoadrenal precursor cells based on its homology to the *Drosophila achaete-scute* genes [97]. Mash-1 (Hash-1 in humans) is expressed in neuronal precursors of both the CNS and PNS [98] and is essential for development of sympathetic, parasympathetic, olfactory and part of the enteric neurons whereas it seems dispensable for development of most of the CNS and sensory neurons of the PNS as well as some chromaffin cells of the adrenal medulla [99-101]. In the absence of Mash-1, multipotent stem cells differentiate into neuronal precursor cells but are not able to further differentiate into mature neurons indicating a role for Mash-1 in promoting neuronal differentiation of already committed cells [101].

Data suggest that transient rather than continuous expression of Mash-1 is important for neuronal differentiation [98, 101-103]. In the rat, *Mash-1* expression can be detected at E10.5 in the developing CNS and from E11 to E13.5 in progenitor cells of the sympathetic ganglia whereafter expression is subsequently down regulated [98]. A similar pattern can be seen in humans, where *Hash-1* is expressed in sympathetic ganglia surrounding the aorta as well as in sympathetic cells invading the adrenal gland and in developing enteric neurons from week 6.5. After embryonic week 7, *Hash-1* is only expressed in the adrenal gland and enteric neuroblasts and by week 10, *Hash-1* expression is no longer detectable in the sympathetic nervous system [104]. In transient transfection experiment using pluripotent mouse cells, Mash-1 expression promoted neuronal differentiation, a process that was greatly enhanced by co-expression of the putative dimerization partner E12 [105]. In addition, during induced differentiation of P19 and neuroblastoma tumor cells *in vitro*, Mash-1/Hash-1 are transiently expressed [103, 106]. In the PNS, Mash-1 expression disappears before or concomitantly with the expression of sympathoadrenal differentiation markers such as neurofilament, SCG10 and tyrosine hydroxylase (TH) [98, 99].

During development, neural crest progenitor cells are subjected to extrinsic factors influencing their capacity to differentiate into autonomic neurons. One such factor is bone morphogenic protein 2 (BMP2), a member of the TGFβ superfamily, which is able to induce expression of Mash-1, promoting autonomic neurogenesis *in vitro*

[107, 108]. In neural crest stem cells, Mash-1 has been shown to induce the expression of the paired homeodomain transcription factor Phox2a and the tyrosine kinase receptor c-ret leading to neuronal differentiation [109]. Phox2a and its close relative Phox2b, whose expression does not depend on Mash-1, are crucial for development of noradrenergic neurons of the PNS. Expression of Phox2 genes *in vitro* and *in vivo* is capable of inducing ectopic sympathoadrenal neurons expressing sympathetic markers such as dopamine beta-hydroxylase (DBH) and TH, both essential in the synthesis of catecholamines, and the pan-neuronal gene SCG10 [110]. Interestingly, germline mutations of Phox2b have been identified in some cases of human neuroblastoma [111, 112]. In addition, Hash-1 is expressed in the majority of human neuroblastoma tumors and cell lines, reflecting the sympathoadrenal and immature phenotype of this tumor.

Neuroblastoma

Neuroblastoma is a childhood tumor originating from sympathetic progenitor cells derived from the neural crest (Fig. 3). It is the most common pediatric solid tumor outside the CNS, and each year 10-15 children in Sweden are diagnosed with the disease. Even though the frequency of neuroblastoma is relatively low, the mortality rate is high compared to other childhood tumors with a five-year survival rate of approximately 50%. The tumors arise at locations of the sympathetic nervous system; primary tumors typically locate to the adrenal medulla or sympathetic chain ganglia. In addition and as a consequence of their sympathetic origin and immature phenotype, neuroblastoma tumors express markers characteristic of sympathetic progenitor cells and in many cases the tumors produce catecholamines. This has been utilized in diagnosis and mass screening of neuroblastoma since it is possible to measure the ratio of dopamine and the two metabolites of catecholamine synthesis, vanilmandelic and homovanillic acid, in serum and urine of patients. Neuroblastoma is a heterogeneous cancer spanning from highly aggressive tumors with poor prognosis to tumors that regress spontaneously.

Tumor classification and prognosis

According to the International Neuroblastoma Staging System (INSS) neuroblastoma tumors can be divided into six groups (1, 2A, 2B, 3, 4 and 4S) based on localization of the primary tumor, lymph node involvement and pattern of metastasis. Stage 1 and 2 tumors are localized with the possible involvement of ipsilateral ("on the same side") lymph nodes whereas stage 3 and 4 include disseminated tumors, often involving metastases to distant lymph nodes [113]. Low stage neuroblastoma tumors are correlated with a good prognosis, as is the case for patients with stage 4S disease despite excessive metastases to the liver and skin. Stage 4S tumors are limited to children younger than 1 year of age at the time of diagnosis and show spontaneous regression either by increased apoptosis or differentiation into benign ganglioneuromas [114]. In addition to tumor stage, age at diagnosis is an important prognostic factor for neuroblastoma. The majority of children diagnosed before the age of one have a good prognosis with tumors of stage 1 and 2, whereas older patients most often have high stage tumors (stage 3 and 4) and a poor prognosis [115].

Genetic aberrations in neuroblastoma

A number of recurrent genetic abnormalities have been identified in human neuroblastoma

[114, 116]. One of the most frequent genetic aberrations is amplification of the proto-oncogene N-myc, found in approximately 20-25% of neuroblastoma tumors and clearly associated to high proliferation and aggressive disease [117, 118]. N-myc was originally identified in human neuroblastoma tumors and cell lines, hence the "N", on its homology to the myc oncogene [119]. The importance of N-myc in the genesis of neuroblastoma has been strengthened experimentally in mice, where overexpression of the oncogene specifically in migrating neural crest cells caused neuroblastoma tumors [120]. Amplification of N-myc is often associated with loss of heterozygosity (LOH) of the short arm of chromosome 1 [121, 122], (1p), and extensive studies have been performed to identify a tumor suppressor gene at 1p.36, the region most often deleted [122-128]. LOH at 1p is also often coupled to gain of chromosomal material on the long arm of chromosome 17 (17q). This can be due to unbalanced translocations, most often with chromosome 1p or 11q, or as a part of whole chromosome gain [129, 130]. Typically, LOH at 1p and gain of genomic material on 17q is associated with a poor outcome (reviewed in [116]). In addition, LOH on the long arm of chromosome 14 (14q) has been detected in several neuroblastoma tumors and thus, 14q has been suggested to harbor a tumor suppressor gene involved in the initiation or progression of neuroblastoma [131, 132].

The neurotrophin receptor TrkA, which is involved in development of the nervous system and is expressed in mature sympathetic ganglia, is highly expressed in favorable neuroblastoma [133, 134]. High TrkA expression is connected to young age and absence of N-myc amplification. Overexpression of TrkA in the neuroblastoma cell line SH-SY5Y, renders the cells responsive to treatment with the ligand NGF, resulting in morphological differentiation and up regulation of neuronal markers.

Neuroblastoma cell lines

Neuroblastoma cell lines have been useful for *in vitro* studies characterizing the phenotype of neuroblastoma cells as well as for investigating neuronal growth and differentiation in general. It is possible to induce neuronal differentiation in some of the neuroblastoma cell lines using the phorbolester TPA, retinoic acid (RA) or a combination of growth factors. During this *in vitro* differentiation, N-myc is down regulated and markers of sympathetic neurons such as NPY (neuropeptide tyrosine Y) and GAP-43 (growth associated protein-43) are up regulated along with marked morphological differentiation as characterized by neurite-like outgrowth [106, 135-137].

Notch signaling in neuroblastoma

Most neuroblastoma tumors and cell lines have been shown to express Hash-1 [104, 106, 138]. This expression indicates that the cells are blocked at an early stage of development of the sympathetic nervous system since Hash-1 is not expressed in mature neurons. During induced differentiation *in vitro* using TPA or RA, Hash-1 is down regulated, followed by neurite outgrowth and expression of the neuronal marker genes GAP-43 and NPY [106, 138-140]. Thus, down regulation of Hash-1 might be necessary for neuronal differentiation to proceed. Grynfeld et al. showed that, in addition to down regulation of Hash-1, Hes-1 expression is transiently up regulated during this induced differentiation [139]. Furthermore, the Id proteins, which are able to dimerize with Hes-1 and thus decrease its DNA binding activity, are down regulated

upon treatment with TPA or RA [140, 141]. Since Hes-1 has been shown to bind the Hash-1 promoter, up regulation of Hes-1 and down regulation of the Id proteins might be involved in the repression of Hash-1 [139, 141, 142].

Overexpression of ICN inhibits neurite outgrowth induced by TPA or RA, and thus Notch activity seems to inhibit neuroblastoma cell differentiation [139]. This is in line with studies in the mouse neuroblastoma cell line N2a, where overexpression of ICN or stimulation of Notch activity by co-culturing with Delta expressing cells inhibits primary neurite outgrowth [143]. In this study the authors showed that while Delta expression in *trans* (i.e. on another cell) inhibits neurite outgrowth by stimulation of the Notch cascade, *cis* expression (i.e. on the same cell) of the ligand promotes neurite outgrowth, possibly a consequence of the antagonistic effect of ligand binding in *cis* as mentioned above.

The most extensive analysis of Notch genes in neuroblastoma so far is the one performed by Van Limpt and co-workers [15]. They detected *Hash-1*, *Notch-3* and *Dlk-1* mRNA in distinctive subsets of neuroblastoma cell lines and suggested that the differential gene expression reflected at what stage of development the tumors originated from, ranging from early neural crest cells to chromaffin cells of the adrenal medulla. During normal development, expression of Dlk in chromaffin precursors of the adrenal medulla is not detectable until week 24 of gestagation, and is thus a late marker of differentiation. In the cell lines studied, *Dlk-1* correlated to expression of *DBH*, indicating a differentiated sympathetic phenotype [15]. Importantly, in a study determining neuroblastoma prognosis using gene expression profiling, expression of *Dlk-1* was shown to be one out of six genes that were highly correlated to a bad prognosis [144]. Whether this expression of *Dlk-1* reflects the origin of the tumors or implies a functional role for Dlk and possibly Notch signaling in promoting a more aggressive phenotype is however not known. The finding that *Dlk-1* is correlated to poor prognosis is intriguing since the studies by van Limpt et al. indicate that *Dlk-1* correlates to a differentiated phenotype, and hence most likely a less aggressive tumor. Interestingly, Dlk maps at 14q32, the region for LOH in 30% of neuroblastoma tumors [145].

Another interesting observation that might connect to Notch signaling is the overexpression of cyclin D1 in neuroblastoma cell lines and tumor samples [146]. As mentioned above the promoter of cyclin D1 contains a binding site for RBP-Jκ, which makes it a possible Notch target [65] and thus high cyclin D1 mRNA and protein levels in neuroblastoma could be a cause of Notch signaling, though currently there are no data proving that this is the case.

Notch signaling in cancer

Notch signaling has been implicated in the genesis of several mammalian tumors. It is important to note that the outcome of Notch signaling is highly cell type dependent, and quite surprisingly, it can act as an oncogene in one tissue whereas it functions as tumor suppressor in another. It has been suggested that if Notch signaling is active in the progenitor cell and has to be down regulated for the cell to terminally differentiate, then Notch functions as an oncogene. Conversely, in cells that Notch activity is required in order for the cells to differentiate, Notch is considered a tumor suppressor and thus loss of activity prevents differentiation (Fig. 5)

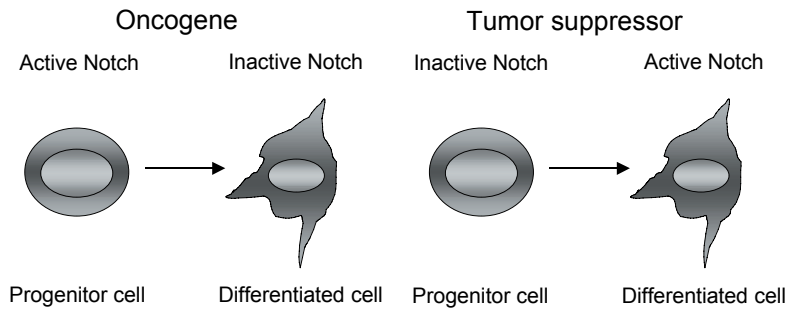

Figure 5. Notch can act either as an oncogene or a tumor suppressor. Constitutive activation in a cell type where Notch is expressed in the proliferating progenitor cell and has to be down regulated in order for differentiation to occur might lead to blocked differentiation and render the cells susceptible for additional transforming events. The opposite is true for cells in which Notch has to be activated for differentiation to occur and thus in this case Notch might function as a tumor suppressor. Modified from [147].

(reviewed in [147]). The exact mechanism behind Notch induced transformation is not clear, but several studies indicate that Notch needs to cooperate with other proteins and signaling pathways involved in tissue growth and homeostasis for transformation to occur.

Notch as an oncogene

The role of Notch as an oncogene was first discovered in a subset of T-cell acute lymphoblastic leukemias (T-ALLs) containing a t(7;9) (q34;q34.3) chromosomal translocation, involving the human Notch-1 gene and the T-cell receptor β (TCRβ) locus [148]. Notch-1 is involved at several steps during lymphocyte development, promoting differentiation into T-cells at the expense of B-cells. The positioning of a truncated Notch-1 allele under control of the TCRβ promoter leads to constitutive expression of ICN, resulting in blocked T-cell development and a subsequent expansion of the double positive ($CD4^+CD8^+$) pool of T-lymphocytes. Work in mice showed that ICN-1 overexpression in bone marrow caused T-cell leukemia [149]. However, the t(7;9) translocation is only found in a subset of T-ALLs, corresponding to approximately 1%, and still almost all T-ALLs express high levels of Notch-1 and Notch-3. This got its explanation in a study by Weng et al. where the authors could show that the Notch-1 receptor expressed in T-ALL cell lines contained mutations in the heterodimerization domain and/or in the PEST domain [150]. Either or of these mutations were also shown to be present in more than 50% of bone marrow samples from primary T-ALL tumors. Experimentally, it was shown that the Notch-1 mutations found in T-ALL augmented the transcriptional activity of the receptor, probably by enhancing γ-secretase cleavage and increasing ICN half-life respectively. Importantly, inhibiting Notch activity by γ-secreatase inhibitors or dominant negative MAML1 peptides, resulted in growth inhibition of Notch-1 transformed cell lines, showing that Notch signaling is not only involved in the initiation of these cancers, but also in their maintenance [150].

An important role for Notch signaling has also been documented in the development of murine mammary cancer. Mouse Mammary Tumor Virus (MMTV) recurrently integrates into the *Notch-4* gene and induces the

expression of a truncated protein containing the transmembrane and intracellular regions of Notch-4 [5]. This results in an active form of Notch-4 in a way similar to Notch-1 activation in T-ALL. The net result of overexpression of active Notch-4 in mouse mammary epithelium *in vivo* is abnormal proliferation and blocked differentiation followed by development of adenocarcinoma [151]. Overexpression of the Notch-4 oncoprotein in mouse mammary epithelial cells *in vitro* results in anchorage-independent growth, matrix invasion and loss of contact inhibition, features of malignant transformation [152]. In addition, constitutive expression of Notch-1 in mouse mammary epithelia induces lactation dependent neoplasms that eventually progress into malignant adenocarcinomas [66]. Despite the well-documented role of aberrant Notch signaling in murine mammary tumors, the function in human breast cancer is still elusive and needs further attention. Though, in a recent study it was shown that the Notch inhibitor Numb was lost in approximately 50% of human mammary carcinomas, thus suggesting that improper regulation of Notch might be involved in human breast cancer [153].

Not only the Notch receptors, but also the ligands and proteins that modify the Notch signaling pathway are involved in the genesis or maintenance of cancer. For example, both Notch-1 and the ligands DLL -1 and JAG-1 are expressed in primary human gliomas and cell lines and seem necessary for their proliferation and survival [154]. In human pancreas Notch signaling appears to prevent cellular differentiation and maintain a pool of undifferentiated progenitor cells during development [155]. Interestingly, components of the Notch pathway (i.e. Notch-1 to –4, Hes-1, Hes-4, HeyL (structurally similar to Hey-1 and –2 but without the C-terminal YRPW motif [156]), Hey-1, JAG-1 and –2 and Dlk-1) are expressed at high levels in human pancreatic cancer as compared to normal pancreas [157]. Studies in normal pancreatic cells revealed that overexpression of Notch-1 and Notch-2 induced acinar-to-ductal metaplasia, an event preceding malignant transformation, probably by expansion of an undifferentiated pool of precursor cells [157]. Further, it was shown that aberrant Notch activity could be induced by, and was a requisite for, oncogenic signaling through the epidermal growth factor receptor (EGFR) stimulated by TGFα [157]. The cooperation between the Notch and EGFR pathways has been implicated in the genesis and continuation of several tumors and will be discussed in the chapter covering crosstalk between Notch and EGFR signaling.

Notch as a tumor suppressor

Apart from its oncogenic activity, several studies have now shown that Notch can function as a tumor suppressor. This has been firmly established in mice, where inducible deletion of Notch-1 in keratinocytes and corneal epithelium results in epidermal and corneal hyperplasia and subsequent skin tumors [158, 159]. During *in vitro* differentiation of keratinocytes, Notch-1 is up regulated and is crucial for inducing the cell cycle inhibitor p21 and early markers of differentiation [159]. In addition, ablation of Notch-1 facilitates chemical-induced skin carcinogenesis. This could be due to down regulation of p21 and inappropriate activation of β-catenin, a key component of the Wnt signaling pathway, known to be involved in basal cell carcinoma [158].

Notch has also been implicated to work as a tumor suppressor in small cell lung cancer (SCLC), in which overexpression of Notch up

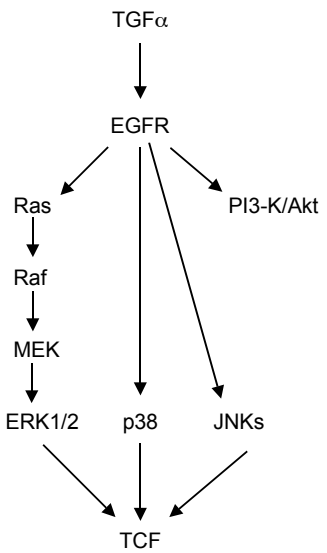

Figure 6. Schematic illustration of signaling through the EGF receptor, showing the three MAPK families: ERK1/2, p38 and JNKs. Some steps in the Ras/MAPK pathway discussed in this thesis are depicted. TCF=transcription factor.

regulates p21 and p27 and causes a G_1 cell cycle arrest [160]. In addition, Notch is able to induce degradation of Hash-1, which is highly expressed in some human neuroendocrine tumors such as SCLC and might contribute to their formation [161-163]. Degradation of Hash-1 would thus maybe be a critical step in mediating the growth inhibitory effect of Notch-1, although this is purely speculative.

Crosstalk between Notch and EGFR signaling

The Notch cascade is known to cooperate with other important signaling pathways such as TGFβ, Wnt and EGFR. Several lines of evidence show that the Notch pathway is intimately coupled to signaling through EGFR or downstream targets in both normal development (e.g. vulval development in *C. elegans* [164]) and in the development and maintenance of cancer.

Signaling through the EGFR

The epidermal growth factor receptor (EGFR, also known as HER1 and ErbB1) is a 170 kDa transmembrane receptor tyrosine kinase (RTK) belonging to the ErbB family of receptors, comprising in total four members (ErbB1-4). The mammalian ligands binding to EGFR include the epidermal growth factor (EGF) and transforming growth factor α (TGFα). Ligand binding to EGFR induces receptor homo- or heterodimerization with other members of the ErbB family, with ErbB2 being the preferential heterodimerization partner. Dimerization results in autophosphorylation of the receptor, eventually activating intracellular signaling cascades such as the Ras/MAPK (mitogen-activated protein kinase) and PI3-K/Akt (phosphatidylinositol 3-kinase) signaling pathways, important for cellular proliferation and survival respectively (Fig. 6).

Mitogen-activated protein kinases (MAPKs) compose a family of protein kinases that phosphorylate specific serines and threonines of target proteins, thereby regulating their activity. There are three well-characterized subfamilies of MAPKs namely the extracellular-regulated kinases (ERK1 and ERK2), the c-Jun N-terminal kinases (JNK1 to –3) and p38 (reviewed in [165]). To become activated the MAPK are phosphorylated by MAPK kinases (MAPKK), which in turn are activated by phosphorylation by MAPK kinase kinases (MAPKKK) [165, 166]. The best understood MAPK pathway leads to activation of ERK1/2. This pathway involves the upstream Raf MAPKKK, which can be activated by the proto-oncogene Ras upon RTK activation such as EGFR. Downstream effects of activated MAPKs include phosphorylation and activation of transcription factors, such as c-Fos and AP-1, involved in a plethora of cellular events (Fig. 6).

EGFR signaling in cancer

Aberrant activation of EGFR or downstream signaling components through various mechanisms such as mutations, overexpression and autocrine or paracrine production of ligands, has been implicated in many human tumors. Activation of EGFR is involved in key events leading to neoplastic transformation such as proliferation, differentiation, metastasis, angiogenesis and inhibition of apoptosis. EGFR activity has also been shown to mediate resistance to chemo- and radiotherapy [167]. Most often, aberrant EGFR signaling in tumors is associated with factors that predict aggressive behavior and poor clinical outcome. Therefore, EGFR and its downstream signaling pathways are tempting targets for cancer therapy. Up to date there are two major classes of anti-EGFR inhibitors: monoclonal antibodies that bind to and block the extra cellular part of EGFR and small-molecule tyrosine-kinase inhibitors (TKIs) that either interferes with the ATP or substrate binding sites, both leading to inhibition of EGFR signaling activity.

Notch and EGFR crosstalk in cancer

The crosstalk between Notch and EGFR signaling appears to be reciprocal, i.e. working in both ways. In breast cancer, Fitzgerald et al. showed that the ERK/MAPK and PI-3 kinase pathways downstream of Ras are required for transformation of mouse mammary cells isolated from transgenic animals overexpressing Notch-4 [168]. Another study by Weijzen et al. showed that instead of Ras being a crucial mediator of Notch, Notch-1 signaling is required for maintaining the neoplastic phenotype of human fibroblasts initially transformed by introduction of hTERT, SV40 large T and oncogenic Ras [169]. They also showed that the expression of Ras and Notch-1 correlated in samples from human breast cancer. When transiently expressing Deltex, which as mentioned before might act as an inhibitor of Notch signaling, in mouse mammary epithelium, oncogenesis driven by Ras is inhibited, implying a regulatory event between Notch and Ras [66]. In addition and as discussed above, Notch is activated by EGFR signaling in pancreatic cancer and seems to be required for the tumor-initiating effects of TGFα [157].

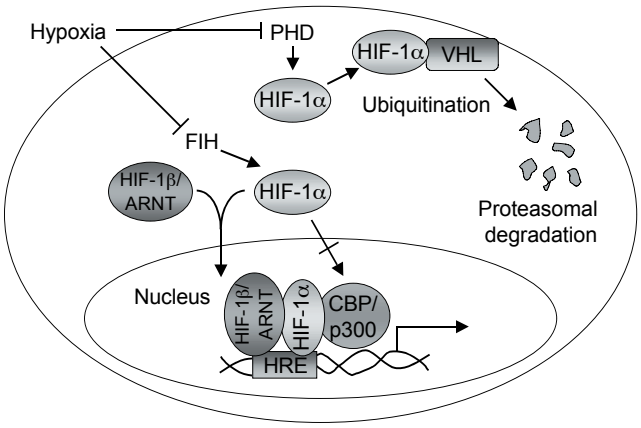

Figure 7. Regulation of HIF-1α. During normoxia, HIF-1α is hydroxylated by PHDs leading to ubiquitination of HIF-1α by interaction with VHL and subsequent degradation by the protasome. In addition, HIF-1α transcriptional activity is regulated by FIH, which inhibits HIF-1α interaction with CBP/p300. Hypoxia inhibits both PHDs and FIH and leads to heterodimerization between HIF-1α and ARNT (HIF-1β) and initiation of transcription. Modified from Giaccia 2004.

Hypoxia

Tumor hypoxia evolves as a consequence of improper oxygen delivery and is a feature of most solid tumors. High rate of proliferation and increased distance to existing blood vessels together with insufficient neo-vascularization contributes to a tumor microenvironment with low oxygen tension. Cancer cells undergo numerous changes that enable them to adapt to and survive during hypoxia. These processes most likely contribute to the malignant phenotype and aggressive behavior of the tumor cells.

Transcriptional response to hypoxia

Many of the cellular responses to hypoxia are mediated by the hypoxia inducible factor (HIF) family of transcription factors. These proteins belong to the basic helix-loop-helix-PAS (bHLH-PAS) family [170] and enclose the hypoxia regulated HIF-1α, -2α, -3α and the constitutively expressed HIF-1β or ARNT. The HIF-α proteins form a transcriptionally active complex with ARNT named HIF-1 during hypoxia and initiate transcription by binding to hypoxia response elements (HREs) in target gene promoters [171, 172]. Targeted deletion of the HIF-1α gene results in decreased expression of many hypoxia regulated transcripts, indicating HIF-1α as the major player in the cellular response to hypoxia [173]. During normal oxygen levels, normoxia, the HIF-1α protein is ubiquitinated by the von Hippel-Lindau (VHL) protein followed by degradation by the proteasomal machinery [172], a process that will be described later (Fig. 7). Recent studies have shown that VHL only binds to HIF-1α after hydroxylation of a key proline in the HIF-1α protein by the prolyl hydroxylases 1-4 (PHD 1-4) [174]. Since the hydroxylation is oxygen dependent this ensures tight regulation of the HIF-1α protein. In addition to being regulated at the protein stability level, HIF-1α is also regulated at the level of transcriptional activity. To initiate transcription, HIF-1α interacts with the co-activator CBP/p300. This interaction involves an asparagine in the HIF-1α protein that is hydroxylated by an asparagine hydroxylase called FIH, for factor inhibiting HIF, at normoxia thereby blocking the HIF-1α interaction with CBP/p300 [175]. During hypoxia, the HIF-1α protein is no longer hydroxylated by the PHDs and VHL does not bind. This results in stabilization of the HIF-1α protein that now can interact with CBP/p300 and initiate transcription from HREs in target gene promoters by forming a complex together with ARNT (Fig. 7). Genes induced by hypoxia include those regulating glucose metabolism and proliferation such as glycolytic enzymes allowing the generation of ATP without the requirement for oxygen, angiogenic factors (e.g. *vascular endothelial growth factor* (*VEGF*) and *erythropoietin (EPO)*) involved in the formation of new blood vessels and production of red blood cells, and growth factors such as *transforming growth factor* α (*TGF*α) [176]. Interestingly, it has been demonstrated that growth factors such as TGFα can induce HIF-1α activity through the Ras/MAPK or PI3-K pathways, thus providing an autocrine loop regulating the hypoxic response (reviewed in [176]). Additionally, the Ras/MAPK pathway has also been shown to enhance the transcriptional activity of HIF-1α. This is at least partially due to ERK1/2 mediated phosphorylation of p300 [177, 178].

Hypoxia, neuroblastoma and Notch signaling

The response to hypoxia enables tumor cells to survive and even proliferate in an environment

lacking oxygen and nutrients. Hypoxia also increases the probability of metastasis and often selects for a more aggressive phenotype. During hypoxic growth *in vitro*, human neuroblastoma cells dedifferentiate and become more immature as shown by up regulation of markers expressed in neural crest sympathetic progenitors such as *c-kit* together with down regulation of neuronal marker genes such as *GAP-43* and *NPY* [179, 180]. This change in differentiation status could be a mechanism for selection of a more malignant tumor phenotype. Indeed, xenograft experiments using human neuroblastoma cell lines pretreated with hypoxia indicated that these cells tended to form tumors more rapidly after injection compared to normoxic treated cells [179].

Interestingly, some of the genes up regulated during the dedifferentiation of hypoxic neuroblastoma cells include *Hes-1* and *Notch-1* [179, 180]. In addition, *Hash-1* is down regulated, indicating that these cells adopt a less differentiated phenotype since Hash-1 is only transiently expressed in sympatho-adrenal precursors and its derivates. Induction of Notch signaling at hypoxia has also been demonstrated during acute pancreatitis (AP), i.e. inflammation of the pancreas, a status often associated with regional hypoxia, in mice. In these experiments it was demonstrated that HIF-1α and hypoxia inducible genes as well as Notch signaling genes (i.e. *Notch-1*, *DLL-1*, *RBP-Jκ* and *Hes-1*) reached maximal expression 8-12 after induction of AP [181]. Furthermore, hypoxia has been shown to induce the expression of presenilin-1 resulting in increased cleavage of APP [182], thus raising the possibility that hypoxia stimulates Notch signaling by enhancing the S3 cleavage. Taken together, activation of Notch signaling might be an important aspect of hypoxic adaptation of neuroblastoma cells, although the mechanism(s) behind this is currently not clarified.

With regard to neuroblastoma it is particularly important to point out that HIF-2α has a developmentally crucial role in the tissues from which the tumor arise since HIF-2α knock-out mice die around E12.5 to E15.5 due to impaired catecholamine production and bradycardia [183]. In human paraganglia, HIF-2α is expressed at week 8.5 [184]. In these cells, Hif-2α is co-expressed with TH, which is known to be induced by hypoxia and, as discussed before, is important in the synthesis of catecholamines.

Chromatin and transcriptional regulation by histone acetylation

The eukaryotic DNA is organized into a structure called chromatin. This is based on the presence of histone proteins that bind to the DNA. Post-translational modifications of the histones have been shown to be involved in transcriptional regulation. These modifications include acetylation and de-acetylation of the histone tails, resulting in a more or less condensed DNA structure, allowing or inhibiting the binding of transcription factors. Dysregulated acetylation and de-acetylation has been implicated in the genesis of tumors, showing the importance of proper regulation of chromatin and transcription and posing a role for targeting acetylation in the treatment of cancer.

The structure of chromatin

The nucleosome, which consists of 146 base pairs of DNA wrapped around a histone core containing two copies each of four different histone proteins (H2A, H2B, H3 and H4), forms the basic repeating unit of chromatin

(reviewed in [185]). In transcriptionally silent cells the chromatin is tightly packed and is called condensed chromatin. Protruding out from the nucleosome complex are the N-terminal tails of the histones. The tails are believed to be involved in mediating contact between adjacent nucleosomes and to DNA and thus play a role in the higher-order organization of chromatin. Histone tails contain conserved lysine residues that can be acetylated resulting in elimination of positive charge, potentially weakening the interaction with the negatively charged DNA and other histones and nucleosomes [186]. This results in a less condensed chromatin structure, possibly allowing transcription factors to access the DNA. Indeed, acetylation of histones has been correlated with nucleosome remodeling and transcriptional activation [187]. Histone acetylation is performed by histone acetyltransferases (HATs), most of which function as transcriptional co-activators recruited to chromatin by interacting with DNA binding proteins [187]. One example of a human HAT is CBP/p300 [188]. On the contrary, histone deacetylases (HDACs) function to remove acetyl groups from histone tails and thus induce transcriptional repression by chromatin condensation. The linker histone H1 is important for chromatin organization but is not modified by acetylation and will not be discussed here.

Histone deacetylases and cancer

Broadly speaking, human HDACs can be divided into three distinct families namely class I HDACs (HDAC1, 2, 3, and 8) which either bind directly to transcription factors or through the co-repressors NCoR and SMRT and mainly seems to be expressed in the nucleus, the class II HDACs (HDAC4, 5, 6, 7, 9, and 10) that can shuttle between the nucleus and cytoplasm and bind hormone co-repressors and certain transcription factors, and finally the class III HDACs which were identified based on their homology to the yeast Sir2 transcriptional repressor (reviewed in [189]).

The link between altered HDAC activity and cancer is best shown in acute promyelocytic leukemia (APL), where translocations between the retinoic acid receptor α (RARα) and either PML (promyelocytic leukemia protein) or PLZF (promyelocytic zink finger), produce fusion proteins that bind to the retinoic acid response elements (RAREs) in the DNA and recruits HDACs, resulting in constitutive repression of RA targeted genes needed for differentiation of myeloid cells [190, 191]. These fusion proteins render the retinoic acid receptor (RAR) insensitive to physiological levels of RA. Though, addition of exogenous RA overcomes the repression exerted by the PML-RARα fusion protein but not the PLZF-RARα. However, by combining RA with inhibitors of HDAC activity it is possible to induce differentiation of PLZF-RARα expressing APL cells both *in vitro* and *in vivo* [190-192].

A wide array of HDAC inhibitors have been shown to possess anti-tumor activity by inducing cell cycle arrest, inhibiting proliferation and stimulating apoptosis and cellular differentiation. It is also plausible that HDAC inhibition might induce expression of silenced tumor suppressor genes. A role for the HDAC inhibitor TSA (trichostatin A) in blocking angiogenesis *in vivo* has also been shown, thus possibly playing an inhibitory role in neovascularization during tumor hypoxia (rewieved in [185]). The anti-angiogenic effect has also been attributed to other HDAC inhibitors, which, in one study, profoundly inhibited *in vivo* tumor growth

of xenografted prostate and breast tumors when combined with inhibitors of the VEGF receptor [193].

Taken together, it is clear that improper regulation of chromatin acetylation and de-acetylation is important in the genesis of cancer and that HDAC inhibitors, alone or in combination with other drugs, might be an attractive tool in tumor treatment.

VPA

Valproic acid (VPA), a drug currently used for long-term treatment of epilepsy in both adults and children, has been shown to possess anti-tumoral activity by inducing cell cycle arrest, cellular differentiation and apoptosis in several *in vitro* and *in vivo* studies (reviewed in [194, 195]). The mechanisms for these actions are not clear but might involve inhibition of glycogen synthase kinase-3β (GSK3β), an important negative regulator of the Wnt signaling pathway, cell-cycle regulatory proteins, down regulation of protein kinase C (PKC) or activation of ERK/MAPK signaling, possibly by increasing DNA binding of the downstream transcription factor AP-1 [194, 195]. In addition it has been shown that VPA functions as an HDAC inhibitor causing hyperacetylation of histones H3 and H4 [196]. It has been proposed that VPA inhibits most class I and II HDACs, with the possible exception for HDACs 6 and 10 [197], and many of the anti-tumoral activities of VPA have been attributed to its HDAC inhibitory effect [194, 195, 197].

VPA and neuroblastoma

One apparent advantage with VPA in treating neuroblastoma is that it is known to be well-tolerated in children. In addition, it is possible to obtain anti-tumoral effects at the concentrations corresponding to those obtained in serum during treatment of epilepsy with no adverse side effects. Several in *vitro* and *in vivo* studies using neuroblastoma cells have shown that VPA induces neuronal differentiation with or without inhibiting proliferation, exemplified by specific up regulation of p21 and p27, and induction of apoptosis [198-202]. Recently it was found that a combination of VPA and IFN-α (interferon-α) synergistically inhibits cell growth *in vitro* as well as in xenografted neuroblastoma tumors [203, 204]. This combination also induces neuronal differentiation, down regulation of N-myc and up regulation of anti-angiogenic factors, collectively suggesting a suppressed malignant potential [204].

Several studies in neuroblastoma cells have shown that VPA increases the AP-1 DNA binding activity *in vitro* [205, 206]. Interestingly, Yuan et al showed that VPA activates the ERK/MAPK pathway known to regulate AP-1 transcription factors through the activation of c-Fos as mentioned above, and that VPA treatment of neuroblastoma cells promoted neurite outgrowth and up regulation of GAP-43, which harbors an AP-1 binding site [207]. In addition, they showed that ERK activity was required for VPA to promote neurite outgrowth. Another study performed in the same cell line, SH-SY5Y, suggested that activation of the ERK/MAPK signaling pathway by VPA was independent of its HDAC inhibitory activity [208].

As described above, several crucial steps of Notch signaling such as CSL mediated repression of Notch target genes, and Hes-1 repression of Hash-1, are regulated by HDAC activity. The role of VPA as an HDAC inhibitor regulating the Notch cascade and its ability to induce neuronal differentiation of neuroblastoma cells will be discussed in "present investigation". In addition, the notion

that VPA can induce ERK/MAPK signaling, and how this might connect to regulation of Hes-1, will also be discussed.

Protein degradation

In recent years it has become clear that protein degradation plays an important role in regulating protein function. The two major ways of protein degradation are the non-lysosomal system, involving the 26S proteasome, and the endosome-lysosome system, important for degradation of endocytosed proteins. Of specific interest for this thesis, is the degradation of the Notch receptor by interaction with E3 ubiquitin ligases and the finding that Notch can mediate proteasome-dependent proteolysis of Hash-1. In addition, proteasomal degradation of HIF-α proteins is crucial in regulating the response to hypoxia as described above.

The ubiquitin-26S proteasome machinery

The non-lysosomal system degrades proteins from the nucleus, the cytosol and the endoplasmatic reticulum (ER) and is primarily carried out by the ubiquitin-26S proteasome machinery. This intracellular proteolysis is performed in two successive steps, the first involving addition of multiple ubiquitin molecules to the target protein and the second degradation of the ubiquitin-tagged protein by the 26S proteasome, releasing free ubiquitin molecules that are recycled (reviewed in [209]). Addition of ubiquitin to the target protein, a process called ubiquitination or ubiquitylation, occurs in three steps (reviewed in [209]). In the first step, an ubiquitin-activating enzyme known as E1 activates free ubiquitin. This process requires ATP and results in the binding of E1 to the ubiquitin molecule. Ubiqiutin is then transferred from E1 to an ubiquitin-carrier enzyme, E2 that associates with an ubiquitin protein ligase (E3). Finally, E3 catalyses the transfer of ubiquitin from E2 to a lysine residue in the target protein. There are several E2 and E3 proteins allowing for expansion and specificity of the reaction. The E3 ligases are divided into HECT domain E3s and RING FINGER E3s depending on which conserved domains they contain. The ubiquitin molecule harbors at least four lysine residues (positions 11, 29, 48 and 63) capable of binding target proteins. In addition, ubiquitin molecules are able to link to each other through binding to these lysine residues forming multi-ubiquitin chains. Depending on how many ubiquitin molecules that are attached to the protein and which lysine residues that are involved, ubiquitination results in different fates for the target protein. Notably, only multi-ubiquitination targets proteins for degradation by the 26S proteasome, and this most likely involves lysine 48 (K48) in ubiquitin. Other processes induced by ubiquitination involve DNA repair, protein translation and endocytosis (reviewed in [209]).

The 26S proteasome complex recognizes the multi-ubiquitin chain and degrades the tagged protein into small peptides. The proteasome is a large multi-catalytic protease composed of two subunits: a 20S core particle that harbors the catalytic activity and a regulatory 19S particle that is responsible for recognizing the ubiqiutinated proteins and allows entry of the substrate into the proteolytic chamber (reviewed in [210]).

Ubiquilin

Ubiquitin family proteins, defined by their structural homology with ubiquitin, are divided into two major classes. The first class, ubiquitin-like proteins (UBLs), can be attached to other proteins by their C-termini

and function in a way similar to ubiquitin whereas proteins of the second class contain domains related to ubiquitin, but are otherwise structurally unrelated and are not conjugated to other proteins (reviewed in [211, 212]). These proteins are referred to as ubiquitin-like domain proteins or UDPs and play essential roles in diverse cellular events such as DNA repair, apoptosis, protein folding and signaling (reviewed in [211, 212]). Interestingly, some UDPs have also been suggested to function as a link between the ubiquitin- and proteasomal pathways by binding to ubiquitin, ubiquitin E3 ligases and certain subunits of the proteasome (reviewed in [212]).

Ubiquilin-1, also referred to as hPLIC-1 or DA41, belongs to the UDP group of ubiquitin-like proteins. The role of ubuquilin-1 is currently not well understood, though several studies have shown that it interacts with E3 ubiquitin ligases and proteasomes and thus may provide a link between ubiquitin and proteasomal degradation [213]. In addition ubiquilin-1 seems to be involved in regulating the expression levels of various proteins as it prevents degradation of p53 and IκBα [213, 214], and promotes accumulation of presenilin-1 and –2 possibly by blocking ubiquitin binding and preventing subsequent proteosomal degradation [215, 216]. Another interesting finding is that the *Xenopus* ubiquilin-1 homologue XDRP1, related to yeast Dsk2, binds to cyclin A and prevents its degradation possibly by function as a molecular chaperone, protecting cyclin A from proteolysis [217].

We have shown that overexpression of ubiquilin-1 leads to accumulation of tissue specific bHLH proteins (paper I), and the role of ubiquilin-1 in Notch signaling will be further discussed in “present investigation”.

The present investigation

Aims

The main objective for this thesis has been to elucidate how Notch signaling is regulated and what role it plays in growth and differentiation of human neuroblastoma cells. More specifically, we wanted to:

Isolate novel proteins to which the downstream Notch target Hash-1 binds, and investigate the impact of their interaction.

Study the effect of the HDAC inhibitor VPA on the regulation of the Notch signaling cascade and the consequences on neuroblastoma cell differentiation and viability.

Investigate the crosstalk between mitogenic EGFR signaling and the Notch cascade both in normoxic and hypoxic neuroblastoma cells.

Results and discussion

Ubiquilin-1 interacts with Hash-1 and affects the levels of neuronal bHLH transcription factors (paper I)

Hash-1 is transiently expressed in the developing SNS and undetectable in mature sympathetic neurons [104]. Therefore, the finding that Hash-1 is expressed in the majority of human neuroblastoma cell lines and tumors implies that these tumors are arrested at an immature stage of neuronal differentiation. In addition, during induced differentiation of neuroblastoma cells *in vitro*, Hash-1 is down regulated suggesting that this is a pre-requisite for differentiation to take place [106, 139]. On the other hand, Hash-1 is also down regulated during the proposed dedifferentiation that takes place during hypoxia, but then probably reflects a regression of differentiation status rather than progression since this is accompanied by a down regulation of virtually all differentiation markers [179]. Thus, precise regulation of Hash-1 expression is important and proteins that interact with Hash-1, and might modulate its function, are of interest.

In a yeast-two hybrid screen using a cDNA library from the human neuroblastoma cell line SH-SY5Y, we identified 31 clones that were able to interact with the 180 N-terminal amino acid residues of Hash-1. One of these clones coded for full-length ubiquilin–1 (hPLIC-1, DA41), a ubiquitin-like domain protein. The interaction between Hash-1 and ubiquilin-1 was further corroborated in mammalian two-hybrid and co-immunoprecipitation experiments. Additionally, from the mammalian two-hybrid experiments it was clear that ubiquilin-1 was able to interact with itself and with pro-neuronal, tissue-specific bHLH proteins other than Hash-1 such as Hes-1 and dHAND but not with the ubiquitously expressed E-proteins E2-2 and E47 or the HLH protein Id1.

When Hash-1 was introduced into CHO cells, a clear accumulation of the protein was detected upon treatment with proteasome inhibitors, and this accumulation was further accentuated upon co-expression with ubiquilin-1. Thus we concluded that Hash-1 is degraded through the 26S proteasomal machinery. Though, in neuroblastoma cells, Hash-1 was surprisingly down regulated when proteasomal activity was inhibited. Since Hes-1 is a potent repressor of Hash-1 we reasoned that a rapid accumulation of Hes-1 might precede the effect of proteasome inhibitors on the Hash-1 protein. Indeed, by Northern blotting we could see that *Hash-1* mRNA was decreased in the presence of proteasome inhibitors, indicating less transcription. Since ubiquilin-1 has been implicated in the ubiquitin proteasomal degradation pathway, we further wanted to investigate whether Hash-1 levels were modulated by the ubiquilin-1 interaction. Transient overexpression of ubiquilin-1 in neuroblastoma cell lines promoted accumulation of both Hash-1 and Hes-1, supporting our interaction data and further substantiating the notion that ubiquilin-1 might be involved in the control of proteasomal degradation of tissue-specific bHLH proteins. A possible mechanism for this might be through preventing ubiquitin binding and subsequent recognition by the 26S proteasome. Another is that ubiquilin works as a chaperone, protecting Hash-1 from degradation as has been suggested for cyclin A in *Xenopus* [217].

As mentioned above, ubiquilin has been shown increase the expression of presenilin-1 and -2 [215, 216]. Since both Hes-1 and Hash-1 reside in the Notch signaling cascade, this is a

very interesting observation since it could mean that ubiquilin, through increased presenilin levels, might induce the S3 cleavage of the Notch-1 receptor and thus lead to activation of the pathway. However, it was recently demonstrated that even if overexpression of ubiquilin indeed leads to accumulation of presenilins, it inhibits their processing which is thought to be crucial for their activity [218]. In addition, ubiquilin decreases the expression of nicastrin and Pen-2, two other components of the γ-secretase complex as mentioned above [218]. These results suggest that, rather than stimulating γ-secretase activity, ubiquilin inhibits it. This would then result in decreased Notch signaling, and thus de-repression and up regulation of Hash-1. In addition, since Notch has been shown to degrade Hash-1 through the proteasome [161], decreased Notch levels in the presence of ubiquilin would lead to increased Hash-1 expression. The inhibition of presenilin and γ-secretase activity by ubiquilin would thus lead to increased Hash-1 levels, which is in line with our observation that ubiquilin promotes accumulation of Hash-1, but this is purely speculative and must be further examined. Another interesting observation from the Notch signaling perspective is that ubiquilin is up regulated by hypoxia in human neuroblastoma and astrocytoma cell lines and seems to be involved in cellular adaptation to ischemia by suppressing cell death [219]. Since neuroblastoma cells seems to adapt to hypoxia through dedifferentiation, possibly involving activation of the Notch cascade, this would be interesting to examine in more detail.

Inhibition of HDAC activity increases ICN expression and leads to neuroblastoma cell differentiation (paper II)

Several steps of the Notch signaling cascade are regulated by HDACs and thus we argued that it would be possible to modulate Notch activity by applying HDAC inhibitors. Many HDAC inhibitors have been shown to possess anti-tumor activity and some have even been used in cancer therapy. Valproic acid (VPA) is a well-tolerated drug that has been used for treating epilepsy in both adults and children without any severe side effects (reviewed in [194, 195]). Several studies have also shown that VPA possess anti-tumor activity in neuroblastoma cells as it induces cellular differentiation and some times also apoptosis [198-202]. Recently it became clear that VPA can act as an HDAC inhibitor and many of its anti-tumoral properties have been attributed to this function [194-197]. Thus, due to its low toxicity and its anti-tumor activity together with the fact that it is already in clinical use, VPA might be an attractive drug for treating neuroblastoma.

In paper II we show that human neuroblastoma cells differentiate morphologically as shown by neurite-like extensions upon treatment with therapeutic levels of VPA. In addition, the neuronal differentiation markers *NPY* and *GAP-43* were also up regulated, supporting neuronal differentiation. Furthermore, VPA treatment also induced cell death as shown by propidium iodide and Annexin V stainings as well as by MTT assays. Treating neuroblastoma cells with VPA resulted in prominent acetylation of histone H3 confirming its activity as an HDAC inhibitor. In line with this, Hes-1 was up regulated in a concentration dependent manner upon VPA treatment. This was probably a consequence of increased transcription as

reflected by augmented promoter activity in luciferase assays.

More intriguingly was the observation that VPA treatment increased ICN expression as demonstrated by specific recognition by an antibody directed towards Val1744, which is only exposed after the activating S3 cleavage of the Notch receptor. In addition, the VPA induced differentiation of SK-N-BE(2) cells was partially repressed by inhibition of γ-secretase, thus implying that ICN was involved in the differentiation mediated by VPA. This is paradoxical to the idea that Notch signaling maintains neuroblastoma cells in an undifferentiated state. Though during induced differentiation *in vitro*, we have observed a transient increase in both Notch-1 and Hes-1 levels, followed by rapid down regulation of Hash-1. Thus the activation of Notch by VPA could merely reflect the initial phase of differentiation. The mechanism behind the VPA induced Notch activation is not known, however one could speculate that VPA either promotes accumulation of ICN by inhibiting receptor degradation or by stimulating γ-secretase mediated cleavage.

In contrast to what we had expected, VPA treatment did not enhance Hash-1 expression, which would have been the case if the HDAC to which TLE binds, was inhibited. Though, not all HDACs are inhibited by VPA and it remains possible that it is these insensitive HDACs that are responsible for repression of Hash-1. Indeed, it has been shown that Hes-1 is able to repress transcription, not only by binding to TLE and thus HDAC through its WRPW motif, but also by recruiting an HDAC called SIRT1 via its bHLH domain [220]. Interestingly, SIRT has been shown to be insensitive to TSA treatment. Thus it is possible that Hes-1 mediates some repression on the *Hash-1* promoter even in the presence of VPA. In addition, and as mentioned above, Notch-1 is able to induce degradation of Hash-1 via the proteasome [161], and since ICN is increased during VPA treatment one could speculate that this would result in decreased Hash-1 expression. Another explanation is that the decreased Hash-1 expression reflects the differentiated phenotype, as Hash-1 is not detected in mature neurons.

Other researchers have reported that VPA induces activation of ERK1/2 in neuroblastoma and that this activates differentiation related genes such as GAP-43 regulated by AP-1 [207]. However, in our experiments we could only detect a very modest activation of ERK1/2. Still the idea that VPA activates ERK1/2 is of particular interest considering the Notch pathway since several reports have shown a reciprocal crosstalk between the Notch and Ras/MAPK signaling cascades. As we show in paper III, Hes-1 can be induced by TGFα, a well known hypoxic target gene, and blocked by inhibitors of EGFR signaling, independent of Notch activity. Thus, if VPA activates ERK1/2 this might result in increased Hes-1 expression, though this remains to be elucidated.

In conclusion, VPA can potentially be used in treating human neuroblastoma as it promotes cell death and differentiation. Furthermore, some of the differentiation related effects might be attributed to Notch signaling, but further studies are required to clarify to what extent the Notch cascade mediates the effect of VPA on neuroblastoma cells. It would also be very interesting to study whether other HDAC inhibitors affect Notch signaling. We have observed that neuroblastoma cells are sensitive to blocking EGFR signaling and an interesting approach would be to combine VPA treatment with inhibitors of this pathway.

Signaling through EGFR and ERK1/2 regulates Hes-1 expression without Notch activation (paper III)

Since it has been shown that the Notch signaling cascade is linked to Ras/MAPK signaling, often dysregulated in human cancers, we wanted to investigate whether these two pathways converge in neuroblastoma cells and how they affect each other. TGFα is a ligand of the EGF receptor and a potent mitogen of human neuroblastoma cells [221]. Therefore we treated the neuroblastoma cell line SK-N-BE(2)c with TGFα and studied the effect on Hes-1 as a readout for Notch signaling activity. Interestingly, Hes-1 was up regulated both at the mRNA and protein levels by TGFα. This induction was time and concentration dependent and occurred concomitantly with phosphorylation of ERK1/2. We could not detect any increase in neither *Notch-1* nor *Notch-3* mRNA levels indicating that the *Hes-1* induction occurred either due to increased activation of the Notch receptors already present or due to direct influence from EGFR signaling. By using two different γ-secretase inhibitors we could show that increased Hes-1 expression was only partially a consequence of Notch activation. Instead, up regulation of Hes-1 by TGFα treatment seemed to depend on activation of ERK1/2, as inhibition of either MEK (the kinase responsible for activation of ERK1/2) or EGFR almost completely abolished Hes-1 expression and decreased *Hes-1* reporter activity. Interestingly, also basal Hes-1 expression seemed to depend on ERK1/2 phosphorylation rather than Notch activity. Though, in contrast to Hes-1 expression in TGFα treated cells, inhibition of EGFR did not affect basal Hes-1 expression whereas inhibition of MEK did. These results indicate that ERK1/2 can be activated independently of EGFR signaling in unstimulated SK-N-BE(2)c cells and that this is involved in basal Hes-1 expression. To further investigate the mechanism behind the ERK1/2 mediated up regulation of Hes-1, we performed luciferase assays using a reporter construct containing eight repetitive binding sites for CSL (8 x CSL). Interestingly, TGFα was equally efficient in stimulating this reporter as the construct containing the Hes-1 promoter suggesting that the Ras/MAPK cascade directly affects CSL transcriptional activity. In line with this observation, γ-secretase inhibitors could not down regulate the reporter activity, suggesting that TGFα affects CSL mediated transcription without the presence of ICN. Also the basal activity of the 8 x CSL construct was in large refractory to γ-secretase inhibition.

These results were indeed surprising since Hes-1 is considered to be a direct Notch target and ICN is thought to be necessary for CSL mediated transcription. Though, as discussed above, gene ablation studies in mice have shown that when knocking out either Notch-1 or RBP-Jκ, the expression of Hes-1 is unaffected (de la Pompa 1997), indicating Notch and/or CSL independent pathways in the regulation of Hes-1. In addition, a recent study in *Drosophila* showed that Su(H), a homologue to CSL, is capable of signaling without the presence of Notch [222]. Furthermore, a translocation in mucoepidermoid cancer involving a protein of unknown function called MECT1 (for mucoepidermoid carcinoma translocated 1) and MAML2 is able to induce Hes-1 expression by a yet unidentified mechanism since both Notch receptor signaling and CSL binding sites seemed dispensable for this induction [223]. Thus it is possible that ERK1/2 by unknown means is involved in stimulating the expression of Hes-1 independently of ICN activity.

To investigate the functionality of the TGFα induced Hes-1 we looked at the Hes-1 target Hash-1. Our results show that Hash-1 is rapidly down regulated in a concentration dependent manner upon TGFα stimulation. Thus, it seems as if Hes-1 is capable of repressing Hash-1. However, the biological significance of Hes-1 induction upon TGFα stimulation remains to be investigated, but it is tempting to suggest a role for Hes-1 in maintaining an undifferentiated neuroblastoma phenotype by repressing Hash-1 and at the same time mediating some of the mitogenic effects of EGFR signaling. It should however be stressed that the cell line used in this study, SK-N-BE(2)c has a relatively low basal Notch signaling activity, as indicated by the modest Hes-1 levels detected. It is possible that the strong effect of Ras/MAPK signaling on Hes-1 is more readily detectable in such a cell line compared to other cell lines with higher basal Hes-1 levels, and hence presumably a more active Notch cascade.

Hes-1 is induced by hypoxic stimulation of EGFR signaling and is refractory to Notch inhibition (paper IV)

Hypoxia is a key feature of solid tumors and results from improper vascularization as cancer cells grow with increasing distance to existing blood vessels. Neuroblastoma cells exposed to hypoxia have been shown to dedifferentiate, possibly a way of adapting to the harsh environment [179]. This dedifferentiation has also been proposed to contribute to the increased malignant potential of the cells [180]. Therefore, it is of interest to investigate what factors are involved in this adaptation to hypoxia. Several components of the Notch signaling cascade have been shown to be up regulated by hypoxia in neuroblastoma cells and we were interested in studying whether this activation was involved in the hypoxic adaptation and possibly dedifferentiation observed. Therefore we grew human neuroblastoma cells at 1% oxygen, hypoxia, and used cells grown at normoxia (21% oxygen) as a control.

We show in paper IV that Hes-1 expression is increased in several human neuroblastoma cell lines after 24 hours of hypoxic growth. This up regulation is most likely due to enhanced transcription as shown by increased luciferase activity using the *Hes-1* promoter coupled to a renilla reporter gene. Further studies in the SK-N-BE(2)c neuroblastoma cell line revealed that the Hes-1 induction occurred already after 2 hours of hypoxia and lasted throughout the course of the experiment. The increase in Hes-1 expression correlated with ERK1/2 activation as well as with HIF-1α protein stabilization, a key marker of hypoxia. Interestingly, and in line with the observations in paper III, we could not detect any effect of γ-secretase inhibition on the Hes-1 levels. Instead the Hes-1 expression was sensitive to both MEK and EGFR inhibitors comparable to the situation in TGFα stimulated neuroblastoma cells at normoxia (paper III). Thus, one could speculate that the Hes-1 expression at hypoxia primarily is regulated by ERK1/2 activation, possibly by stimulation of EGFR signaling through up regulation of growth factors such as TGFα.

Since previous studies have shown that Notch-1 is up regulated at hypoxia in neuroblastoma cells [179], it is still possible that Notch activation is involved in Hes-1 induction in a time- and cell type specific manner. A tempting scenario would be that the immediate effect on Hes-1 expression is mediated by growth factors such as TGFα and Ras/MAPK activity whereas long-term

effects are mediated by increased Notch signaling activity. This would be likely since others have shown that presenilin is increased by hypoxia [182], which might be associated with enhanced Notch receptor processing. To investigate this possibility we therefore grew SK-N-BE(2)c cells at hypoxia and added either the γ-secretase inhibitor DAPT or the MEK inhibitor U0126 two hours before harvest. Interestingly, addition of U0126 affected the Hes-1 levels at all time points studied whereas DAPT treatment left the Hes-1 induction unaffected. Thus, from these experiments we concluded that both immediate and long-term Hes-1 induction in SK-N-BE(2)c cells was mediated through ERK1/2 activation at hypoxia.

As discussed in paper III, the cell line used in these studies has a low basal Notch activity. It is therefore possible that Hes-1 is regulated in a different way in cells with higher Notch receptor expression. A first attempt to clarify whether Notch receptor levels influence the hypoxic regulation of Hes-1, SK-N-BE(2)c cells were transfected with either intracellular Notch (ICN) or full length Notch (FLN) together with the 8 x CSL luciferase reporter construct and treated the cells with the γ-secretase inhibitor L-685,458. Interestingly, hypoxia caused reporter gene activation to a much larger extent when the FLN construct was introduced compared to the ICN construct. This implied that increased processing of the receptor might occur at hypoxia. Indeed, when treating the cells with the γ-secretase inhibitor, it was possible to inhibit most of the hypoxia induced reporter activity in the FLN transfected cells but not in the ICN cells. In addition to increased processing, some accumulation of ICN at hypoxia might occur since the FLN induction was not completely abolished with γ-secretase inhibition and since luciferase activity was slightly induced by ICN transfection. The mechanism behind this possible accumulation is not known, however, it could involve down regulation of proteins that negatively affects Notch signaling such as Numb or Itch involved in turnover of ICN.

In conclusion, these results show that Hes-1 is mainly regulated by increased activity of the Ras/MAPK signaling cascade at hypoxia. This is in line with our observation in paper III showing that TGFα can induce Hes-1 expression in an ERK1/2 dependent manner. In addition, hypoxia might also induce Notch signaling through elevated processing of the receptor. From a conceptual point of view this observation might be important since this would also lead to activation of Notch target genes other than Hes-1. The role of Notch signaling activity in dedifferentiation and neuroblastoma cell adaptation to hypoxia remains however to be elucidated.

Conclusions

Ubiquilin-1 interacts with Hash-1 and promotes its accumulation, possibly by protecting it from degradation by the 26S proteasomal machinery.

VPA activates Notch signaling through increased ICN and Hes-1 expression. In addition, VPA induced differentiation of human neuroblastoma cells is partially mediated through Notch activity.

Hes-1 is regulated by TGFα induced Ras/MAPK signaling independent of Notch activity.

Hypoxia induces Hes-1 expression independent of Notch signaling activity and is dependent on signaling through the EGFR.

Populärvetenskaplig sammanfattning

Uppkomsten och utvecklingen av en tumör beror på att det reglerade förhållandet mellan celldelning och celldöd är satt ur spel. Neuroblastom är en tumör som drabbar i genomsnitt 10-15 barn per år i Sverige. Tumören uppstår från celler som bildar det sympatiska nervsystemet och som av någon anledning är blockerade i sin normala utmognad, differentiering, till nerver. Det sympatiska nervsystemet kontrollerar funktioner som inte kan styras av viljan, såsom svar på stress och fara. Neuroblastom kan delas in i ett flertal grupper beroende på var i kroppen tumören uppkommer och hur differentierad den är. De olika grupperna har också olika prognos, där mer differentierade tumörer korrelerar till bättre överlevnad.

Eftersom neuroblastom uppstår ur omogna sympatiska nervceller uttrycker tumören också gener som annars bara uttrycks under fosterutvecklingen. En av dessa kodar för ett s.k. bHLH (basiskt helix-loop-helix) protein som heter Hash-1. Hash-1 är en transkriptionsfaktor, d.v.s. den reglerar uttrycket av andra gener varav ett flertal är involverade i differentiering av det sympatiska nervsystemet. Hash-1 uttryck är nödvändigt för utmognaden av sympatiska nervsystemet, men uttrycks ej i mogna neuron. Det är möjligt att Hash-1 är involverad i den blockerade differentieringen av det sympatiska nervsystemet som leder till uppkomst av neuroblastom. Därför är det av intresse att studera hur Hash-1 regleras. I arbete I visar vi att Hash-1 binder till ubiquilin-1, ett protein som skyddar andra proteiner från nedbrytning. Hash-1 bryts ner via den s.k. proteasomen och i närvaro av ubiquilin-1 ackumuleras Hash-1. Däremot försvinner Hash-1 uttrycket då man inhiberar proteasomen. Detta beror troligtvis på att ett annat bHLH protein, Hes-1, som negativt reglerar uttrycket av Hash-1, under dessa förhållanden uppregleras.

Både Hes-1 och Hash-1 ingår i den så kallade Notch kaskaden. Notch är en receptor som sitter i cellmembranet och som kan binda till andra proteiner, s.k. ligander, på intilliggande celler. Då Notch binder en ligand, klyvs en del av receptorn av och transporteras in i cellens kärna där den styr uttrycket av målgener. Ett av proteinerna som direkt regleras av Notch är Hes-1. Generellt sätt uttrycks Notch i omogna celler och verkar bibehålla dessa i ett odifferentierat stadium. Notch receptorn är involverad i uppkomsten av ett flertal tumörer där felaktigt överuttryck hindar cellernas normala utmognad. I andra fall kan frånvaron av Notch leda till cancer. Det är möjligt att på kemisk väg få neuroblastomceller att mogna till nervceller. Vi har tidigare visat att Hash-1 nedregleras och Hes-1 först uppregleras för att sedan försvinna under denna differentieringsprocess. Dessutom har vi sett att överuttryck av Notch receptorn verkar hindra differentieringen och därför möjligen bibehåller cellerna i ett omoget stadium. Därför kan regleringen av Notch kaskaden vara viktig vid uppkomsten av neuroblastom.

Ett flertal steg i Notch kaskaden regleras av s.k. histon deacetylaser, HDACs. Dessa fungerar så att de bibehåller DNA't i ett stadium så att transkription inte kan ske. Man kan på kemisk väg hindra HDAC aktiviteten och detta har visat sig ha positiv effekt vid behandlingen av ett flertal tumörer. VPA, är en drog som används kliniskt då man behandlar epilepsi. Dessutom fungerar VPA som en

HDAC inhibitor och har visat sig hindra tillväxt av neuroblastom och också inducera neuronal differentiering. I arbete II visar vi att VPA leder till differentiering av SK-N-BE(2) neuroblastomceller. Dessutom visar vi att VPA aktiverar Notch kaskaden på två sätt, dels genom klyvning av receptorn, och dels genom uppreglering av Hes-1. Om vi på kemisk väg hindrar Notch aktiveringen, får vi också mindre differentiering, varför Notch verkar vara inblandad i denna process. Hur detta sker är dock inte känt, men vi tror att VPA härmar de första stegen i den kemiskt inducerade differentieringen. Eftersom VPA redan används kliniskt och har få bieffekter skulle det vara möjligt att använda VPA vid behandling av neuroblastom.

I solida tumörer såsom neuroblastom är det vanligt att hypoxi, lågt syretryck, uppkommer i områden där blodtillförseln är otillräcklig. Neuroblastomceller som utsätts för hypoxi har vistats anpassa sig till den yttre miljön genom att bli mindre differentierade och därför också mer maligna. Vi är intresserade av vilken roll Notch kaskaden spelar vid cellens anpassning till hypoxi. Tidigare studier har visat att Notch induceras vid hypoxi och vi visar i arbete IV att detta även gäller Hes-1. Notch kaskaden samarbetar med andra för cellen viktiga signaleringsvägar, varav några är involverade i uppkomsten av cancer. En av dessa vägar är signalering via den s.k. EGF receptorn (EGFR). Det är sedan tidigare väl känt att tillväxtfaktorer som stimulerar EGFR induceras vid hypoxi. I arbete III visar vi att Hes-1 kan regleras direkt av EGFR kaskaden då man stimulerar neuroblastomceller med EGFR liganden, TGFα. Vi visar att denna reglering sker oberoende av Notch. I arbete IV visar vi att Hes-1 uppregleringen vid hypoxi beror av den ökade EGFR aktiviteten och även här är oberoende av Notch. Kopplingen mellan EGFR signalering och Notch kaskaden är mycket intressant eftersom det finns ett flertal droger som hindrar EGFR signalering och om man kan påverka Notch kaskaden med dessa så kan man kanske också hindra tillväxten av neuroblastom.

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