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Notch signaling in neuroblastoma and renal cell carcinoma

Jonas Sjölund



LUND UNIVERSITY
Faculty of Medicine

Academic dissertation

By due permission of the Faculty of Medicine, Lund University, Sweden to be defended at the main lecture hall, Pathology building, Malmö University Hospital on Friday the 15th of February, 2008, at 09.15 for the degree of Doctor of Philosophy, Faculty of Medicine.

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| <p>Abstract</p> <p>The Notch signaling pathway governs cell functions essential for normal development and organogenesis. Aberrant Notch signaling has been implicated in tumorigenesis by perturbing control of proliferation, differentiation, apoptosis, migration and angiogenesis.</p> <p>Neuroblastoma is an embryonal tumor derived from cells of the sympathetic nervous system, arrested at an immature stage of differentiation. Notch signaling is thought to play an important role during the development of the sympathetic nervous system, and prior studies have indicated that dysregulated Notch signaling might be associated with the perturbed differentiation that characterize neuroblastoma cells. Here we show that the histone deacetylase inhibitor valproic acid induced differentiation of neuroblastoma cells, in conjunction with activation of Notch-1 signaling. In addition, we also noted that the expression of the Notch target HES-1 was primarily regulated by ERK-MAP kinase signaling in a neuroblastoma cell line.</p> <p>Clear cell renal cell carcinoma (CCRCC) is characterized by activation of the hypoxia inducible factor (HIF) pathway due to functional loss of the von Hippel-Lindau tumor suppressor gene. However, cooperating oncogenic events parallel to the HIF pathway remains poorly described. Our results presented here imply a growth-promoting role for Notch signaling in CCRCC cells, seemingly independent of HIF-regulated cellular events. Treatment of nude mice bearing CCRCC xenografts, with daily injections of a Notch inhibitor (γ-secretase inhibitor) in cycles of 3 days followed by 4 days without treatment, retarded tumor growth and minimized the adverse effects commonly associated with γ-secretase inhibition. In addition, analysis of primary CCRCC samples revealed that Notch-1 and Jagged-1 were expressed at significantly higher levels compared to normal kidney tissue samples. Finally, we found that inhibition of Notch signaling decreased the migratory potential of CCRCC cells. This effect might be linked to a cross-talk between Notch and TGF-β signaling in CCRCC cells.</p> | | |
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Signature Jonas Sjölund Date 11th of January, 2008

”Jaha, nähä”
Håkan Axelson

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

- I** Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells
Marie-Thérèse Stockhausen, Jonas Sjölund, Christina Manetopoulos, and Håkan Axelson
Br J Cancer 92: 751-759, 2005
- II** Regulation of the Notch target gene Hes-1 by TGF- α induced Ras/MAPK signalling in human neuroblastoma cells
Marie-Thérèse Stockhausen, Jonas Sjölund, and Håkan Axelson
Exp Cell Res 310: 218-228, 2005
- III** Suppression of renal cell carcinoma growth by inhibition of Notch signaling *in vitro* and *in vivo*
Jonas Sjölund, Martin Johansson, Sugata Manna, Carl Norin, Alexander Pietras, Siv Beckman, Elise Nilsson, Börje Ljungberg, and Håkan Axelson
J Clin Invest 118: 217-228, 2008
- IV** Notch-1 signaling increases CCRCC cell migration via the TGF- β signaling pathway
Jonas Sjölund, Anna-Karin Boström*, Sugata Manna*, Aristidis Moustakas, Erik Fredlund, and Håkan Axelson
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*These authors contributed equally to this work.

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Abbreviations

| | |
|---------------|---|
| ANK domain | ankyrin domain |
| APL | acute promyelocytic leukemia |
| ARNT | aryl hydrocarbon receptor |
| BCC | basal cell carcinomas |
| bHLH | basic helix-loop-helix |
| BMP | bone morphogenic protein |
| CCRCC | clear cell renal cell carcinoma |
| CREB | cAMP response element binding |
| CREBBP | CREB-binding protein |
| CSC | cancer stem cells |
| CSL | CBF-1/Suppressor of Hairless/Lag-1 |
| DCIS | ductal carcinoma in situ |
| Dlk-1 | Delta homolog-like-1 |
| DLL | Delta-like |
| DSL domain | Delta, Serrate, Lag-2 domain |
| EBV | Epstein–Barr virus |
| EGF | epidermal growth factor |
| EMT | epithelial-to-mesenchymal transformation |
| ER | endoplasmic reticulum |
| ERK | extra cellular signal regulated kinase |
| FBW7 | F-box and WD40 domain protein-7 |
| FIH-1 | factor inhibiting HIF |
| GAP43 | growth associated protein 43 |
| GSI | γ -secretase inhibitor |
| GSK-3 β | glycogen synthase kinase-3 β |
| HAND-2 | heart- and neural crest derivatives-expressed 2 |
| HASH-1 | human achaete scute homolog 1 |
| HAT | histone acetyl transferase |
| HD | heterodimerization domains |
| HDAC | histone deacetylase |
| HES | Hairy/Enhancer of Split |
| HEY | Hairy-related |
| HIF | hypoxia inducible transcription factor |
| HNSCC | head and neck squamous cell carcinoma |
| HPV | human papilloma virus |
| ICN | Intracellular Notch |
| IFN- α | interferon- α |
| IL-2 | Interleukin-2 |
| INSS | International Neuroblastoma Staging System |
| JNK | c-Jun amino-terminal kinases |
| KSHV | Kaposi's sarcoma-associated herpesvirus |
| LNR | Lin Notch Region |
| MAML | Mastermind-like |
| MAPK | mitogen-activated kinase |
| MASH | mouse achaete scute homolog |
| MEC | mucoepidermoid carcinoma |
| MMTV | mouse mammary tumor virus |
| mTOR | mammalian target of rapamycin |
| NB | neuroblastoma |

| | |
|--------------|--|
| NCOR | nuclear receptor co-repressor |
| NPY | neuropeptide tyrosine Y |
| PDGF | platelet derived growth factor |
| PHOX-2B | paired-like homeobox 2B |
| PI3K | phosphatidylinositol-3-kinase |
| PLZF | promyelocytic zinc finger |
| PNET | primitive neuroectodermal tumors |
| PNMT | phenylethananide N-metyltransferase |
| POFUT-1 | O-fucosyltransferase-1 |
| PS | presenilin |
| RA | retinoic acid |
| RAM23 domain | RBP-Jκ associated molecule domain |
| RAR-α | retinoic acid receptor α |
| RCC | renal cell carcinoma |
| SCC | squamous cell carcinoma |
| Shh | sonic hedgehog |
| SIF | small intensely fluorescent |
| SKIP | Ski-interacting protein |
| SMAD | small mothers against decapentaplegic |
| SNS | sympathetic nervous system |
| TAD | transactivation domain |
| T-ALL | T-cell acute lymphoblastic leukemia |
| TGFBR | TGF-β serine/threonine kinase receptor |
| TGF | transforming growth factor |
| TH | tyrosine hydroxylase |
| TLE | transducin-like enhancer of split |
| TNM | tumor-node-metastasis |
| TPA | phorbol-12-myristate-13-acetate |
| VEGF | vascular endothelial growth factor |
| VHL | von Hippel-Lindau |
| VPA | valproic acid |
| WNT | wingless |

Introduction

“All in all, a kind of hopeless monster is produced which can not develop beyond the embryonic stage”. This is what Donald Poulsen wrote in 1945 when studying Notch mutant fly embryos, which really pinpoints the importance of Notch signaling during development. Since then we have learnt that Notch signaling is one of the most important developmental regulators also in mammalian development, together with a distinct set of additional pathways, which governs the extremely complex process of embryogenesis, by regulating cellular fate selection, migration, proliferation and apoptosis. It is therefore not surprising that these embryonal pathways have been implicated in cancer, where corrupt versions of their developmental roles are aberrantly recapitulated. Since 1991, when it was shown that a Notch receptor was targeted by a chromosomal translocation in T-cell leukemia, the interest in the role of this pathway in cancer has gradually increased.

In this thesis, I have studied the role of Notch signaling in two very different tumor types; neuroblastoma, a childhood tumor of the sympathetic nervous system and clear cell renal cell carcinoma, an epithelial kidney tumor that primarily occurs late in life. In neuroblastoma, we have studied how valproic acid and mitogenic growth factor stimulation modulate the Notch signaling pathway. In clear cell renal cell carcinoma, we have described a previously unrecognized growth promoting role of Notch signaling in this tumor, an observation that might be of potential future therapeutic importance. Finally, we have identified a cross-talk between the Notch and the TGF- β pathways that might regulate the migratory potential of the kidney cancer cells.

Background

Architecture of the Notch signaling pathway

The pioneering work on the *Notch* gene was done in the beginning of the 20th century using the workhorse of genetics, *Drosophila melanogaster*. Genes involved in embryonic development were identified by generating random mutations in fruit flies and Thomas Hunt Morgan and collaborators identified a strain of *Drosophila* carrying notches at their wing margins (1). It was later shown that this strain was heterozygous for the *Notch* gene (2). The Notch signaling pathway has been a subject of intense investigation since the mid-80s employing *Drosophila* and *Caenorhabditis elegans* as model systems to describe the basic features of this cascade. Subsequent studies in higher ordered species have established that the Notch signaling pathway is a highly conserved pathway for juxtarine signaling across the animal kingdom (3).

Since, this thesis is focused on summarizing the current knowledge of the link between Notch signaling and cancer, I will describe the current knowledge of Notch signaling in mammalian cells. For a detailed description of Notch signaling in *Drosophila* and *C. elegans*, I suggest a number of recent reviews that more thoroughly covers the complexity of this research field (3-5).

The Notch receptors

In mammals, four Notch receptors (Notch-1 to -4) categorized as type I transmembrane proteins have been identified (6-9) (Figure 1). The Notch receptors are initially translated as single precursor proteins. The O-fucosyltransferase-1 (POFUT-1) functions as a chaperone and is believed to facilitate the transport of the receptor from the endoplasmic reticulum to the Golgi apparatus (5). In addition, POFUT-1 adds fucose sugar residues to the receptors, which are further glycosylated within the Golgi apparatus by the Fringe family (Lunatic, Manic, and Radical) of glycosyltransferases, creating larger glycan moieties. All in all, these modifications modulate the capability of spe-

cific ligands to activate the Notch receptors (5). In addition, the level of calcium also appears to be a factor that regulates the affinity between the Notch ligands and receptors (3). In the trans-Golgi network the receptors are cleaved at site 1 (S1) by a furin-convertase in order to generate signaling-competent receptors and thus appear as non-covalently associated heterodimers at the cell surface (3).

The mature heterodimer residing at the cell surface is composed of a large extracellular segment and a smaller part comprising a short ectodomain, a single-pass transmembranal domain and an intracellular domain. The extracellular domain is further subdivided into distinct parts, e.g. tandemly arranged epidermal growth factor (EGF)-like repeats involved in ligand binding, a negative regulatory region (NRR), consisting of three cysteine-rich Lin Notch Region (LNR) repeats and the heterodimerization domain, which prevents ligand-independent activation of the receptor (10, 11). The intracellular domain consists of a RBP-Jκ associated molecule (RAM23) domain, an ankyrin repeat domain (ANK), which are essential for mediating interaction with downstream effector proteins, two nuclear localization sequences, a transactivation domain (TAD) and a C-terminal PEST domain enriched for proline, glutamate, serine and threonine residues that is involved in protein stability. Of the four Notch receptors, Notch-1 (Figure 1) and Notch-2 display the closest similarities with 36 EGF-like repeats in their structure while Notch-3 and Notch-4 are slightly divergent (10, 11). There are a numerous proteins involved in regulating intracellular trafficking of the Notch receptors and thus regulate the sheer number of Notch receptors at the cell membrane available for ligand activation (12, 13). NUMB, assisted by the AP2 clathrin-adaptor complex subunit protein α -Adaptin, may target Notch receptors for endocytosis (12). Furthermore, the ubiquitin ligases Deltex, AIP4 (human homolog of mouse Itch), NEDD4 and Cbl may mediate ubiquitylation, endocytosis, and degradation of the Notch receptors (12). Since dysregulation of any of the proteins regulating Notch trafficking may lead to aberrant Notch signaling activity and hence pathogenesis, this as an

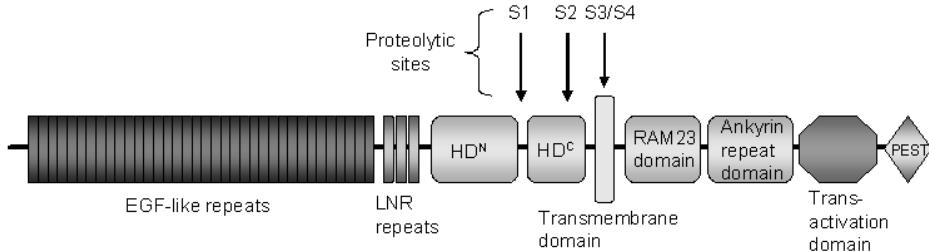


Figure 1. The domains and cleavage sites of the Notch-1 receptor. The tandemly arranged epidermal growth factor (EGF)-like repeats are involved in ligand binding. The three cysteine-rich Lin Notch Region (LNR) repeats and the heterodimerization domain (HD^N and HD^C, N and C-terminal parts of the heterodimerization domain (HD)) prevent ligand-independent activation of the receptor. The intracellular domain consists of a RBP-Jκ associated molecule (RAM23) domain, an ankyrin repeat domain, which are essential for mediating interaction with downstream effector proteins, two nuclear localization sequences, a transactivation domain and a C-terminal PEST domain enriched for proline, glutamate, serine and threonine residues that is involved in protein stability. The receptors are cleaved at site 1 (S1) by a furin-convertase, at S2 by the metalloprotease and disintegrin proteases (ADAM-10 or ADAM-17) and at S3 and S4 by the γ -secretase complex. Adapted from (78).

active area of research which most likely will lead to many important discoveries within the near future (12).

The Notch ligands

Five typical ligands (Delta-like-1 (DLL-1), DLL-3, DLL-4, Jagged-1 and Jagged-2), which in similarity to the Notch receptors are categorized as type I transmembrane proteins, have been identified in mammals (14-18). The ligands possess Delta, Serrate, Lag-2 (DSL) domains and tandem EGF-like repeats in their extracellular domain, which are involved in receptor binding and activation. In addition the ligands harbor a small intracellular domain. Jagged-1 and -2 are slightly larger than the DLL ligands and contain cysteine-rich domains in addition to the aforementioned structural segments (5). Apart from the well-characterized Notch ligands mentioned above, atypical or non-canonical ligands like Delta homolog-like-1 (DLK-1), F3/contactin, NB-3, Delta and Notch-like EGF-related receptor and microfibrillassociated glycoprotein-1 and -2 have recently been described (19-24). The atypical ligands lack the DSL domains but they still retain their capacity to modulate the Notch signaling activity by binding to the Notch receptor. Their functions remain however to a large extent unknown and they will not be covered in further detail in this thesis, except for DLK-1.

Receptor activation

Interaction between receptor and ligand on adjacent cells leads to three consecutive proteolytic cleavages (S2, S3 and S4) of the receptors (Figure 1) (12). Following ligand binding, E3 ubiquitin ligases (mind bomb-1 and -2 and neurlized-1 and -2) ubiquitinate the ligand, that subsequently is subjected to epsin-mediated endocytosis (13). Very recent data clearly shows that mind bomb-1 is the E3 ubiquitin ligase responsible for ligand endocytosis during mammalian development (25). The ligand internalization is thought to promote a physical dissociation of the receptor and transendocytosis of the extracellular part of the receptor heterodimer into the sending cell. This causes a conformational change in the NRR of the Notch receptor leading to Notch cleavage at S2 on the extracellular side by the metalloprotease and disintegrin proteases (ADAM-10 or ADAM-17) (12, 26). The signal-producing cleavages at S3 and S4 within the transmembrane domain, resulting in the liberation of the intracellular domain of the Notch receptor (ICN) from the cell membrane, are executed by the γ -secretase complex. The S4 cleavage also generates a small hydrophobic peptide ($N\beta$), whose function remains elusive (12). Interestingly, since Gupta-Rossi et al revealed that monoubiquiti-

nation and endocytosis of Notch are a prerequisite for its activation (27), the cellular compartment where the activating cleavage of the Notch receptor takes place is still open for discussion. Recent studies also suggest that Notch ligands might be susceptible for ADAM- and γ -secretase-mediated cleavages, in analogy with the Notch receptors (28, 29). The functional role, at least in mammalian cells, of the intracellular ligand fragment generated upon these cleavages remains unclear.

The enzymatic γ -secretase complex is composed of four membrane proteins, presenilin (PS) which is the catalytic component of γ -secretase complex, Pen-2, nicastrin, and Aph-1 (30). The processing of the amyloid precursor protein, which results in the generation of β -amyloid peptides that are implicated in the onset and progression of Alzheimer's disease, is also a γ -secretase dependent process. This has led to the development of several small molecule γ -secretase inhibitors (GSIs) as potential therapeutic agents for treatment of this

neurodegenerative disorder (30). Since GSIs also inhibit the Notch signaling cascade, these inhibitors have been extremely useful for our understanding of the processes regulated by the Notch signaling pathway. The potential role of GSIs in treating cancers where the Notch pathway has been implicated, as well as the downsides with this therapeutic approach, will be discussed further ahead.

The transcriptional switch

Once released from the cell membrane upon the γ -secretase mediated cleavage imposed by ligand binding (31, 32), ICN undergoes translocation to the nucleus where it forms a transcriptional activating complex on promoters of a selected set of target genes (Figure 2) (33). This transcriptional activating complex includes a DNA-binding transcription factor CBF-1/Suppressor of Hairless/Lag-1 (CSL) and a coactivator of the Mastermind-like (MAML) family (34, 35). In the absence of ICN, CSL is bound to the DNA at the core sequence GTGGGAA and represses tran-

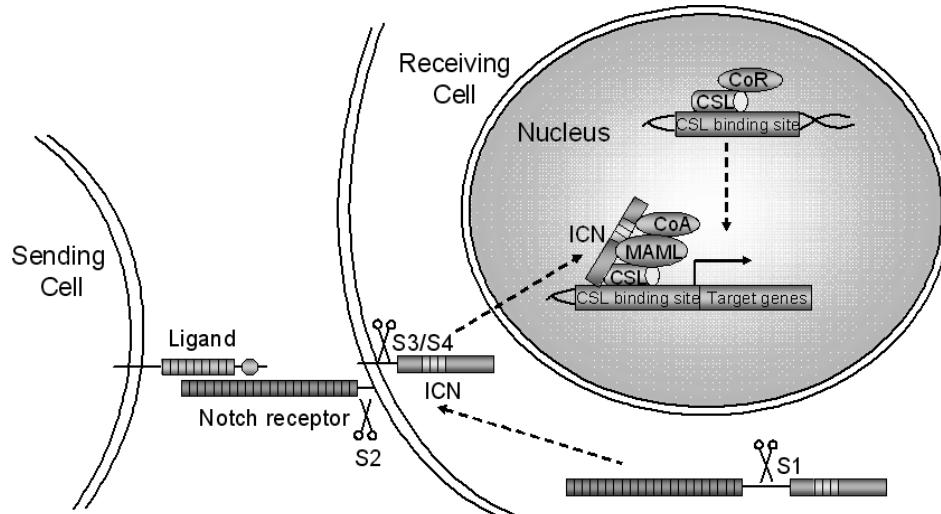


Figure 2. The core axis of Notch signaling. In the trans-Golgi network, receptors are cleaved at site 1 (S1) by a furin-convertase in order to generate signaling-competent receptors and thus appear as non-covalently associated heterodimers at the cell surface. Notch signaling is initiated by ligand-receptor interaction between adjacent cells. The interaction results in consecutive cleavages (S2 and S3/S4) of the receptor, which are executed by metalloproteinase and disintegrin proteases (S2) and a γ -secretase complex (S3/S4), subsequently leading to the release of the intracellular domain Notch (ICN). ICN translocates into the nucleus and binds the CSL transcription factor, thereby displacing corepressors (CoR) and recruiting coactivators (CoA), such as MAML proteins, where after transcription is initiated.

scription of Notch target genes by association with corepressors such as SPEN (other aliases SHARP, MINT), KyoT2, nuclear receptor co-repressor 2 (NCOR-2), CBF1 interacting corepressor (CIR), SIN3A and Hairless (36-38). By binding of histone deacetylases (HDACs) to CSL and corepressor complexes, the chromatin of the target promoters are held in a transcriptionally inert form. The transcriptional switch is initiated with the docking of ICN on CSL through a binding site in the RAM23 domain, which in turn assists the binding of the ANK domain to CSL (39). The allosteric changes within CSL imposed by ICN binding is suggested to aid corepressor displacement from CSL and assist recruitment of MAML (38). Recent structural studies have revealed that the ANK domain of ICN and CSL creates a composite binding interface which forms a groove that allow binding of MAML (40, 41). With the formation of a ternary complex composed of ICN, CSL, and MAML, transcription is induced by the recruitment of histone acetyl transferases (HATs) and other proteins necessary for initiation of transcription, such as CREB-binding protein (CREBBP), CBP/p300 and RNA polymerase II (38). The role of the bifunctional cofactor Ski-interacting protein (SKIP), which was reported to interact either with CSL and corepressors or ICN and CSL (42), has recently been questioned since it only appears to be recruited to target genes promoters simultaneously with ICN (43). Thus, a more detailed understanding of the role of SKIP in the transcription-activating complex awaits further studies. Besides the obligatory role as a coactivator in ICN mediated transcription, MAML also appears to be one of the factors limiting the intensity and duration of ICN-driven transcription. The transcription complex is rapidly turned over due to MAML-mediated recruitment of a cyclin C and cyclin-dependent kinase 8 (CyC:CDK8) complex (43), which promotes hyperphosphorylation of ICN. The hyperphosphorylated ICN is then ubiquitinated by the F-box and WD40 domain protein-7 (FBW7) E3 ubiquitin ligase and targeted for proteasomal degradation (43). Following ICN degradation, CSL associates with corepressors (38). ICN stability might

also be regulated by other proteins. For example, phosphorylation of ICN in the nucleus by glycogen synthase kinase-3 β (GSK-3 β) has been linked to ICN degradation (44), however this modification appears to take place off DNA.

On the complexity of the signaling output
We know that Notch signaling is a focal point during development and maturation of almost all tissues. In addition, Notch also appears to control cell behavior in self-renewing systems throughout adult life (45). As previously mentioned, the prototypical view of Notch signaling is as an inhibitor of differentiation, keeping cells in an immature state, awaiting inductive signals at later stages thus allowing for cells to adopt secondary fates. Classical examples of this inhibiting mode of action include Notch's role in early neurogenesis and myogenesis (45). However, recently Notch signaling has also been shown to have a direct instructive part in directing differentiation towards specific fates. For example, in gliogenesis Notch signaling directly promotes the differentiation of many glial subtypes (46, 47). In addition, there are now several observations indicating opposing roles for Notch signaling not only on differentiation, but also on a variety of other cellular processes such as proliferation, apoptosis and cell migration (48). Furthermore, the role of the Notch cascade does not only vary between completely different cell types, but also differ in the same cell type in a context dependent manner (49). Ultimately the question arises; how is this mechanistically relatively unsophisticated pathway able to deliver such widely divergent signals? The picture that emerges from the literature during recent years, and probably the most obvious answer to this question, is that Notch signaling and regulation is far more complex than originally assumed (3). For example, the two receptors with very similar biochemical features, Notch-1 and Notch-2, are functionally interchangeable in some instances, while in others they appear to elicit very different responses (49-52). This regulation of Notch activity in a context-specific manner indicates (intrinsic) cellular differences in processes or factors which are poorly understood. Based

on the current understanding of the pathway, it is plausible that factors such as cellular concentrations of the various ligands and receptors, cross-talk with other signaling pathways, trafficking (compartmentalization and endocytosis), post-transcriptional modification, accessory transcription factors, ICN concentration, and epigenetic status of the promoters of target genes are important (3, 48, 53).

There are also some evidences pointing towards the existence of a noncanonical pathway, where Notch signaling may occur in a CSL-independent fashion and where CSL-signaling occurs in a Notch-independent manner, which certainly adds an extra layer of complexity. Notch-1 has been shown to activate the GTPase R-RAS independent of CSL transcription (54). Furthermore, induction of phosphatidylinositol-3-kinase-Akt (PI3K-Akt) signaling by Notch signaling has been linked to the function of Deltex-1 independently of CSL (55). The Epstein-Barr virus (EBV) protein, Epstein-Barr virus nuclear antigen 2, binds CSL and displaces the corepressor complex in a manner similar to ICN (56, 57). The Kaposi's sarcoma-associated herpesvirus (KSHV) lytic regulatory protein RTA also binds CSL and recently it has been suggested that RTA promotes DNA binding by CSL (58, 59). The mechanisms whereby the EBV and KSHV proteins modulate CSL-dependent transcription are multi-faceted and it seems as the transcriptional responses to EBNA2 and RTA are both distinct and overlaps with those of ICN (56). The ability of these viral proteins to regulate CSL transactivation has been suggested to play an important role during EBV-associated B-cell transformation and in KSHV-associated primary effusion lymphoma and Kaposi's sarcoma (56). The MAML proteins may also have roles that are independent of ICN/CSL binding activities. Although MAML proteins have been considered Notch-specific coactivators, there are several reports showing that MAML proteins may function as a coactivator for human papilloma virus (HPV) E6 oncoproteins, β -catenin, p53 and the muscle differentiation-related transcription factor MEF2 (60-63). Furthermore, muco-epidermoid carcinomas (MEC), that arises in salivary and bronchial glands, are associated

with a specific recurring chromosomal translocation t(11;19)(q14–21;p12–13) (64). This translocation results in the expression of a fusion protein that consists of the cAMP response element binding protein (CREB)-binding domain of the CREB-regulated transcriptional coactivator MEC translocated 1 (MECT1, also called CRTC1, WAMTP1, or TORC1) and the TAD of MAML2 (64-66). The fusion protein appears to regulate Notch targets (*HES-1* and *HES-5*) through a CSL independent mechanism (65, 66). It is possible that transcriptional regulation of *HES-1* occurs in part through the capacity of the MECT1-MAML2 fusion protein to constitutively activate CREB, but this has not been formally proven and remains debated (67-69). In addition, recent data indicate that the *MECT1-MAML2* fusion oncogene is not specific to MEC carcinomas and that there are novel fusions partners for the *MAML2* gene (70-75).

The Notch target genes

Given the plethora of processes affected by the Notch pathway there are surprisingly few well characterized Notch target genes (76, 77). Surely, the definition of target genes in an unambiguous manner has been complicated by the highly tissue and context dependent nature of the Notch signaling pathway. By employing novel genomic approaches many more tissue-specific target genes will without any doubts be defined, regulated by combinatorial cues from several signaling pathways.

Of the direct targets that appear to be regulated by the Notch cascade in several tissues are the basic helix-loop-helix (bHLH) transcriptional repressors of the *Hairy/Enhancer of Split (HES)* and *Hairy-related (HEY)* families the best characterized (76, 77). It is however clear that these more universal target genes are not the sole mediators of Notch signaling in any tissue. Examples of tissue-specific targets include *NFKB*, *p21^{Cip1}*, *pre-T α* , *Cyclin D1*, *SKP-2*, *c-Myc*, *GATA-2* and -3 (78-80). After a brief description of the primary targets *HES* and *HEY*, the role of some of the tissue specific target genes, will be discussed in the section covering the tumor biological aspects of Notch signaling.

The HES and HEY families of transcription factors

Seven *HES* (*HES-1* to *HES-7*) and three *HEY* (*HEY-1*, -2, -L) genes have been identified in mammals (76, 77). Notch activation seems to induce the expression of all three *HEY* proteins, but only three of the seven *HES* proteins (*HES-1*, *HES-5* and *HES-7*) (76). Like other bHLH transcription factors, the *HES* and *HEY* proteins harbors a DNA-binding basic region and a helix-loop-helix dimerization domain (76). However, the *HES* and *HEY* proteins also contains some unique residues and domains which separate them from other bHLH factors. They share a domain denoted Orange, which consists of two conserved α -helices (76). The Orange domain is believed to confer some extra protein interaction abilities and has also been shown to repress transcription (76). The *HES* proteins contain an invariant proline residue in their basic region which allows for binding to both E- and N-boxes at promoters of their target genes (77). This proline residue is changed to a glycine in the *HEY* proteins, which is thought to mediate a preferential E-box binding capacity (76). A WRPW domain at the C-terminal end of the *HES* proteins is associated with two functional properties; mediating part of the target gene repression recruitment of Transducin-like Enhancer of Split (TLE) corepressors as well as degradation of these proteins (77). In the *HEY* proteins the WRPW tetrapeptide is replaced with a YRPW tetrapeptide (*HEY-1* and *HEY-2*) or a YXXW tetrapeptide (*HEY-L*), which are unable to recruit TLE corepressors (76). The proposed modes of transcriptional repression executed by the *HES* and *HEY* proteins can broadly be divided into active or passive repression. Active repression involves homo- or heterodimer formation and transcriptional repression by DNA binding (76). One of the classical examples of this mode of action is the binding of *HES-1* to the promoter of the proneural gene *human aschaete scute homolog 1* (*HASH-1*, *MASH-1* in mouse) which leads to repression of its transcription (see also the neuroblastoma section) (81). As indicated above, the *HES* and *HEY* proteins use different factors to actively repress their target

genes. TLE proteins associated to the *HES* proteins are thought to attract HDACs and inactivate the chromatin (77). The *HEY-1* and *HEY-2* proteins, on the other hand, appear to recruit N-CoR and mSIN3A corepressor complexes (containing HDAC-1) by their bHLH domains (76). The bHLH domains of *HES-1* and *HEY-2* have also been shown to interact with an additional HDAC called SIRT (82). Furthermore, *HES* and *HEY* proteins can form heterodimers which have markedly elevated DNA binding affinities and have greater repression activities compared to the respective homodimers (76). Passive repression is transcriptional regulation without DNA-binding. This can be achieved by formation of dominant-negative heterodimers with E-proteins, ubiquitous partners of many tissue specific bHLH proteins (76). For example, *HES-1* might form non-DNA-binding heterodimers with E47, thereby disrupting the formation of *MASH-1/E47* heterodimers, which normally are able to activate the transcription of proneural genes (77, 83).

To further complicate the function of *HES* proteins, it has been shown that *HES-1* can be converted to a transcriptional activator through induction of Ca/calmodulin-dependent protein kinase II δ mediated phosphorylation switching *HES-1* mediated repression of the *MASH-1* gene to activation (84). There are also data showing that *HES* and *HEY* proteins have opposite effects on the activity of RUNX-2, which is a central regulator of bone development (76). Thus one can imagine that although these factors appear to be regulated by Notch in several tissues, the functional outcome of these bHLH factors are also highly context-dependent.

As being primary Notch targets, the *HES* and *HEY* proteins have been extensively used as surrogate markers of Notch signaling activity. It should however be pointed out that *HES-1* seems to be regulated in a Notch-independent manner under some circumstances (see below). To complicate things more, other means of detecting Notch activation may also hold some flaws. For example, the antibodies that detect the cleaved form of the Notch receptors might also be imperfect tools, since high levels of Notch-1 activation

do not always imply a functional role as shown in studies of intestinal stem cells and in proximal progenitors during nephron segmentation (85, 86). With these caveats in mind it seems likely that a combinatorial experimental approach, in which several different aspects of Notch signaling is analyzed, is required for a correct functional assessment of Notch signaling activity.

Cross-talk between Notch and EGFR/RAS/MAPK or TGF- β signaling pathways

There is a bevy of papers providing evidence for the existence of both antagonistic and agonistic relationship between Notch signaling and other pivotal cellular signaling pathways, such as TGF- β , WNT, NF κ B, JAK/STAT, PI3K-Akt, VHL-HIF, Hedgehog and EGFR/RAS/MAPK signaling pathways (87-89). It is likely that these pathways form a complex network that broadens or limits the response normally provided if each cascade would be considered linear. I will specifically focus on two pathways, the transforming growth factor β (TGF- β) and the EGFR/RAS/MAPK pathways since they are relevant for Papers II and IV. It should be stated that the molecular mechanisms behind these cross-talks remains poorly described. As pointed out elsewhere, it remains however likely that many of the so called context-dependent effects of the Notch signaling pathway can be explained by such cross-talks, making better insights into these interactions an urging issue.

EGFR/RAS/MAPK signaling pathway

The EGF receptor (EGFR, also called HER-1, erbB1) belongs to the human epidermal receptor family (HER) of receptor tyrosine kinases, which in addition includes HER-2 (neu, erbB2), HER3 (erbB3), and HER-4 (erbB4) (90). The two best characterized EGFR-ligands are EGF and transforming growth factor α (TGF- α). Ligand binding causes EGFR to dimerize or to heterodimerize with another receptor of the HER-family, resulting in activation of downstream path-

ways instrumental in proliferation and survival, such as mitogen-activated protein kinase (MAPK) and PI3K-Akt (90). There are multiple MAPK pathways in mammals including extra cellular signal regulated kinases 1 and 2 (ERK-1 and -2), c-Jun amino-terminal kinases 1 to 3 (JNK-1 to -3), p38- α , - β , - γ and - δ and ERK-5 (91). ERK-1 and -2 are primarily activated in response to mitogens, whereas JNKs, p38s and ERK-5 are primarily activated by cytokines and growth factors (91). The MAPKs are generally activated sequentially acting protein phosphorylation by kinases. MAPKKK (MAPK kinase kinase) activation leads to phosphorylation and activation of a MAKK (MAPK kinase), which then activates the MAPK by phosphorylation at threonine and tyrosine residues (91).

From a cancer perspective the ERK-1 and -2 MAPK cascade seems most relevant (92). Ligand binding to the HER receptors leads to activation of RAS-proteins (H-, K- and N-RAS), through the recruitment of son of sevenless (SOS), a RAS-activating guanidine nucleotide exchange factor, to the plasma membrane. SOS converts RAS from an inactive GDP-bound state to an active GTP-bound state. RAS in turn activates MAPKK component of the ERK-1 and -2 cascade which comprise RAF-kinases (c-RAF-1, A-RAF and B-RAF). They subsequently activate the MEK-1 and -2 MAPKKs, which in turn phosphorylate and activate ERK-1 and -2. These kinases in turn phosphorylate and regulate various transcription factors, such as the Ets family of transcription factors (93). The EGFR/RAS/ERK cascade promotes a number of processes that in its oncogenic form promote different aspects of tumorigenesis, such as cell proliferation, survival, differentiation and metastasis (94). Accordingly, this pathway constitutes one of the most frequently mutated cascades in cancer. These alterations include ligand and EGFR overexpression, EGFR mutations, RAS mutations, and B-Raf mutations in a variety of human cancer (94). A number of therapies based on inhibition of this pathway are currently in clinical trials or have been approved for treatment of cancer. The approaches include monoclonal antibodies, small molecule tyro-

sine kinase inhibitors of EGFR and small molecule MAPK inhibitors (94).

Cross-talk between the Notch signaling and EGFR/RAS/MAPK signaling pathways

The cross-talk between Notch and EGFR/RAS is complex and below follows some examples of the often contradictory nature of this cross-talk. Ectopic expression of ICN-1 in melanoma cells, small cell lung cancer cells leads to enhanced activation of ERK-1 and -2, whereas Notch-1 activation in endothelial cells leads to suppressed ERK-1 and -2 phosphorylation (95-97). There are also reports suggesting that Notch signaling might mediate certain aspects of RAS induced transformation of fibroblasts and kidney epithelial cells, as well as in mouse mammary epithelium and primary breast tumors (98) (the latter will be discussed in further details below). Moreover, TGF- α induces the expression of the Notch primary target HES-1 by γ -secretase-dependent Notch activation in mouse pancreatic explants (99). Studies have also shown that IL-6 in breast cancer cells and TGF- α /EGFR in head and neck squamous cell carcinoma (HNSCC) cells, induces the expression of Jagged-1 in a MEK/ERK-dependent manner (100, 101). There is also a report suggesting that Notch activation can induce HER2 expression in 293 cells (102). Finally, one of several proteins targeted by the proteolytic activity of the γ -secretase complex is the HER4 (ErbB4) receptor (103). Two recent reports also showed that reduction in γ -secretase activity resulted in increased EGFR expression (104, 105).

The TGF- β signaling pathway

The TGF- β super family of growth factor includes TGF- β isoforms, activins, bone morphogenic proteins (BMPs), nodal and growth and differentiating factors (GDFs) (106). These factors regulate a multitude of cellular processes including cell growth, adhesion, migration, cell-fate determination, differentiation and apoptosis. Dysregulation of TGF- β signaling has been associated with a wide variety of pathological conditions, such

as cancer, fibrosis and wound-healing disorders (106). Below follows a simplistic view of the TGF- β /small mothers against decapentaplegic (SMAD) signaling pathway (Figure 3). Binding of biologically active TGF- β to type II dimers of the TGF- β serine/threonine kinase receptor (TGF β RII), results in recruitment of two type I TGF- β receptor (TGF β RI) serine/threonine kinases (107, 108). With the formation of a heterotetrameric receptor complex, the TGF β RII is able to phosphorylate a juxtamembrane domain of the TGF β RI, which enables the recruitment of receptor regulated SMADs (SMAD-2 and -3). Subsequently, the TGF β RI receptor phosphorylates SMAD-2 and -3, which allow them to bind

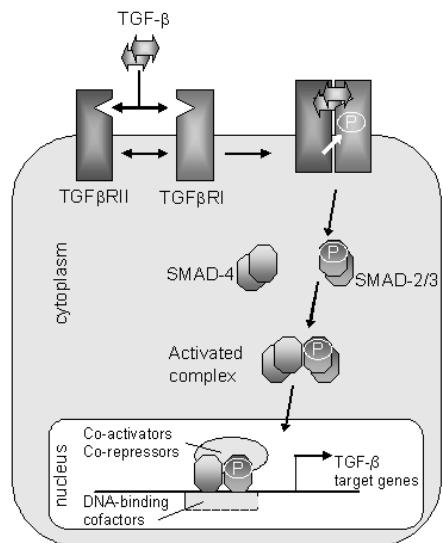


Figure 3. Binding of biologically active TGF- β to type II dimers of the TGF- β serine/threonine kinase receptor (TGF β RII), results in recruitment of two type I TGF- β receptor (TGF β RI) serine/threonine kinases. With the formation of a heterotetrameric receptor complex, the TGF β RII is able to phosphorylate a juxtamembrane do-main of the TGF β RI, which enables the recruitment of receptor regulated SMADs (SMAD-2 and -3). Sub-sequently, the TGF β RI receptor phosphorylates SMAD-2 and -3, which allow them to bind SMAD-4. This complex thereafter translocates to the nucleus to act as transcriptional regulators. The SMAD complex interacts with DNA and transcription factors, coactivators or core-pressors in a target-gene dependent manner. Adapted from (108).

SMAD-4. This complex thereafter translocates to the nucleus to act as transcriptional regulators. The SMAD complex interacts with DNA and transcription factors, coactivators or corepressors in a target-gene dependent manner (107). Negative feedback loops, involving inhibitor SMADs (SMAD-6 and SMAD-7), tightly control the TGF- β signaling pathway. In addition, TGF- β is also able to signal through the ERK, JNK, p38 and PI3K-Akt signaling pathways (106). The TGF- β signaling pathway is thought to play a complex dual role in carcinogenesis of many tumors (108). Thus, TGF- β inhibits the growth of normal epithelium, and also inhibits the early stages of epithelial neoplasia through induction of the CDK4/6 inhibitor p15^{ink4b} and CDK2 inhibitor p21^{cip1} and by repression of α -Myc (108). Mutations or loss of TGF- β receptors and SMADs can render cells insensitive to TGF- β mediated cytostatic growth arrest while enabling other responses that support tumor progression (108). Moreover, breast cancer and glioblastoma cells often retain a functional TGF- β signaling pathway and instead lose the ability to respond TGF- β cytostatic effects through other means. At this stage increased TGF- β levels may instead activate gene responses that promote tumor growth, epithelial-to-mesenchymal transformation (EMT), invasion, angiogenesis and metastasis. TGF- β in the tumor microenvironment may also be an advantage for the tumor cells through its powerful immunosuppressive effects (108).

Cross-talk between the Notch and and the TGF- β signaling pathways

Here I will mostly address TGF- β and Notch signaling interactions, but a cross-talk between BMP and Notch signaling has also been reported. A number of papers have shown that TGF- β and BMP induced the expression of Notch target genes including HES-1, HEY-1, and HES-5 (109-112). One mechanism behind this induction seems to depend on interaction between ICN-1 and SMAD-3 upon TGF- β stimulation (109). However, the immediate-early upregulation of Notch target genes

through direct SMAD-dependent activation is in some cell types followed by a second, Notch receptor dependent wave of induction (112). Of particular importance for this thesis is the observation that a TGF- β -dependent upregulation of Jagged-1 have been observed in renal cells both *in vitro* and *in vivo* (112, 113). On the other hand, TGF- β has also been shown to negatively regulate the expression of the Notch-3 receptor in fibroblasts (114).

There are also some reports addressing Notch signaling effects on the TGF- β signaling cascade. During mouse embryonic heart development, Notch signaling is required for the expression of TGF- β 2 and TGF- β receptors (115). Moreover, recent reports show that TGF- β and Notch signaling share a large number of positively and negatively regulated target genes (116).

With regard to the functional outcome of the Notch and TGF- β cross-talk, accumulating evidences show that the two pathways jointly regulate two events at the heart of the tumorigenic process, EMT and proliferation. EMT is a phenotypical change whereby epithelial cells disassemble their junctional structures, start acquiring mesenchymal characteristics, remodel their extracellular matrix and become migratory (117) (more on EMT see Notch and breast cancer). TGF- β and Notch seems to cooperate in inducing EMT both *in vitro* and *in vivo* (112, 118).

In cervical carcinoma cells, ICN-1 suppresses the growth inhibitory TGF- β signal through sequestration of CBP/p300 from SMAD-3 (119). Likewise, in breast cancer cells Notch-4 signaling was reported to insensitize these cells to the growth inhibitory actions of TGF- β (120). On the other hand, Niimi et al showed that Notch signaling was required for TGF- β -induced growth arrest of epithelial cells of different origins (116).

Altogether, the communication between two such context-dependent signaling pathways seems to appear at a variety of levels resulting in seemingly conflicting outcomes.

The Notch pathway in cancer

Dysregulation of Notch signaling has been implicated in the tumorigenic process of a large number of tumors, but in many instances this is based solely on expression analyses, and further experimental evidences are required in order to fully elucidate the role of the pathway (121, 122). For the sake of clarity, I have focused on four tumor types in this thesis: T-cell acute lymphoblastic leukemia (T-ALL), breast cancer, skin cancer and brain tumors. They also serve to exemplify different aspects of dysregulated Notch signaling, which ranges from oncogenic block of differentiation and augmented growth capacity to a growth inhibiting tumor suppressor function.

Notch and T-cell acute lymphoblastic leukemia

Interest of a potential role of the Notch cascade in cancer began with the discovery of *Notch-1* as a target for a chromosomal translocation in T-ALL, an aggressive tumor form derived from T-cell progenitors (123). The (7;9)(q34;q34.3) translocation juxtaposes a truncated form of the *Notch-1* gene to the TCR- β enhancer. This results in enhanced expression of the constitutively active ICN-1 protein. However, this translocation only occurs in less than 1% of all T-ALL cases (78). Subsequent studies in mice, transplanted with cells expressing ICN-1 revealed that this truncated form of the Notch receptor is a powerful inducer of murine T-ALL (124). Definitive proof for a much more profound role for Notch-1 in human T-ALL than was originally believed, came in 2004 (125). Weng et al undertook a mutational screen of human T-ALL samples, since previous findings showed that T-ALL cell lines without any known abnormality in Notch (i.e. translocation) were growth attenuated upon treatment with GSIs (125). The mutational screen revealed two types of oncogenic somatic mutations of *Notch-1* in approximately 55% of primary T-ALLs. The first type appear in part of the Notch-1 gene encoding the N- and C-terminal halves of the heterodimerization domains (HDs) and occur in 40-45% of the T-ALL

tumors (125). This type of mutations can be further subdivided into two classes (I and II), that both causes ligand-independent cleavage at site S2, which is a prerequisite for the final γ -secretase mediated cleavage (126). The most common class I mutations, which is located in the part of the gene that encodes the hydrophobic core of the HD probably result in subunit dissociation or alternatively, a conformational change that exposes the S2 site (78, 126). The rare class II mutations lead to the introduction of a second unprotected S2 site outside the NRR (126). The second type of mutations are present in approximately 20-30% of the primary T-ALLs and cause a shift in the reading frames which results in a deletion of the C-terminal PEST domain and hence increases ICN-1 stability (125). It has been postulated that these mutations also causes the removal of several phosphorylation sites that normally are targeted by proteins that regulate degradation, e.g. FBW7 (78). Thus, a recent study showed that one of the sequences (WSSSP), which is lost by the second type of *Notch-1* mutations, is associated with normal regulation of ICN-1 stability (127). In the seminal work by Weng et al, it was also reported that 10-20% of the T-ALL samples have mutations in both the HD and PEST domains (125). These simultaneous mutations were always located on the same *Notch-1* allele, suggesting a synergistic enhancement of Notch-1 signaling by the two mutations (125). More recently, several groups reported that *FBW7* is mutated in human T-ALL cell lines and patient samples, lacking mutations involving the PEST domain (128-130). These mutant forms of FBW7 are unable to regulate ICN-1 stability and thus results in increased Notch-1 activity.

The gain-of-function mutations of Notch-1 have been confirmed in several different tumor materials and they are present in combination with all the various genetic aberrations associated with T-ALL pathogenesis (78). In murine models, similar gain-of-function mutations of *Notch-1* have been reported in most genetic backgrounds prone to development of T-ALL (such as Tal-1 or Hox-11 overexpressing mice) (78). Although enforced expression of ICN-2 and ICN-3 lead to T-ALL in animal

model systems, neither the *Notch-2* gene nor the *Notch-3* gene harbor sporadic mutations in human or murine T-ALL (78). It should be noted that the *Notch-3* gene is a direct target of Notch-1 signaling in T-ALL cells (131, 132), but the exact role of Notch-3 in T-ALL development remains debated (78). Moreover, *Notch-1* mutations have not been found in B-cell ALL (B-ALL) and are exceedingly rare in acute myeloid leukemia (AML) (78).

Together, all these studies suggest that Notch-1 mutations can either be the primary initiating event or a secondary collaborating event in T-ALL and a strong selection pressure to activate the Notch-1 signaling cascade seems to be at hand during T-ALL induction and progression (78).

The specific transforming capacity of Notch-1 mutations in T-ALLs, which resembles various stages of pre-T-cell development, is most likely a reflection of non-redundant Notch-1 functions during normal pre-T-cell development (78). Notch-1 signals are essential for T-cell fate specification from a multipotent precursor and further on during pre-T-cell development up to and including a critical checkpoint (called DN3 β -selection) in the thymus (78). During this checkpoint, pre- β -selection cells (DN3a) undergo a proliferative burst that requires at least two signals, one that is generated by Notch-1 activation and the other by expression of the pre-T-cell receptor (78). Once the thymocytes have reached the post- β -selection stage, the Notch-1 signals are rapidly downregulated in these more mature cells (called DN3b), which then continue to differentiate to the double-positive stage (CD4+ and CD8+) in a Notch-independent manner (78). However, in the murine T-ALL models (and in many human T-ALLs), Notch signaling is kept at high levels beyond the β -selection checkpoint and thus the T-ALL cells appear to be arrested at the DN3b and/or the double-positive stage of development (78, 125, 133). Given the Notch-1 functions during normal pre-T-cell development, where Notch-1 signaling has to be silenced for further maturation of the pre-T-cells, it was therefore quite surprising that GSI-treatment of T-ALL cells primarily re-

sulted in growth arrest, without effects on differentiation (78).

Recently, several studies further explained important aspects of Notch-1 signaling in T-ALL by showing that *c-Myc* is a direct downstream target gene in these cells (131, 132, 134). Reintroduction of *c-Myc*, a key regulator of cell growth, into T-ALL cells that have been growth restrained upon GSI treatment, completely rescues their growth. By using gene expression and chip-on-chip data, Ferrando and colleagues also showed that Notch-1 and c-Myc share many targets that directly are involved in regulating cell growth and anabolic metabolism (131). It was also postulated that the Notch/c-Myc signaling axis might reflect an aberrant recapitulation of a normal developmental relationship. In normal T-cell progenitors, Notch signaling upregulated *c-Myc* in DN3a cells, and with the drop of Notch-1 signaling in DN3b cells *c-Myc* levels also became substantially lower (132, 135). This suggests a model where Notch signaling in the normal situation upregulates *c-Myc* to allow expansion of DN3a cells for a limited period, whereas in T-ALL cells constitutive Notch-1 activation and hence *c-Myc* expression results in indefinite proliferation (78, 132).

An additional pro-growth pathway targeted by Notch in T-ALL cells appears to be the PTEN/PI3K-Akt/mTOR pathway (136, 137). It was shown that the Notch target gene HES-1 repressed the expression of *PTEN* in T-ALL cells (137). *PTEN* downregulation resulted in increased PI3K-Akt signaling activity and probably subsequent mammalian target of rapamycin (mTOR) activation, which in analogy with the c-Myc effects promoted cell metabolism and growth. The authors also showed that the growth arrest upon Notch inhibition could be rescued by enforced expression of a constitutive active form of AKT (137). In another study, it was reported that Notch signaling positively regulated the mTOR pathway, albeit through a pathway independent of the PI3-Akt cascade (136). The discrepancies between these two studies warrant further studies. Nonetheless, it is also noteworthy that *Notch-1* deficient normal T-cell progenitors that are arrested at the DN3a stage can be rescued by overexpression of

constitutively active AKT, which in turn promotes cell growth probably via mTOR activation (138), once again suggesting that Notch-1 functions in T-ALL cells is a reflection of a subverted normal functional relationship (78).

Additional complex interactions between Notch signaling and p53, NF- κ B, IKAROS and E2A have all been found in a variety of T-ALL models, which might turn out to be important determinants of oncogenic Notch functions in this tumor form (78).

T-cell acute lymphoblastic leukemia and γ -secretase inhibitors

Despite some uncertainties about the true nature of the molecular network downstream of (or parallel to) Notch signaling in T-ALLs, the use of Notch inhibitor therapy in treatment of T-ALL patients seems very attractive (78). There are however several factors that needs to be taken into account in order for this treatment option to be successful in clinical trials. Systemic use of the GSI leads to massive diarrhea, mainly due to an expansion of mucus-secreting goblet cells (139, 140). This is a consequence of the important role of Notch signaling in normal homeostasis along the crypt-to-villus axis in the small intestine (45). Gain-of-function and loss-of-function of Notch pathway elements within the small intestine of mice have revealed that Notch signaling maintain crypt progenitors in a proliferative undifferentiated state and also regulates a binary cell fate decision of transit amplifying cells, where Notch signaling appears to dictate differentiation towards the absorptive enterocyte cell fate (45, 141, 142). Thus, inhibition of Notch signaling results in loss of proliferating transient amplifying cells and to production of an excess of secretory cells (such as goblet cells) at the expense of enterocytes (141, 142). In addition, GSI effects observed in mice included a modest arrest of T-cell development (140). Considering the plethora of γ -secretase regulated proteins, the side-effects of GSI treatment are fairly limited and importantly, most likely can be attributed to the role of Notch signaling in the respective organ system. However, a very recent publication showed that mice with genetic reduction

of γ -secretase activity below certain threshold levels developed splenomegaly and squamous cell carcinoma (SCC) (143) (related to Notch-1 functions in skin see Notch and skin cancer). The authors of this report also provided data, indicating that γ -secretase inhibitors might have more off-target side effects than previously appreciated (143). The full spectrum of adverse effects upon chronic GSI treatment is not known and the list of side-effects will most likely be extended. Moreover, the different molecular types of GSIs available will probably have some distinct side-effects and these have not been properly evaluated. There might however be several solutions for avoiding these adverse effects, such as direct tumor targeting, adjusting dosing schedules, or by delivering GSIs together with other substances that limits off-target tissue toxicities. The development of more specific inhibitors against each individual Notch receptor might also reduce toxicity in tissues where the targeted receptor may play a redundant role (78). Irrespective of the approach taken to shut down Notch signaling, our current knowledge-base speaks almost invariably in favor for combinatorial drug strategies. In the case of human T-ALL, Notch inhibition primarily results in growth arrest and restrained metabolism, thus implicating that single-agent trials with GSIs may "only" have cytostatic effects (78). Combinational approaches have already been tried on T-ALL cells, where it appears that GSIs and Rapamycin (a mTOR inhibitor) or GSIs and NF- κ B pathway inhibitors act synergistically (136, 144). Obviously, the ultimate goal would be to find a combination of drugs that result in cytotoxic effects.

Finally, about half of the human T-ALL cell lines harboring HD mutations do not respond to GSIs (125). As previously mentioned, it was recently discovered that *FBW7* is mutated in T-ALL cell lines and primary T-ALL samples (128, 130, 145). *FBW7* normally targets ICN for degradation, however other substrates for *FBW7* includes cyclin E, c-Jun and intriguingly c-Myc. Importantly, all T-ALL cell lines with *FBW7* mutations did not respond to GSI treatment and it was postulated that this resistance should be attributed to increased stabilization of the c-Myc protein

(129, 130). Even though ICN stability is increased in FBW7 mutated cells, c-Myc levels are suggested to be held at sufficient levels due to the aborted degradation, rendering Notch-1 transcriptional effects indispensable (129, 130). Intriguingly, *PTEN* mutations were also found in the same cell-lines that harbored FBW7 mutations (137). Upon *PTEN* loss, the PI3K-Akt pathway delivers growth promoting or survival signals independently of Notch-1/HES-1 signaling axis (137). These insights into mechanisms of GSI resistance further underscore the needs for multi-target treatment regimes.

Another unsettled issue that requires further attention is the prognostic value of *Notch-1* mutations in T-ALL. Two studies have reported that Notch-1 mutations predicted favorable long-term survival in children with T-ALL, whereas a third study could not find any significant difference of survival according to *Notch-1* mutations in pediatric T-ALL patients (146-148).

The role of the Notch cascade in AML and a variety of B-cell malignancies is controversial. There are several studies indicating that Notch signaling promotes proliferation of malignant B-cells, but there are also studies implying that Notch signaling induces growth inhibition and apoptosis in the same cells (149). In AML, there are also conflicting reports, in this case regarding whether the Notch cascade promote or block AML cell differentiation (78). Obviously, only additional studies by the persistent will provide insights into these matters.

Notch and breast cancer

The earliest findings suggesting a role of Notch signaling in breast oncogenesis were reports showing that the genes coding for the *Notch-1* and *Notch-4* receptors were located at Mouse mammary tumor virus (MMTV) insertion sites (150, 151). The integration of the MMTV provirus leads to expression of truncated constitutively active forms of the Notch-1 and Notch-4 receptors in mouse mammary gland tumors (150, 151). Further studies revealed that expression of ICN-1, ICN-3 or ICN4 in mouse mammary epithelial cells in transgenic mice blocks ductal and

lobulo-alveolar mammary gland development and eventually leads to the induction of mammary tumors (48). Although these mouse models would suggest that dysregulated Notch might play a role in human breast cancer, the clinical significance of this pathway in human tumors remained obscure. This at least in part explained by limited knowledge of the various Notch components role in normal mammary development (152). Nevertheless, the studies in mice have surely founded the increased attention given to the role of Notch signaling in human breast cancer during recent years.

In the first study of human breast cancer tissues, Weijzen et al reported that seven primary ductal carcinomas showed positive Notch-1 staining whereas adjacent normal tissue exhibited little or no Notch-1 staining (153). In a larger cohort of patients, it was reported that that elevated levels of Notch-1 may be associated with poorly differentiated tumors and hence poor prognosis of the patients, while increased Notch-2 levels were associated with well-differentiated tumors and thus better prognosis (52). A third study used *in situ* hybridization to analyze gene expression in a cohort from 184 patients with invasive primary ductal breast cancer and showed that high levels of *Notch-1* and *Jagged-1* expression significantly correlated with poor survival for patients with advanced breast cancer (154). Furthermore, coexpression of *Jagged-1* and *Notch-1* defined a subclass of breast cancer with very poor outcome (154). In a follow-up study, it was shown that prognostication in the very same cohort could be enhanced by assessing both mRNA and protein expression levels for Jagged-1 (155). Interestingly, in a very recent study high *Jagged-1* mRNA expression was also associated with reduced disease free survival in patients with lymph node negative breast cancer (156). The Notch ligands DLL-3 and DLL-4 also appeared to be overexpressed in medullary breast carcinoma samples (157). This breast cancer type is however associated with a relatively good prognosis. Importantly, firm evidences for active Notch signaling in human breast cancer have been shown in two studies, where accumulation of ICN-1 and expression of Notch down-

stream targets in a wide range of breast carcinomas and cell lines were detected (158, 159). In a very recent study, increased ICN-1 staining in ductal carcinoma in situ (DCIS) samples was significantly associated with recurrence at 5 years (160). Since DCIS is a pre-invasive lesion this result may suggest that enhanced Notch signaling is a relatively early event in the pathogenesis of human breast cancer.

Since gain-of-functions mutations in Notch receptors so far seems to be unique to T-ALL, what then is the mechanism by which Notch signaling is maintained in breast cancer? A number of reports have addressed this question. Weijzen et al reported a correlation between elevated RAS staining and strong Notch-1 staining in four of seven investigated breast tumors and further suggested that ICN-1 accumulation was dependent on RAS signaling through p38 MAPK (153). However, the experimental data in this study were based on transformed human foreskin fibroblasts and human embryonic kidney cells and not breast cancer cells, which make the clinical significance of their findings rather elusive. More convincing data was presented in a paper showing that the Notch signaling antagonist NUMB was expressed at reduced levels in about 50% of primary breast tumor samples (158). In samples showing low NUMB expression, Notch signaling was elevated. This inverse expression pattern was confirmed both in cultured primary tumor cells derived from these samples, and in established breast cancer cell lines. Moreover, an inverse correlation was seen between NUMB expression levels and GSI-sensitivity in cultured primary cells (158). One obvious mechanism in breast cancer, as well as in all other tumor-types without gain-of-function-mutation in the Notch-1 receptor, is that an overexpression of Notch ligands and/or receptors would allow for an enhanced autocrine or juxtacrine Notch activation. The mechanism driving Jagged-1 expression in breast tumor cells is currently not known. However, ectopic expression of wingless 1 (WNT-1) in primary human mammary epithelial cells leads to upregulation of Notch ligands of the DLL family (157).

Several studies have provided insights into the molecular consequences of aberrant

Notch signaling in breast cancer. In analogy with T-ALL, *c-Myc* is a direct transcriptional target of ICN-1 and appears to play an indispensable role in ICN-1-induced murine mammary tumorigenesis, probably by affecting proliferation rather than blocking differentiation (161, 162). A positive correlation between ICN-1 and *c-myc* expression in 38% of the human breast tumors examined was reported. It should however be noted that the immunostaining data did not correlate with clinical parameters, such as tumor pathological type or stage (161, 162). Notch signaling was shown to be required for WNT-1 induced transformation of primary human mammary epithelial cells both *in vitro* and *in vivo* (157). The proliferative potential of both primary tumor cells and established cell lines are suppressed by GSI treatment and enforced expression of NUMB (158, 159). An anti-apoptotic role of Notch signaling have also been described in both normal human breast cells, as well in a human breast cancer cell line, most likely mediated by inhibition of p53-mediated apoptosis upon cytotoxic drug stimulation (159). A very recent paper by Leong et al, provided very interesting data that may at least in part explain why Jagged-1 and Notch-1 expression are associated with a poor prognosis in breast cancer (163). They showed that Jagged-1 mediated Notch activation promoted breast cancer cell metastasis through initiation EMT both *in vitro* and *in vivo* (163). EMT is a differentiation and/or morphogenic process where cells undergo a developmental switch from a locostatic epithelial state to a migratory mesenchymal state (117). EMT is believed to be a fundamental process during embryogenesis facilitating the generation of new tissue types. Recapitulation of the EMT process has been implicated as a central event in chronic inflammation, fibrosis and cancer progression. Central events include, but are not limited to, disassembly of the junctional structures of epithelial cells through down-regulation of E-cadherin by transcriptional repressors such as Snail, Slug and Twist. E-cadherin repression is followed by induced expression of mesenchymal cell proteins such as Vimentin and N-cadherin, further remodeling of the extracellular matrix and thus the

cells acquire a migratory phenotype (117). Leong et al showed that Slug is a direct target of the Notch signaling pathway in breast cancer cells leading to E-cadherin repression and both enhanced tumor growth and increased metastasis in a xenograft model (163). Jagged-1 and Notch-1 expression correlated positively with Slug in primary breast cancer samples and they further suggested that this relationship may account for a general effect on tumor progression, since a positive correlation between *Jagged-1* and *Slug* could be found in a wide range of human cancers. Moreover, they provided evidence that the Notch-Slug signaling axis enhanced cell survival in the absences of cell matrix adhesion, so called anoikis (163).

Recent evidence might also support a role for Notch signaling in proposed so called “cancer stem cells” (CSCs), “cancer initiating cells” or “tumor initiating cells” of the breast (100, 160, 164). Normal stem cells and/or progenitor cells, which have been described in more or less detail in almost every organ of the human body, are characterized by their self-renewal capabilities, and are maintained through symmetric and asymmetric cell divisions (165, 166). Asymmetric division produces one daughter stem cell and one committed progenitor, which is the feature primarily but not solely responsible for the self-renewal and multipotency of the stem cells (165). The committed progenitor or transit amplifying cell may undergo a limited number of cell divisions and then differentiate to various cell phenotypes. In analogy, CSCs are thought of as a rare population of tumor cells within a cancer that retain the capacity to self-renew and to differentiate into the wide array of tumor cells that constitute the bulk of the tumor (165). Albeit this is an attractive model it should be pointed out that the CSC research field is a work in progress (166). Nevertheless, CSCs have been identified, by means of their slow-cycling properties, by expression of cell surface markers that are characteristics of their normal stem cell counterparts and also their capacity to exclude Hoechst dye, by having multi-drug resistance transporters. Serial transplantation in animal models *in vivo* and their ability to proliferate in suspension as spherical structures *in vitro* for many genera-

tions, are two features frequently used as evidence for that isolated cells indeed are CSCs (167, 168). From a therapeutic perspective, it has been postulated that while chemotherapy may kill the majority of tumor cells within a tumor, the CSCs are inherently treatment resistant. Thus, the surviving CSCs may then form new tumors after treatment has been finished. Accordingly, finding a way to target the CSCs may have great impact on current cancer treatment protocols (165, 167, 168). With regard to Notch signaling and putative CSCs in the breast, Notch signaling appears to be important for the proliferation and self-renewal of both normal mammary stem cells and putative breast CSCs. More specifically, Dontu et al showed that Notch activation by soluble ligands enhanced mammary stem cell self-renewal and early progenitor proliferation, as judged by a substantial increase in secondary mammosphere formation, which could be blocked by adding anti-Notch receptor antibodies (169). Furthermore, when secondary mammospheres were embedded in matrigel the resulting branching morphogenesis could be inhibited by GSI treatment and a Notch receptor antagonist (169). With regard to the CSCs, Fernie et al provided data showing that mammosphere formation by cells isolated from DCIS lesions was inhibited upon GSI treatment or incubation with a Notch receptor-neutralizing antibody (160).

Skin cancers: Notch as a tumor suppressor and an oncogene?

Self-renewal of the skin is a continuous process throughout life. Terminally differentiated keratinocytes are continuously shed from the skin surface, whereas immature cells within the basal layer of the epidermis produce progeny that continuously differentiate and migrate through the different layers of the epidermis (45). Notch receptors and ligands are expressed in human and murine keratinocytes and Notch-1 signaling leads to cell cycle withdrawal and terminal differentiation of these cells (121). Consistent with this, deletion of *Notch-1* in murine epidermis results in extensive epidermal hyperplasia and the mice spontaneously develop cutaneous basal cell carcinomas (BCCs) at 8-12 months of age (170).

Furthermore, *Notch-1*-deficient mice were more susceptible to chemical induced carcinogenesis, which led to the formation of cutaneous lesions resembling both BCC and SCC (170). These results suggested that Notch-1 function as a tumor suppressor in mouse skin. In mouse keratinocytes, Notch-1 tumor suppressor functions can be attributed to (RBP-JK)-dependent up-regulation of p21^{Cip1} and repression of both Sonic Hedgehog (Shh) and WNT signaling (121). It also seems like Notch-1 loss-of-function needs to be complemented with EGFR and/or RAS activation in order to induce the more aggressive SCCs (104, 170). However, it has also been postulated that the SCC phenotype might be induced when blocking all Notch receptors expressed in the epidermis, whereas the BCC phenotype is seen only upon *Notch-1* knockdown. The evidence for this hypothesis relies on experiments showing that conditional transgenic mice generated to express the pan-Notch inhibitor, dominant-negative Mastermind-Like-1 (DNMAML1) exclusively develop SCCs (171).

Emerging data also suggest that Notch-1 functions as a tumor suppressor in human skin. In a very small set of human BCCs, *Notch-1*, *Notch-2* and *Jagged-1* levels were reduced compared to normal skin tissue (172). A more recent report showed that *Notch-1* was reduced in a panel of skin and oral SCC cell lines relative to normal human primary keratinocytes (173). *Notch-2* expression was also reported to be decreased, albeit less consistently. Furthermore, in primary skin SCCs samples *Notch-1* expression was also found to be reduced relative to normal epidermis, with a parallel reduction in levels of the primary Notch target HES-1 (173). Since loss of heterozygosity or deletions of *Notch-1* has not been reported in BCCs or SCCs, down-modulation of Notch signaling in these tumors has been suggested to be due to compromised p53 function (173). Moreover, in human keratinocytes, Notch-1 activation leads to little or no increase of p21^{Cip1} expression, and seems to result in a more long-term suppression of growth and induction of differentiation (121). G. Paolo Dotto and colleagues have suggested that *Notch-1* tumor suppres-

sion functions might be attributed to negative regulation of the proposed self-renewal factor p63 and the pro-oncogenic ROCK1/2 and MRCK α kinases (173, 174). As in murine keratinocytes, suppression of Notch signaling collaborates with RAS in oncogenic transformation of primary human keratinocytes to SCC cells (173). Altogether, these data support a tumor suppressive role for Notch signaling in BCCs and SCCs.

In contrast to the tumor suppressive role of Notch in the keratinocyte-derived skin tumors (BCC and SCC), Notch has an oncogenic role in the development of the melanocyte-derived skin tumor melanoma (175, 176). Melanomas are highly aggressive and believed to arise through uncontrolled proliferation of melanocytes. In the normal setting, Notch signaling supports the survival of melanoblasts and melanocyte stem cells (175, 176). Consistent with this, apoptosis was induced upon GSI treatment of melanoma cell lines mediated by upregulation of NOXA and Bim through a p53-independent mechanism (177). Apart from promoting survival, constitutively active Notch-1 enabled melanoma cells to gain metastatic capability through a β -catenin-dependent mechanism, but Notch activity had little effect on metastatic melanoma (178). Increased Notch activity was also reported to promote melanoma progression by activating the MAPK and PI3K-Akt pathways and by up-regulating N-cadherin expression (95). Importantly, multiple Notch ligands, receptors and downstream targets are upregulated in primary human melanomas (178). Since most functional studies have been done by overexpression of ICN-1 or treatment with pan-Notch inhibitors, genetic loss-of-functions models are highly warranted in order to determine the individual role of each Notch component in this fatal disease (121).

Brain tumors with a special focus on Notch signaling in medulloblastoma

Given the fundamental importance of Notch signaling in the developing and adult central nervous system (CNS), it is perhaps not surprising that dysregulation of this pathway during recent years have been implicated in oncogenesis across a range of CNS tumors, e.g.

meningiomas, choroids plexus papillomas, gliomas (ependymomas, oligodendrogiomas, and astrocytomas (including anaplastic astrocytoma and glioblastoma multiforme)) (179-185).

The cerebellar medulloblastomas and extracerebellar primitive neuroectodermal tumors (PNETs) constitute the embryonal brain tumors. These tumors are thought to arise from neural progenitors and/or stem cells and have been associated with activation of the WNT and Hedgehog "stemness" pathways (186, 187). Overexpression of components of a third "stemness" pathway, i.e. Notch, was reported by two groups simultaneously (49, 188). Fan et al showed that both PNETs and medulloblastomas expressed elevated mRNA levels of the *Notch-2* receptor and found that 15% of the analyzed tumors presented with *Notch-2* gene amplification (49). While the *Notch-1* receptor levels were not elevated in medulloblastomas compared to normal cerebellum, the PNETs expressed significantly higher levels of *Notch-1* compared to medulloblastomas. Furthermore, Fan et al reported that Notch-2 and Notch-1 had opposite functional effects on the tested cell lines. Notch-2 was the pro-tumorigenic of the two receptors, which might be explained by their disparate function during development, where Notch-2 expression is associated with proliferating progenitors while Notch-1 expression is correlated with a postmitotic, differentiating stage (49). It should be noted that in the different types of gliomas a complex expression pattern of the various Notch components exists, which most likely indicates that non-redundant roles for some of these components exists in other types of brain tumors as well (181-185). Hallahan and colleagues reported more frequent *Notch-1* than *Notch-2* overexpression in primary medulloblastomas, which is contradictory to the aforementioned results reported by Fan et al and also their own results obtained in a medulloblastoma mouse model (188). In the medulloblastoma mouse model with hyperactivation of the Shh pathway, the *Notch-2* receptor but not *Notch-1* was elevated in the medulloblastomas of the transgenic mice. However, both groups reported elevated levels of Notch downstream

targets and Fan et al showed that HES-1 protein expression in medulloblastoma patients was associated with significantly shorter patient survival (49, 188). Importantly, both group showed that GSI inhibition resulted in decreased growth of medulloblastoma cells (49, 188). In a follow-up study by Fan and colleagues, it was shown that Notch inhibition lead to cell cycle exit, apoptosis and induction of neuronal differentiation markers in medulloblastoma cell lines (189). Hallahan et al also provided data showing that GSI-treatment of mice with medulloblastoma xenografts led to decreased proliferation and increased apoptosis of the tumors (188). The connection between the Hedgehog and Notch pathways has been substantiated by more recent studies, which also suggest that WNT signaling in some way cooperates with Hedgehog and Notch (190-192). These notions do however require further experimental evidences. Di Marcotullio et al showed that NUMB mRNA levels were significantly reduced in human medulloblastomas compared to normal adult cerebellar tissues (191). Furthermore, NUMB was shown to suppress the Hedgehog pathway by targeting the Hedgehog primary target Gli for ubiquitination and degradation. Although, evidence for an enhanced Notch signaling activity in NUMB-low medulloblastoma tumors were not provided by Di Marcotullio et al, it is conceivable that lower levels of the Notch-antagonist NUMB would also lead to enhanced activity of this pathway. An alternative model might be that Hedgehog signaling activates Notch signaling, which in turn downregulates NUMB (191, 193). The elucidation of how this complex cross-talk is wired will represent a future challenge. Putative CSCs have been identified in many different brain tumors including a population of CD133-positive and/or nestin-positive (both are markers of neural stem cells) medulloblastoma CSCs (194). Subsequently it was shown that Notch signaling was required for the maintenance of the medulloblastoma CSCs, which most likely is a reflection of Notch functions in normal CNS stem cells (189). Notch inhibition abolished the side-population in medulloblastoma cell lines and decreased the number of CD133 expressing

cells in the same cell lines (189). Importantly, depletion of the stem-like subpopulation in the medulloblastoma cell lines by Notch inhibition dramatically reduced the ability of these cell lines to form tumor xenografts (189). Furthermore, a nestin-positive stem-like subpopulation was more sensitive to GSI-induced apoptosis than nestin-negative more differentiated cells. With regard to the other brain tumors, some data also indicate a role for Notch signaling in maintenance of the CSCs of gliomas (195-197).

Altogether, these investigations clearly imply that targeting Notch signaling in medulloblastoma as well as in other brain tumors might hold great promises, especially since this pathway also seems to be critical for the maintenance of the tumor-initiating cells in some of these tumors.

Notch and angiogenesis

Apart from substantial evidence underscoring the role of the Notch cascade in tumor cells, accumulating data has also shown that Notch signaling has essential functions in regulation of tumor angiogenesis.

Several components of the Notch pathway are expressed in the vasculature at various stages of development and genetic studies in mice clearly implies essential roles for various Notch components during vascular development (198, 199).. Importantly, haploinsufficiency of *DLL-4* in mice resulted in embryonic lethality due to severe vascular abnormalities (200). *DLL-4* and *vascular endothelial growth factor (VEGF)* are the two only known examples where loss of a single allele causes this dramatic phenotype (199, 200). Experiments *in vitro* showed that *DLL-4* regulates multiple endothelial cell functions and established that *DLL-4* have essential roles in angiogenesis. Mice carrying a lacZ-reporter coupled to a *DLL4* promoter showed that *DLL-4* is expressed in smaller arteries and microvessels within tumors (199, 200). In human tumors, *DLL-4* expression is increased in tumor vessels of clear-cell renal cell carcinoma (CCRCC), superficial and basal bladder carcinomas, breast cancer and glioblastoma (201-204). Recently, a series of seminal papers provided substantial insight into the role of *DLL-*

4/Notch signaling in normal and pathological angiogenesis (199, 205-207). Inhibition of *DLL-4/Notch* signaling resulted in excessive angiogenic sprouting and branching (206, 207). More specifically, *DLL-4/Notch* inhibition resulted in that more endothelial cells adopted a tip-cell fate at the expense of stalk-cell fate and promoted endothelial cells to migrate in response to VEGF (205). In mice tumor models, systemic treatment with soluble *DLL-4-Fc* or anti-*DLL-4* antibody lead to increased tumor vessel density. Surprisingly and contra-intuitively, this unrestrained angiogenesis upon blockade of *DLL-4* decreased tumor growth (206, 207). *DLL-4/Notch* inhibition of tumor growth was due to the development of poorly functional vessels and hence decreased tumor perfusion. Furthermore, blockade of *DLL-4* in combination with anti-VEGF therapy had synergistic anti-tumor activity and *DLL-4* inhibition alone could inhibit the growth of tumors that were resistant to VEGF inhibition (206, 207). Unlike chronic systemic GSI treatment, the *DLL-4* inhibition approaches were not associated with any apparent toxicity in the treated mice (207). In summary, these data have established that *DLL-4/Notch* pathway represents a new target in anti-angiogenic therapy.

In addition, Jagged-1 has also been implicated in having a role in neovascularization of tumors. HNSCC cells expressing Jagged-1 appeared to activate bordering endothelial cells and thereby stimulated angiogenesis (101).

Neuroblastoma

Neuroblastoma (NB) is an embryonal malignancy that originates from precursor cell of the peripheral (sympathetic) nervous system (208). NB is the most frequently diagnosed neoplasm during infancy and is responsible for around 15% of all childhood cancer deaths (209). Since NBs are derived from neural crest cells of the sympathetic-adrenal lineage, it can arise anywhere in the sympathetic nervous system (SNS) (209). The most common site is within the adrenal glands and it has been estimated that about half of the NB patients will have metastases at presentation (210). The overall survival rates are less than 40% despite

aggressive multimodal therapy (209). NBs are characterized by their variability in clinical behavior, ranging from spontaneous regression, differentiation into benign ganglioneuromas to rapidly growing and aggressive tumors (210). The most significant genetic aberration in NBs is amplification of the NB *MYC* oncogene (*MYCN*) (211), which has an overall prevalence of around 20% in NB tumors (209). The oncogenic properties of *MYCN* have been demonstrated in transgenic mice where overexpression of *MYCN* in neuroectodermal cells resulted in formation of NB-like tumors (212).

Table 1. The INSS staging system.
Adapted from (209).

| | |
|-----------|--|
| 1 | Localised tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically |
| 2A | Localised tumour with incomplete gross excision; representative ipsilateral non-adherent lymph nodes negative for tumour microscopically |
| 2B | Localised tumour with or without complete gross excision, with ipsilateral non-adherent lymph nodes positive for tumour. Enlarged contralateral lymph nodes should be negative microscopically |
| 3 | Unresectable unilateral tumour infiltrating across the midline, with or without regional lymph node involvement; or localised unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement |
| 4 | Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, or other organs (except as defined by stage 4S) |
| 4S | Localised primary tumour in infants younger than 1 year (as defined for stage 1, 2A, or 2B), with dissemination limited to skin, liver, or bone marrow (<10% malignant cells) |

NB patients are stratified into low-, intermediate-, or high-risk categories in order to assign treatment intensity (209). The risk stratification system is based on several clinical and biological features, including age at diagnosis, tumor histopathology, International Neuroblastoma Staging System (INSS) stage (Table 1), tumor cell ploidy and *MYCN* Amplification status (209). Importantly, all stages of NB in patients younger than 12 months have a better prognosis than patients older than 12 months of age (209). Thus, age at diagnosis is an important feature of risk stratification. The most important histopathological feature is cellular differentiation, which dictates a favorable or an unfavorable classification, where a low level of tumor cell differentiation corresponds to more aggressive NBs and worse prognosis (210).

Low-risk disease

Surgical removal of the tumor is the most common treatment strategy of low-risk disease and results in survival rates of more than 95% for stage 1 disease. Low risk disease also includes some stage 2 disease, which also display high survival rates. Interestingly, there is a high rate of spontaneous regression in some infants with stage 4S disease and these patients are also associated with a favorable outcome (210).

Intermediate- and high-risk disease

Stratification of NB patients with regional disease is made by *MYCN* amplification and DNA index (210). Patients with *MYCN* amplification have much poorer 3-year survival rates (around 10%) compared with those with no amplification (around 93%) (213). In addition, patients with hyperdiploid tumors have considerably higher survival rates compared with those with diploid tumors (213). Knowledge of these parameters may imply that in patients with biologically favorable regional tumors, chemotherapy can be reduced considerably (210). Other DNA aberrations not included in the risk stratification that may be clinically relevant include allelic loss at 1p or 11q and the frequent unbalanced gain of 17q material (209). However, little is known about the genes targeted by these genomic changes.

High risk disease includes patients with stage 4 NB irrespective of the biology in children older than the age-cut point and unresectable disease NB with *MYCN* amplification in all age groups (210). Treatment of this group of patients include local radiotherapy and intense chemotherapy (agents include cisplatin, etoposide, doxorubicin, cyclophosphamide and vincristine) (209, 210). Cis-retinoic acid, which is known to induce terminal differentiation of NB cells *in vitro* (214), is also used in treatment of patients (209).

Neuroblastoma, a developmental error?

There are many factors clearly implying that NB is a consequence of normal developmental processes gone awry (215). Firstly, NB is detected in early life or *in utero* and no environmental factor has been linked to tumor development (215). Secondly, the cancer genes frequently altered in adult human cancer (e.g. RAS, p53, RB) are rarely mutated in NB and molecular players associated with NB pathogenesis such as *MYCN*, paired-like homeobox 2B (PHOX-2B), Hypoxia-inducible factor 2 α (HIF-2 α), TWIST, tyrosine kinase receptor-A and -B have all been implicated in the development of the SNS (209, 215, 216). Finally, recent data indicates that fetal neuroblasts, which have been suggested to be the cell of origin of NB, express many genes in common with NBs (217). Interestingly, in the same study it was also demonstrated that immature self-renewing neural stem cells have more genes in common with NBs than with the more mature, yet primitive, normal neuroblasts.

Development of the SNS

Neural crest cells are transitory cells that detach from the neural fold of the neural tube and initiate extensive migration throughout the embryo (218). Neural crest cells exhibit multipotency and can segregate into several lineages, such as sensory and sympathetic neurons, chromaffin cells, small intensely fluorescent (SIF) cells, peripheral glial cells and melanocytes. Sympathetic neurons, chromaffin cells, and SIF cells constitute the three SNS cell types (218). Sympathetic neurons,

and during development SIF cells, make up the sympathetic ganglia. Chromaffin cells are the main cell type of the adrenal medulla and extra-adrenal paraganglia (219). The sympathetic neurons and chromaffin cells are believed to be derived from a common sympathoadrenal progenitor, which develops from neural crest cells that aggregates in the vicinity of the dorsal aorta (218). The sympathoadrenal cells then migrate from the dorsal aorta to the sympathetic ganglia and the adrenal gland, where they terminally differentiate into sympathetic neurons or chromaffin cells, respectively (218). An important notion is that the immature neuronal cells, i.e. neuroblasts that have migrated to the medulla of the developing human adrenal gland, apparently discontinue their differentiation program and disappear during development (220). The differentiation process of sympathoadrenal cells is thought to be initiated by BMP signals from the dorsal aorta, which might induce neuronal and catecholaminergic differentiation of the cells. Expression of enzymes necessary for production of noradrenaline (tyrosine hydroxylase (TH) and dopamine B-hydroxylase) is initiated, as well as expression of specific neuronal markers (218). A large number of transcription factors control the differentiation of sympathoadrenal precursor cells, by participating in regulation of the expression of enzymes and neuronal markers. These transcription factors include, MASH-1, PHOX-2B, heart- and neural crest derivatives-expressed 2 (HAND-2, also known as dHAND), GATA-2 and -3 (218). It seems like MASH-1 and PHOX2B are the first transcription factors that are expressed upon initiation of differentiation of the sympatho-adrenal precursors (218, 221). Subsequently, the bHLH factor HAND-2, homeodomain transcription factor PHOX-2A and zinc-finger factors GATA-2 and -3, are expressed together with neuronal proteins and further on the noradrenergic enzymes are being produced. Terminally differentiated sympathetic neurons extend long neurites; maintain expression of neuronal markers and enzymes for synthesizing noradrenaline. Terminally differentiated chromaffin cells have secretory vesicles and harbor enzymes for synthesizing

noradrenaline, while neuronal markers are downregulated and these cells exclusively express adrenaline synthesizing enzyme phenylethanolamine N-methyltransferase (PNMT) (218).

With regard to NBs and their SNS origin, the pan-sympathetic marker TH has been reported to be expressed in virtually all the tumors (220). Furthermore, in support of their origin from immature sympathetic precursors, NBs show rather low expression of neuronal markers such as growth associated protein 43 (GAP43), neuropeptide tyrosine Y (NPY), Chromogranin-A and -B, but express HASH-1 (human homologue of MASH-1) and dHAND, that normally are only transiently expressed to control sympatho-adrenal differentiation (220).

Notch in the SNS

Apart from the fairly well-established role of MASH-1 in the development of the SNS, contribution of each individual component in the Notch pathway has not been extensively studied (222).

Gene-targeting of *MASH-1* in mice causes loss of most sympathetic precursors during development (223, 224). Moreover, *MASH-1* does not only seem to be a survival factor, since more recent knock-out models have shown that mutation of *MASH-1* also interfere with differentiation of the neuronal progenitors (225).

Notch genes and ligands are expressed in the SNS and neural crest during development (226, 227). Notch-1, CSL, or HES1 and HES5 or NUMB and NUMBL null mice all exhibit premature neuronal differentiation and a loss of neuroepithelial progenitors (228-232). These studies generally suggest that Notch maintains neuronal progenitors in an undifferentiated state. However, *in vitro* cultured post-migratory neural crest stem cells undergo gliogenesis upon DLL-1 induced Notch activation, accompanied by an irreversible loss of the potential for neuronal differentiation (233, 234). Sean J. Morrison and colleagues recently provided *in vivo* data on this matter (235). Conditional deletion of *CSL* in mouse peripheral nervous system and CNS progenitors resulted in profound effects on gliogenesis.

Interestingly, it was also reported that undifferentiated neural progenitors in most locations, including sympathetic ganglia, were not depleted in these mice (235). This was suggested to be due to the relatively late conditional deletion of *CSL*, after the onset of neurogenesis. Thus, the role of Notch signaling seems to be two-fold during neuronal development, initially promoting the maintenance or generation of neuronal progenitors and later promoting gliogenesis (235). Data from frog, zebrafish and chick model systems suggest that Notch signaling is important during early neural crest formation (neural crest induction), together with WNT, BMP and FGF (236). Analysis of *Delta-1* mutant mice has also suggested that Notch-signaling also effects neural crest migration as well as differentiation (237). Very recently, evidence was provided for a role of Notch signaling in neural crest progenitors in sympathetic ganglia in the chick embryo (238). This study argues that Notch signaling is involved in segregation (proportion of neurons and glial cells) and maintenance of progenitor cells during very early stages of sympathetic ganglion development in order to prevent premature generation of neurons. Moreover, the authors also suggested that from embryonic day 5 and onwards, Notch components are downregulated and the observed increase in neuron numbers from day 5 might be due to proliferation of sympathetic neurons rather than differentiation of sympathetic neurons from pluripotent progenitors (238). However, since species specific functions of Notch signaling with regard to the SNS development has been reported, these results need to be confirmed in mice (236).

Is there a role for Notch signaling in neuroblastoma?

Since accumulating data show that Notch signaling have an important role during the development of SNS, it has been assumed that Notch signaling or its target transcription factors could be involved in the pathogenesis of the SNS-derived tumor NB (239). Knowledge about the expression pattern during human SNS development is currently to a large extent missing. However, primary NB tumors have

been shown to express the Notch associated pro-neural bHLH factor HASH-1 (as mentioned before) and the atypical DSL-lacking Notch ligand DLK-1. However, no correlation between HASH-1 expression and tumor stage was found (240, 241). *DLK-1* is highly expressed in a subset of NBs associated with a differentiated and benign phenotype (242). Two additional studies has corroborated that elevated DLK-1 is most likely observed in tumors with favorable outcome (217, 243). Intriguingly, and in stark contrast to the above studies it has also been shown that elevated *DLK-1* expression served as a strong prognostic marker for adverse NB (244).

Since some NB cell-lines retain characteristics of neuronal progenitor cells, it is possible to induce differentiation of these cells *in vitro*, using agents such as phorbol ester (TPA) and retinoic acid (RA). Upon induced differentiation of these cells, *HASH-1* is downregulated, accompanied by induced expression of neuronal marker genes, such as *NPY* and *GAP-43* along with neurite extension (241, 245-247). During this differentiation process, *HES-1* expression is transiently upregulated, suggesting that downregulation of *HASH-1* via HES-1 repression might be important for NB cell differentiation (245). However, constitutive expression of ICN-1 in these cells inhibited induced differentiation(245). These observations are in line with studies on cultured mouse cortical neurons and N2a mouse NB cells, where Notch inhibits neurite extension (248-250). Furthermore, when NB cells were exposed to low oxygen tension, hypoxia, reduced expression of a number of neuronal and neuroendocrine marker genes such as *NPY*, *GAP-43* and *Chromogranin-A* and -*B* were observed. In addition, the SNS markers *HASH-1* and *HAND2* were also downregulated, whereas putative neural crest associated markers *Notch-1*, *Notch-3*, *HES-1*, *HEY-1* and *KIT* were upregulated (219, 220, 251, 252). Therefore hypoxia is thought to impose a more immature and hence more malignant phenotype of NB cells (219). Interestingly, Gustafsson et al reported that hypoxia activated Notch downstream target genes and promoted the undifferentiated state of neuronal cells and muscle precursors in a Notch-

dependent manner (87). Moreover, HIF-1 α interacted with ICN-1, which resulted in enhanced transcription of Notch target genes (87). Whether such a mechanism is also play a role during hypoxia-induced dedifferentiation of neuroblastoma cells remains to be determined.

The regulation of chromatin by HATs and HDACs

In the eukaryotic cell, DNA is tightly packed in the nucleus in the form of chromatin, which consists of DNA, histones and non-histone proteins (253). The nucleosome represents the basic unit of chromatin, and each nucleosome comprises a histone octamer surrounded by 146 base-pairs of DNA wrapped around the histone core. The histone octamer consists of an H3-H4 histone tetramer and two H2A-H2B histone dimers. Relaxed versus compact chromatin architecture is of fundamental importance in gene regulation. The structure of chromatin can be altered by post-transcriptional modifications of the N-terminal tails of the histones through acetylation, methylation, phosphorylation and ubiquitination (253).

Acetylation and deacetylation of histones is dynamically maintained by two groups of enzymes, histone deacetylases (HDACs) and histone acetyl transferases (HATs, e.g. p300/CBP). Generally, acetylation of histones is associated with a relaxed chromatin structure, enabling DNA-binding transcription factors to have greater access to the DNA. Deacetylation of histones maintains the chromatin in the compact state and is associated with gene repression or silencing (253). There are three main families of HDACs. The class I HDACs (HDAC-1, HDAC-2, HDAC-3 and HDAC-8) are detected almost exclusively in the nucleus and well known examples include NCOR and SMRT. The class II HDACs (HDAC-4, -5, -6, -7, -9 and -10) are able to shuttle between the nucleus and cytoplasm in response to certain cellular signals, while SIRT-1 to -7 comprise the class III HDACs and are nicotinamide adenine dinucleotide-dependent enzymes (253).

Histone deacetylase inhibitors and cancer

The anti-tumoral properties of HDAC inhibitors include induction of cell-cycle arrest, differentiation, and/or apoptosis. HDAC inhibitors main therapeutic properties have been ascribed to their capacity to induce genes that have been epigenetically silenced in tumor cells (254).

Acute promyelocytic leukemia (APL) represents one classical example of altered HDAC activity and tumorigenesis. In APL, where chromosomal translocations between retinoic acid receptor α (RAR- α) and either PML (promyelocytic leukemia protein) or promyelocytic zinc finger (PLZF), produce fusion proteins that binds retinoic acid response elements (RAREs) (253, 255). These fusion proteins have high affinity for HDACs, which results in repression of RAR-targeted genes normally needed for differentiation of myeloid cells in the presence of physiological levels of RA. However, addition of exogenous RA enable HATs to replace HDACs bound to the PML-RAR- α fusion protein, but is still unable to prevent PLZF-RAR- α repression. To overcome the PLZF-RAR- α differentiation block in APL cells, it is necessary to combine RA treatment with HDAC inhibitors (253, 255, 256).

Increased histone acetylation of genes encoding p21^{cip1} and p16^{INK4A} in tumor cells, are also examples of how HDAC inhibitors may possess growth inhibiting properties (254). HDAC inhibitors have also been reported to have antio-angiogenic activities that might be due to effects on both the tumor cells and endothelial cells. Several HDAC inhibitors are currently in clinical trials for treatment of hematological malignancies (254). Recently FDA approved a HDAC inhibitor (Vorinostat, also known as suberoylanilide hydroxamic acid or Zolinza) for treatment of cutaneous manifestations of T-cell lymphoma (257).

Valproic acid

VPA (valproic acid) is an established drug for long-term treatment of epilepsy in both adults and children (258). VPA is generally well tolerated and bioavailability is close to 100%. The adverse effects include liver failure

(1:15000) and teratogenicity (neural tube defects), which occur in a frequency around 1% to 2% (258). In the beginning of this century, VPA was demonstrated to posses HDAC-inhibitory activity (259, 260). VPA inhibits a broad spectrum of HDACs (Class I HDAC-1 to -3 and class II HDAC-4, -5, and -7), and also regulates other chromatin modulation proteins (261). Like other HDAC inhibitors, the anti-neoplastic effects of VPA have been best described in hematological malignancies. The effect of VPA in these tumor types appears to be partially related to induction of p21^{cip1} to achieve cell cycle-arrest and differentiation (261). In addition, VPA promotes apoptosis by both intrinsic and extrinsic pathways, and also seems to modulate immune response and angiogenesis. However, the precise mechanisms for the actions of VPA remain poorly defined and may include non-HDAC effects (261). Furthermore, the biological effects of VPA *in vitro* are highly dependent on the differentiation status and genetic background of the cells tested (261). Another interesting notion comes from studies on hematopoietic stem cell and leukemic progenitor cells from AML samples, where VPA stimulated the expansion of hematopoietic stem cells and also supported the growth of leukemic progenitor cells in the majority of samples tested (262-264).

Nevertheless, several clinical trials have been conducted with VPA, often in combination with cytotoxic drugs, RA or demethylating agents. These trials, also on treatment refractory solid tumors, showed promising clinical responses (265, 266).

VPA and neuroblastoma

The low toxicity profile of VPA in children treated for epilepsy and its potential anti-cancer effects makes VPA an interesting candidate for the treatment of NBs. There are several studies addressing VPA-induced effects on NB cells, both *in vitro* and *in vivo*. *In vivo* studies have shown that VPA suppressed the growth of NB xenografts in nude mice (267, 268). Histological examination of the xenografted tumors indicated that VPA induced differentiation and apoptosis in the treated mice (267). It has also been observed

that a combination between VPA and interferon- α (IFN- α) resulted in a synergistic anti-growth effect on NB xenografts (269). Recently, Susan L. Cohn and colleagues reported that VPA significantly inhibited angiogenesis in NB xenografts and that this effect could be further enhanced by combining VPA with an angiogenic inhibitor (268). *In vitro* data have shown a variety of effects of VPA on NB cells. Several studies have shown that VPA induces apoptosis, neuronal differentiation and negatively affects proliferation of NB cells (270-274). The molecular mechanisms behind these effects seems to be highly cell-type specific. Upregulation of p21^{Cip1}, p27^{Kip1}, GAP-43 and B-cell lymphoma 2, and downregulation of MYCN have been observed upon VPA stimulation of NB cells (268, 270, 271, 274, 275). Recently, Condorelli et al reported that VPA activated the p53 protein via hyperacetylation and nuclear relocalization, which might explain the observed upregulation of p21^{Cip1} upon HDAC inhibition (276). They also provided data showing that VPA induced cell death of SH-SY-5Y NB cells at high VPA concentrations and caused cell-cycle arrest at low concentrations without any effect on differentiation (276). However, in contrast, there are also studies showing that VPA increased survival and differentiation via ERK/MAPK activation in the very same cell line (272, 275).

Renal cell carcinoma

Epidemiology and etiology

Renal cell carcinoma (RCC) arises from the renal epithelium and accounts for nearly 3% of all adult malignancies globally (277). In the United States, 51190 (31590 male and 19600 female) new cases and 19600 (8080 male and 4810 female) deaths of renal cancer (including renal pelvis cancer) are estimated to have occurred in 2007 (278). In Sweden, 1094 new cases (654 male and 440 female) of renal cancer (not including renal pelvis cancer) were discovered in 2006 and roughly 50% of these patients will die from the disease (279). The disease usually occurs at the age of 60-70 and its incidence appears to be increasing, which might partially be explained by incidentally

discovered lesions during imaging for unrelated conditions (277). Approximately 25% of patients have advanced disease at the time of diagnosis, and around 30% undergoing resection of localized RCC will develop recurrence (280). There are a variety of risk factors claimed to be associated with RCC, such as smoking, obesity and hypertension (277). For example, smoking is thought to cause 20-30% of all RCCs (281). In addition, around 4% of all RCCs are associated with inherited syndromes (280).

Tumor types

Renal cell tumors can be sub-divided according to histology, genetics and their putative cell of origin (282, 283). Benign tumors will not be covered below, but include metanephric adenoma and adenofibroma, papillary adenoma, and renal oncocytoma (282). Malignant tumors are subdivided into clear cell RCC (CCRCC) (also called common RCC or conventional RCC), papillary RCC, chromophobe RCC, collecting duct carcinoma and unclassified RCC (282).

CCRCCs are thought to be of proximal tubular origin, but this remains debated (277, 284). CCRCCs account for around 75% of renal cell cancers and are typified by having cells with clear cytoplasm (282). These tumors are characterized by loss of function of the *von-Hippel-Lindau (VHL)* tumor suppressor gene (285) (see below).

Papillary RCCs make up 10-15% of all RCCs and are thought to be of distal tubule origin (277). These tumors are characterized by their papillary growth pattern (282). Papillary RCC are further subdivided into two subgroups, where type 1 is a low grade tumor with cells having a pale cytoplasm and type 2 is a high grade tumor with eosinophilic cytoplasm (277, 286). Hereditary papillary RCCs are related to activating mutations of the *MET* proto-oncogene (papillary RCC type 1) and loss of the putative tumor suppressor gene *fumarate hydratase* (papillary RCC type 2) (283).

Chromophobe RCCs arise from the intercalated cells of the renal tubuli and account for approximately 5% of renal cancers in surgical series (277, 282). This subtype is associated

with the putative tumor suppressor gene *Birt-Hogg-Dubé (folliculin)* (283).

Collecting duct carcinomas are rare (1% of renal cancers), characterized by irregular channels lined by highly atypical epithelium and by having no consistent genetic abnormalities (282).

Unclassified RCC is a category used when a RCC does not fit into anyone of the other sub-types (282).

Prognostic factors

The tumor-node-metastasis (TNM, created by the American Joint Committee on Cancer (AJCC) staging system) is the most commonly used prognostic tool for RCCs (277, 287). The system incorporates tumor size, extension of RCC beyond the renal capsule, involvement of renal vein or vena cava, lymph node involvement and distant metastasis (Table 2). Five-year survival, ranges from 91-100%, 74-96%, 59-70% and 16-32% for stage I through stage IV disease (277). However the prognostic accuracy of this system remains debated (287). Other factors not included in the TNM staging system include tumor grade according to Fuhrman and performance status of the patients (287).

Symptoms and diagnosis

The classical manifestation of RCC, a triad of hematuria, flank pain, and abdominal mass, is now uncommon. As previously stated, roughly half of the RCCs are detected during incidental radiographic examination (277). In symptomatic patients, hematuria (50% of patients) is most common, followed by pain and abdominal mass both being present in 40 % of patients. Modern imaging is able to correctly assess the malignant status of RCC in more than 90% of cases. The lung is the most common site of metastases, followed by bone, liver and brain. Staging and evaluation is needed to be completed before treatment (277).

Treatments of RCC

Surgical excision is the principal treatment of choice for localized RCC. Open radical nephrectomy is the standard surgical procedure, although laparoscopic and partial

nephrectomies are increasing in acceptance. For a limited number of patients metastasectomy may be beneficial (277).

RCC is basically refractory to chemotherapy and radiation, but these therapies can be used for palliation (277). Two immunotherapeutic approaches with cytokines are currently used for the treatment of advanced RCCs. High dose interleukin-2 (IL-2) showed up to a 20% response with 7% of patients achieving a complete response (288, 289). Although, some of the patients with complete responses have

Table 2. The TNM/AJCC staging system and stage grouping for RCC. Adapted From (277).

| Primary tumor | |
|--------------------------|---|
| T1a | Tumor 4 cm or less in greatest dimension, limited to the kidney |
| T1b | Tumor more than 4 cm but not more than 7 cm in greatest dimension, limited to the kidney |
| T2 | Tumor more than 7 cm in greatest dimension, limited to the kidney |
| T3a | Tumor directly invades adrenal gland or perirenal and/or renal sinus fat but not beyond Gerota's fascia |
| T3b | Tumor grossly extends into the renal vein or its segmental (muscle-containing) branches, or vena cava below the diaphragm |
| T3c | Tumor grossly extends into vena cava above diaphragm or invades the wall of the vena cava |
| T4 | Tumor invades beyond Gerota's fascia |
| Regional lymph nodes (N) | |
| N0 | No regional lymph node metastases |
| N1 | Metastases in a single regional lymph node |
| N2 | Metastasis in more than one regional lymph node |
| Distant metastasis (M) | |
| M0 | No distant metastasis |
| M1 | Distant metastasis |
| Stage grouping | |
| Stage I | T1N0M0 |
| Stage II | T2N0M0 |
| Stage III | T1-2N1 or T3N0-1 |
| Stage IV | T4 (any N or M) or N2 (any T or M) or M1 |

not relapsed, IL-2 treatment is both an expensive and considerable toxic treatment approach (277). INF- α demonstrates response rates around 5-15%, but duration is usually limited to a few months (277).

The evolving understanding of the molecular and tumor biological factors behind the development of CCRCC, have led to the development of several new exciting therapeutic approaches, that will be discussed below.

The von Hippel-Lindau tumor suppressor gene and clear cell renal cell carcinoma

The VHL disease, termed after the German ophthalmologist Eugene von Hippel and the Swedish pathologist Arvid Lindau, is an inherited disorder that manifests in tumor formation in multiple organs (290). Patients with the VHL disease are at increased risk of developing CCRCCs, hemangioblastomas, pheochromocytomas and pancreatic islet cell tumors (283, 285, 290). VHL disease affects 1 in 35000 individuals in the United States and is transmitted in an autosomal dominant manner (283). Studies in the late 1980s, implicated chromosomal aberrations involving both sporadic and hereditary RCC (291, 292). In 1993, the *VHL* gene was identified at chromosome 3p25 by positional cloning (293). In accordance with Knudson's two-hit hypothesis, VHL patients have germline mutations of one allele in nearly 100 % of the cases and tumors develop in a susceptible cell with the somatic loss or hypermethylation of the second allele (285, 294). CCRCCs are thought to arise from cells lining pre-neoplastic renal cysts. Loss of heterozygosity studies and immunohistochemical analyses of VHL patients have shown that these pre-neoplastic cells have lost the function of the remaining allele (284, 295). Mice with conditional inactivation of *VHL* in renal proximal tubules also develop renal cysts (296). Interestingly, these mice do not develop CCRCCs, which might imply that additional oncogenic events are needed for the development of CCRCC. Approximately 60% of the VHL patients develop multiple solid and cystic renal lesions, which have to be kept under close surveillance and nephron-sparing surgery are used to control the risk of disease progression (283). However, only a small part

of CCRCCs are of hereditary origin, but analyses of sporadic CCRCCs have shown that *VHL* gene is somatically mutated in approximately 50% of CCRCCs and hypermethylated in another 10-20% (285). Thus, the hallmark of CCRCC is inactivation of the *VHL* tumor suppressor gene.

The *VHL* gene product, pVHL (VHL protein) was shown to possess tumor suppressor activity, since reintroduction of wild-type *VHL* into *VHL*-deficient CCRCC cells inhibited the capacity of these cells to form tumors in mouse xenograft assays (297). A number of studies through the years have shown that the tumor suppressor activity of pVHL largely can be attributed to its role in an ubiquitin ligase complex that target the bHLH-Per/Arnt/Sim hypoxia inducible transcription factors-1 α and -2 α (HIF- α) factors for proteasomal degradation (298, 299).

The HIF- α transcription factors are instrumental for normal cells during adaptation to acute or chronic hypoxia by regulating genes involved in glucose uptake and metabolism, angiogenesis and erythropoiesis (285). Under normoxia, HIF- α subunits are constitutively transcribed and translated, but rapidly degraded through direct interaction with an E3 ubiquitin ligase containing pVHL, elongin-B, elongin-C, r_bx-1 and cullin-2 (Figure 4). The pVHL-complex only recognizes the HIF- α proteins after an oxygen-dependent hydroxylation of the HIF- α subunits by prolyl hydroxylases (300, 301). The HIF- α subunits are also regulated by an oxygen-dependent hydroxylation at their TAD-domain by an asparaginyl hydroxylase called factor inhibiting HIF (FIH-1) (302). This hydroxylation attenuates the recruitment of the p300/CBP required for HIF- α mediated transcription. Under hypoxia, unhydroxylated HIF- α become stabilized and translocates to the nucleus where it dimerizes with its constitutively expressed partner HIF-1 β (also called aryl hydrocarbon receptor, ARNT). This transcription factor complex binds a specific DNA sequence (hypoxia-response element) and recruits coactivators and regulates target genes (285). In CCRCCs with defective pVHL, accumulation of HIF- α proteins occurs irre-

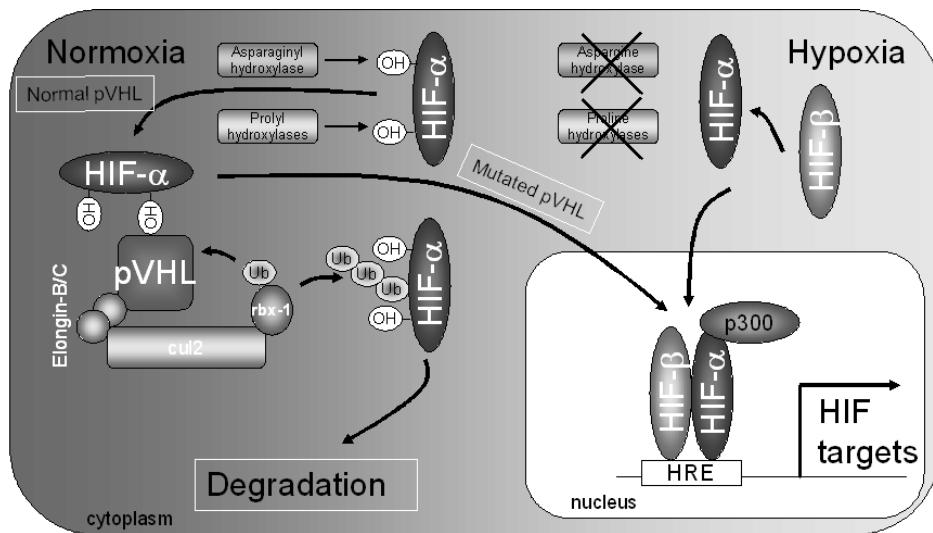


Figure 4. Under normoxia, HIF- α subunits are constitutively transcribed and translated, but rapidly degraded through direct interaction with an E3 ubiquitin ligase containing pVHL, elongin-B and -c, rbx-1 and cullin-2 (cul2). The pVHL-complex only recognizes the HIF- α proteins after an oxygen-dependent hydroxylation of the HIF- α subunits by prolyl hydroxylases. The HIF- α subunits are also regulated by an oxygen-dependent hydroxylation at their transactivation domain by an asparaginyl hydroxylase called factor inhibiting HIF (FIH-1) (302). This hydroxylation attenuates the recruitment of the p300/CBP required for HIF- α mediated transcription. Under hypoxia, unhydroxylated HIF- α become stabilized and translocates to the nucleus where it dimerizes with its constitutively expressed partner HIF- β (also called aryl hydrocarbon receptor, ARNT). This transcription factor complex binds a specific DNA sequence (hypoxia-response element, HRE) and recruits coactivators (p300) and regulates target genes. In CCRCCs with defective pVHL, accumulation of HIF- α proteins occurs irrespective of oxygen tension resulting in overproduction of HIF- α targets, such as VEGF, PDGF- β and TGF- α . Adapted from (280).

spective of oxygen tension resulting in over-production of VEGF, platelet derived growth factor β (PDGF- β), TGF- α , along with their receptors (303-305). The highly vascular phenotype of CCRCCs likely reflects the over-production of angiogenic factors, such as VEGF and PDGF- β , in addition to other putative HIF- α targets such as TGF- β and DLL-4 (204, 285). With regard to TGF- β signaling in CCRCC, there are conflicting reports regarding the functionality of the pathway, as well as how the expression of these proteins relates to progression of the disease (306-308). Other notable targets that are upregulated upon pVHL loss include cyclin D1 and the invasion and metastasis promoters chemokine receptor-4 and its ligand stromal derived factor-1 (285). Recently, it was shown that pVHL loss was associated with E-cadherin repression which might be necessary for an EMT process

during disease progression (309, 310). Of the two HIF- α isoforms, HIF-2 α appears to be the more oncogenic in CCRCC (285). Suppression of HIF-2 α retarded the growth of xenotransplanted CCRCC cells, whereas over-expression enhanced the growth of these tumors (311, 312). Primary CCRCCs express both HIF-1 α and HIF-2 α , or HIF-2 α exclusively (313, 314). In addition, in kidneys from patients with VHL disease, increased HIF-1 α is apparent in the earliest pre-neoplastic cysts, whereas elevated HIF-2 α expression is associated with more advanced lesions (284). Altogether, there is compelling evidence showing that pVHL loss is instrumental for CCRCC pathogenesis.

New treatments for RCC

Several new treatment options of advanced RCC, primarily aimed at targeting the cellular

consequences of VHL loss, have been approved during recent years.

Sunitinib (SU11248, Sutent) is an oral multitargeted kinase inhibitor, whose targets include VEGF receptors (VEGFR-1, -2 and -3), PDGF receptors (PDGFR- α and - β) and stem cell factor receptor (c-KIT) (315). In a randomized phase II study, the median progression free survival was significantly longer with Sunitinib (11 months) compared to IFN- α (5 months) (316). In addition, Sunitinib was also reported to be well tolerated.

Sorafenib (BAY 43-9006, Nexavar) is also an oral multitargeted kinase inhibitor, whose targets include RAF-1 kinases (B-RAF and RAF-1), VEGFR-1 to -3, PDGFR- β and c-Kit (315). In a randomized phase II study, the median progression-free survival was significantly longer in the Sorafenib group (5.5 months) compared to the placebo group (2.8 months) (317). Sorafenib was also reported to be well tolerated.

Temsirolimus (CCI-779, Torsel) is a kinase inhibitor of mTOR, which is administered intravenously (315). In a phase III trial, patients treated with Temsirolimus had significantly improved overall survival (10.9 months) compared to those treated with IFN- α (7.3 months) (318). Moreover, Temsirolomus was reported to be better tolerated than IFN- α .

Notch signaling in kidney development

Expression analyses of Notch pathway components have revealed that multiple ligands, receptors and downstream targets are expressed during mouse kidney development (319-322). Assessments of Notch functions have revealed essential roles during nephron patterning, when glomerular epithelial cells (podocytes), proximal tubules and distal tubules are formed (323). McCright et al reported defects in glomerulogenesis in mice homozygous for a hypomorphic *Notch-2* mutation (324). *In vitro* experiments with kidney organ cultures treated with a GSI, suggested that Notch signaling was required for the formation of podocytes and proximal tubules (325). Likewise, in mice with genetically reduced γ -secretase activity it was observed that the podocytes and proximal tubules were lost

(326). Recently, Kopan and colleagues showed that conditional deletion of *Notch-2* in the kidney resulted in complete loss of podocytes and proximal tubules (85). Intriguingly, ICN-1 was present in the nuclei of putative proximal progenitors but could not compensate for *Notch-2* deficiency.

The present investigation

Paper I – HDAC inhibition of neuroblastoma cells leads to Notch-1 receptor activation

Prior Knowledge

Transcriptional repression by CSL and the HES and HEY transcription factors are mediated by HDAC activity (38, 76). Valproic acid (VPA) is a well tolerated drug used for treatment of epilepsy (258). VPA has been shown to possess HDAC inhibitory properties and to induce differentiation and apoptosis of neuroblastoma (NB) cells (259, 267, 270).

Results

In both NB cell lines tested, VPA induced morphological differentiation as indicated by neurite-like extensions. In addition, the neuronal differentiation markers *NPY* and *GAP-43* were upregulated upon VPA treatment, indicative of neuronal differentiation. VPA also induced cell death in a dose-dependent manner. Furthermore, Notch signaling activity was augmented by VPA, as indicated by enhanced ICN1 expression, *HES-1* promoter activity, *HES-1* expression and possibly *HASH-1* downregulation. Finally, VPA induced differentiation of SK-N-BE(2) NB cells was partially repressed by treatment with a Notch inhibitor.

Context and caveats

The effects on differentiation and apoptosis by VPA were in line with previous studies on VPA and NB (270, 271). More intriguingly, was the observation that VPA stimulated Notch signaling activity and that the VPA induced differentiation of SK-N-BE(2) cells was partially attenuated by Notch inhibition. Since we have assumed that Notch signaling is involved in maintenance of NB cells in an undifferentiated state, this result may seem paradoxical. However, during induced differentiation *in vitro* a transient increase in both Notch-1 and Hes-1 levels can be observed, followed by rapid down-regulation of *HASH-1* (245). Thus activation of Notch by VPA

could just be a reflection of the initial phase of differentiation. More recent data on the other hand, suggest that the effects could be cell-type specific. Liao et al showed that GSI treatment of SH-SY-5Y cells resulted in induced differentiation of these cells as monitored by an induction of *GAP43* and an increase in neurite length (327). In addition, another group reported that mouse NB cells transfected with siRNA against *Notch-1* resulted in neurite extension (328). Treatment of NB cells with an alternative HDAC inhibitor (BL1521) also resulted increased Notch signaling activity, as judged by increased expression of *HES-1*, *HEY-1* and *HEY-L* (329).

Even though the role of Notch signaling in NB is enigmatic, it is rewarding to note that our observation that treatment with VPA activated Notch signaling in NB cells, instigated studies leading to clinical trials using VPA for treatment of neuroendocrine carcinoid tumors. Previous studies have shown that Notch signaling is growth inhibiting in this tumor cell type (122). In a recent study, VPA was shown to activate Notch-1 in carcinoid tumor cells and an increase in full-length Notch-1 expression was also noted (330). Furthermore, this activation was accompanied by suppression of *HASH-1* and dose dependent growth inhibition. Based on these results a phase II clinical study to assess the effectiveness of VPA for treatment of patients with carcinoid cancer has been initiated (330).

Paper II – Notch independent HES-1 regulation by ERK-1 and -2 in neuroblastoma cells

Prior Knowledge

Notch and RAS/MAPK pathway interactions have been observed both in the context of development as well as during tumorigenesis. The EGFR ligand, TGF- α , has been shown to stimulate NB cell proliferation (331). In addition, analysis of *Notch-1* and *CSL* mutant mice revealed no change in HES-1 expression at early stages of development (228), suggesting that HES-1 regulation does not depend solely

on Notch/CSL signaling in this specific context.

Results

TGF- α stimulation induced HES-1 at both the mRNA and protein level in a dose-dependent manner. Concomitantly with the HES-1 induction, there was a decrease in HASH-1 levels upon TGF- α treatment. The upregulation of HES-1 by TGF- α could be abolished by inhibiting EGFR or by inhibiting MEK further downstream in the EGFR signaling pathway. Furthermore, basal HES-1 expression was dependent on ERK-1 and -2 activity but not on EGFR activation. ERK-1 and -2 maintenance of HES-1 expression was most likely CSL dependent, but Notch signaling independent.

Context and caveats

These results provide further evidences to the notion that HES-1 expression is not only regulated by Notch signaling activity. This has been observed in a number of studies, and is also discussed in earlier sections of this thesis. For example, it has been shown that JNK signaling regulates *HES-1* expression through a Notch independent mechanism in endothelial cells (332). Furthermore, the TGF- β signaling pathway is also able to directly regulate Notch target genes (112). In paper IV, we also noticed that HES-1 could be induced upon TGF- β stimulation in CCRCC cells, thus further substantiating the general conclusion that signaling pathways cross-regulate each other and form complicated networks. These studies also entail that Notch signaling under some circumstances can be induced by soluble factors in the extracellular environment, and thus not only by Notch-ligands on adjacent cells. We did not address the functional consequences of the induced HES-1 levels upon TGF- α stimulation, but we noticed that HES-1 probably retains its capacity to repress the proneuronal transcription factor *HASH-1*. Since cell-cycle exit represent a fundamental step to trigger differentiation, it is tempting to speculate that TGF- α stimulation upregulates HES-1 in order to maintain an undifferentiated state and at same time promote prolifera-

tion. The reason for the high basal ERK-1 and -2 activity in these cells are unknown, but either factors in the serum of the cell media or some kind of activating mutation in the RAS/MAPK pathway could be feasible explanations. A recent study has also shown that 18 of 18 primary NBs and the majority of cell lines tested expressed the EGFR receptor (333), which indicates that EGFR signaling may be an important growth promoting pathway in these tumors.

Paper III – Notch signaling is a novel growth promoting pathway in clear cell renal cell carcinoma

Prior Knowledge

Functional loss of the von Hippel-Lindau (*VHL*) tumor suppressor gene, which leads to unregulated expression and activation of the hypoxia inducible factors (HIFs), is a hallmark feature of clear cell renal cell carcinoma (CCRCC) (280). Hypoxia has been shown to potentiate Notch signaling in neuronal and myogenic progenitors, as well as in NB cells (87, 220, 251). In addition, the Notch signaling pathway plays a prominent role during the development of the mammalian kidney (85).

Results

The Notch signaling pathway was found to be actively signaling in CCRCC cells, seemingly independent of the VHL/HIF pathway. Inhibition of Notch signaling attenuated the growth of CCRCC cells and was associated with elevation of CDK inhibitors p21^{cip1} and/or p27^{kip1}. In addition, siRNA experiments showed that the growth promoting effect could be attributed to the Notch-1 receptor. We also found that Notch inhibition restrained the growth of freshly isolated CCRCC cells. Analysis of primary tumor samples revealed that Notch-1 and Jagged-1 were expressed at significantly elevated levels compared to normal kidney tissue samples. Treatment of nude mice bearing CCRCC xenografts, with daily injections of a γ -secretase inhibitor in cycles of 3 days followed by 4 days without treatment, retarded tumor growth. This treatment schedule induced no

side effects as assessed by animal weight and histological examination of the intestines.

Context and caveats

Previous studies have identified elevated expression of *Notch-3* and *Jagged-1* in CCRCCs (334, 335), but when we initiated this work the biological significance of the Notch signaling pathway in CCRCC cells was unknown. Firstly, we were rather surprised that the Notch signaling activity was not a consequence of the pseudohypoxic phenotype of CCRCC cells, since previous studies have indicated that reduced oxygenation induced activation of the Notch pathway. Gustafsson et al, provided data showing that enforced expression of FIH-1 (factor inhibiting HIF), a well-known negative inhibitor of HIF transcriptional activity, significantly reduced Notch signaling activity at both normoxia and hypoxia (87). However, a more recent paper showed that FIH-1 indeed hydroxylated the Notch-1 receptor, but this did not affect the Notch-1 signaling activity (336). From a clinical perspective, the lack of cross-talk between pVHL/HIF pathway and Notch pathway in CCRCC cells may also be therapeutically beneficial, since the novel multi-targeted kinase inhibitors used for treating CCRCC patients and Notch inhibitors thus affect independent pathways.

The molecular mechanisms behind the growth inhibiting effects of Notch inhibition remains in part elusive, since we could not pinpoint the molecular connection between elevation of p21^{cip1} and/or p27^{kip1} and treatment with γ -secretase inhibitors. As pointed out several times in his thesis, the highly context-dependent nature of Notch target gene activation complicates our understanding of the oncogenic properties of this signaling pathway. We could for example exclude important Notch target genes in T-ALL, such as *c-Myc* and *Skp2* as mediators of the growth inhibiting effect in CCRCC, and further studies are with no doubts required for clarification of these matter. In paper IV we describe experiments aimed at resolving this issue. The *in vivo* experiments using the mouse xenograft assay showed a strong growth inhibiting effect of DAPT treatment that was not expected

based on the relatively modest growth inhibition noted *in vitro*. Since Notch inhibition is emerging as a promising novel anti-angiogenic treatment (as discussed earlier in this thesis), it is conceivable that the pronounced growth inhibitory effect on CCRCC xenograft growth is a consequence of a combined effect on both tumor cell growth and angiogenesis. More detailed analysis of the CCRCC tumors that arose in the DAPT treated mice is therefore required.

Paper IV – Notch signaling promotes migration of CCRCC cells by hijacking the TGF- β pathway

Prior Knowledge

In paper III we provide evidences supporting the idea that dysregulated Notch activity might play a pathogenic role in CCRCC. The full spectrum of downstream targets and processes regulated by Notch in this tumor form is unknown. Microarray studies in CCRCC have mainly been focused on defining prognostic signatures or identifying genes associated with inactivation of the *VHL* tumor suppressor gene.

Results

Gene expression analysis of Notch-inhibited CCRCC cells indicated that the Notch signaling cascade mediated regulation of a subset of genes previously associated with the TGF- β signaling pathway. TGF- β stimulation of CCRCC cells led to an enhanced phosphorylation of the downstream effector SMAD-2 and increased the activity of a luciferase reporter with TGF- β regulatory sequences, whereas Notch inhibition reduced phosphorylation of SMAD-2 and decreased the activity of the TGF- β responsive reporter. A detailed analysis of the gene expression profiles showed that the Notch cascade impinges on the regulation of TGF- β target genes associated with migration and invasion, while TGF- β targets associated with cytostasis where largely unaffected. Silencing of Notch-1 expression led to a significant decrease in CCRCC cell migration, and conversely TGF- β activation promoted CCRCC cell migration.

Context and caveats

As described in previous sections of this thesis links between Notch and TGF- β signaling have previously been reported (109, 112, 114). However, most reports so far have indicated that the Notch cascade is downstream of the TGF- β signaling pathway, i.e. that regulation of Notch signaling is an integral part of TGF- β controlled processes. Our observation that inhibition of Notch signaling attenuates the TGF- β pathway indicates that the hierarchy of the two pathways, at least to some extent, is reversed in CCRCC cells. Alternatively, this aspect of Notch/TGF- β cross-talk might have been overlooked in previous studies using other cell systems. Another important aspect of this paper relates to the functionality of the TGF- β pathway in CCRCC cells. Previous studies have implicated that the TGF- β pathway is not functional in CCRCC cells (303, 337). This is most likely a very important issue, since it is known that expression of TGF- β is highly elevated in CCRCC cells as a functional consequence of the pseudo-hypoxic phenotype of the CCRCC cells (338, 339). Thus, an intriguing question has been how the CCRCC cells circumvent the cytostatic effects of TGF- β signaling. Since our microarray experiments of DAPT treated CCRCC cells not only clearly indicated that this treatment led to a downregulation the TGF- β target gene signature, but also indicated the presence of basal TGF- β signaling, we first assessed the functionality of the TGF- β pathway using a variety of techniques. These experiments clearly confirmed a basal signaling activity that could be potentiated by exogenous TGF- β stimulation. These results are particularly intriguing with regard to the widely used 786-O cell line, since it has been reported that the 786-O cells lacks one of the TGF- β receptors (TGF β RII) and therefore do not respond to TGF- β (303). Further studies are required for clarification of these matters, including analyses of TGF- β receptor expression, in CCRCC. Nevertheless, our experiments clearly show that the TGF- β pathway is fully functional in two CCRCC cell lines (including

the 786-O cell line). We are currently performing assessment of the expression of TGF- β pathway components using a tissue microarray platform containing more than 300 primary CCRCC samples. This platform will also aid in clarifying the scientific debate on whether the TGF β RII receptor status has an impact on prognosis (306-308).

Interestingly, Zavadil et al showed that TGF- β and Notch cooperated during epithelial-to-mesenchymal-transition in normal epithelial cells a process that is recapitulated during pathological states such as fibrosis and cancer (112). It has also been shown that TGF- β 1 stimulation promotes RCC bone metastasis *in vivo* (340). If our observation that Notch inhibitors can be used not only to block Notch signaling, but also to dampen migration/metastasis associated TGF- β signaling, this treatment modality definitely seems to affect more tumor promoting systems than we initially expected. It will therefore be important to determine to what extent the assumed tumor promoting properties of TGF- β and Notch overlap or are interdependent in CCRCC.

Conclusions

The HDAC inhibitor, VPA, activates the Notch signaling pathway in neuroblastoma cells

The Notch target Hes-1 is primarily regulated by ERK-1 and -2 signaling in a neuroblastoma cell line

Notch signaling promotes growth of clear cell renal cell carcinoma cells both *in vitro* and *in vivo*

Notch signaling promotes migration of CCRCC cells, most likely, by feeding in to the TGF- β signaling pathway

Populärvetenskaplig sammanfattning

Cancer är fortfarande en till stora delar svårbotad sjukdom, men forskningen på området gör hela tiden upptäckter som leder till en bättre behandling. Vi har studerat två väldigt olika tumörformer, neuroblastom och njurcancer, men vi har forskat på samma protein i båda cancerformerna. Neuroblastom är en ovanlig cancerform som uppstår i det sympatiska nervsystemet hos små barn. Det sympatiska nervsystemet aktiveras när människan blir utsatt för psykisk eller fysisk stress. Njurcancer däremot drabbar oftast äldre männskor (50 till 70 år) och den uppstår från specialiserade celler i njuren som normalt sett hjälper till att sköta kroppens vätske- och saltbalans.

Det protein vi har studerat är en så kallad receptor som heter Notch, som sitter på ytan på cellerna. Den tar emot information från intilliggande celler som uttrycker proteiner (ligander) som kan aktivera receptorn. När receptorn aktiveras skickas en signal in i cellen. Denna signal talar om hur cellen skall bete sig som ett svar på den omgivning en given cell har vid en given tidpunkt. Notch-signalerings har ett flertal centrala funktioner under utvecklingen av olika vävnader under fostertiden, då den talar om för cellerna när det är dags att mogna ut och sluta dela på sig. I början på 1990-talet kom de första indikationerna på att Notch-receptorn också kunde vara inblandad i cancer. Studierna av Notch-signalerings roll, både under fosterutveckling och i cancer komplickeras av det faktum att det inte bara finns fyra varianter av receptorn (Notch1-4) utan också fem olika ligander. Det är också mycket tydligt olika celltyper svarar på olika sätt när Notch-receptorn aktiveras, vilket bland annat beror på att det finns många olika signalvägar in i cellen och att dessa påverkar varandra på ett ytterst komplicerat sätt. En rad substanser har tagits fram som hindrar Notch-signalerings, vilket gör att det finns goda möjligheter att gå från laboratoriet till klinisk verklighet, under förutsättning att felaktigt reglerad Notch-signalerings på något sätt bidrar till tumöruppkomst.

Neuroblastom är som sagt var en tumör som drabbar väldigt små barn och dessvärre kan sjukdomen hos många patienter vara väldigt svårbotad. Det finns därför ett mycket stort behov av nya behandlingsmetoder. För att kunna göra detta måste man förstå varför tumören har uppkommit i detalj, d.v.s. vilka gener som bidrar till tumörcellernas förmåga att överleva och dela sig. Eftersom tumören uppkommer hos barn är det troligt att de system som styr bildandet av det sympatiska nervsystemet på något sätt har spårat ur. Många studier har visat att Notch-signalerings är viktig under denna process och våra studier har inriktats på att förstå om denna signalleringsväg på något sätt är störd i neuroblastomceller, samt om man kan förhindra tumörcellernas delningsförmåga genom att korrigera denna störning. I det första arbetet testade vi om en substans, valproat syra (VPA), som sedan länge använts för behandling av epilepsi, också påverkar Notch-signalerings och därmed också cellernas förmåga att gå från det omogna stadium med hög celldelning som de fastnat i, till ett mer moet och därmed också mer långsamt delande stadium. Vi fann att så var fallet, behandling med VPA ledde till att cellerna blev mer mogna och att förändring av Notch-signalerings kan spela en roll i denna process. Eftersom VPA är en drog som redan används för andra sjukdomar finns det en möjlighet att man kan använda den, tillsammans med andra, mer kraftfulla substanser, för behandling av neuroblastompatienter. I ett andra arbete fokuserade vi hur Notch-signalerings samverkar med en annan viktig signalväg, som styr cellens delningshastighet. Detaljerad kunskap om hur olika signalvägar påverkar varandra är en förutsättning för att vi ska kunna använda kombinationer av olika substanser på ett så effektivt sätt som möjligt.

I studierna av njurcancer behandlade vi samma frågeställningar som i studierna av neuroblastom, d.v.s. bidrar felaktigt reglerad Notch-signalerings till tumöruppkomst och kan man hindra tumörcellernas delningsförmåga genom att stänga av denna signal in i cellen. I ett första arbete visar vi att kontrollen av

Notch-signalering tycks ha gått förlorad i njurcancerceller. Vi noterade också att cellernas delningförmåga minskade då vi hämmade signaleringsvägen. Dessa resultat baserades dock enbart på studier av odlade njurcancerceller och det var därför viktigt att testa Notch-hämmaren på möss som bar på små njurcancertumörer. Dessa studier gjordes alltså för att kunna visa på behandlingseffekter i en modell som skall försöka efterlikna situationen hos en cancerpatient. När man har testat den här substansen på både människor och djur har man upptäckt att den ger biverkningar i form av magproblem. Detta beror att Notch-receptorn, som vi försöker blockera med hämmaren, också har en viktig funktion i den normala tarmen hos både djur och mänska. Blockerar man signalvägen under lång tid så uppstår stora magproblem helt enkelt. Vi testade därför att ge substansen i tre dagar för att sedan göra ett uppehåll på fyra dagar. Teorin bakom denna idé bygger på att det är en stor omsättning på cellerna i tarmarna, så vi spekulerade i att tarmen hinner återhämta sig på fyra dagar innan det är dags för nästa dos. Vi fick mycket goda effekter i dessa försök, vilket gör att vi tror att Notch-hämning kan bli ett möjligt komplement till andra behandlingsterapier för njurcancer.

I det sista arbetet studerade vi i detalj vilka effekter en avstängning av Notch-kaskaden har i njurcancerceller. I detta arbete kunde vi visa att en annan signalväg (TGF- β), som bl.a. påverkar cellers förmåga att röra sig, och därmed också spelar en viktig roll då metastaser (cancertumörer som spritt sig till andra vävnader) bildas, påverkas av Notch-kaskaden. I experiment där cellernas förmåga att röra sig mäts, kunde vi visa att rörelseförmågan delvis var beroende av den aktiverade Notch-signaleringen i tumörcellerna, och att vi genom att behandla njurcancercellerna med en Notch-hämmare kraftigt minskade rörelseförmågan hos cellerna. Även om dessa resultat har erhållits grundade på studier av odlade tumörceller, vilket endast tillåter ganska trubbiga analyser av tumörcellernas rörelseförmåga, ger de en första indikation på att Notch-hämning kanske också kan påverka tumörcellernas förmåga att sprida sig. Denna slutsats måste dock givetvis testas i modellsystem som

på ett bättre sätt återspeglar den komplexa situation som uppstår då tumörer utvecklas och sprids i en levande organism.

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I just want to start off with a sentence that if I was pretentious, or more precisely just would admit my more pretentious sides, would have written on the first page.

"I tried living in the real world instead of a shell but I was bored before I even began"
Stephen Patrick Morrissey

I believe that I will never have the opportunity to make a record, so here are my liner notes:

Producer, lead-guitar and my supervisor:
Håkan may seem like an average almost professor type; living in Södra Sandby, playing computer-chess, reading thick books by dead Russian authors, playing tennis, and listening to Arvo Pärt and The Goldberg-variations. However, he does not drive a BMW; he likes alternative rock and pop music, as well as surfing. All of these shifting skills and features are reflected in his professional life. He is a dynamic, enthusiastic, multi-talented scientist and creates a stimulating and challenging atmosphere.

Håkan, I greatly appreciate that you believe in individual freedom and decision-making, since too much control may stifle creativity. It has also been a fantastic time from a social viewpoint and I will for sure miss all our conversations about everything and nothing. Your sense of humor is fantastic. I'm also sorry for given you a lot of work at late nights (the nice pictures!) and the sudden death of your car. By the way, you are also an excellent target-player in floor ball. All in all, a soft, funny and witty cowboy-scientist who takes

good care of his family and everyone else.
You're the best!

Executive producer, acoustic guitar and clogs:
Sven may seem like an average professor, when just taking a short glimpse at his main interests such as collecting art and very old letters. However, knowing that he also during the last years has taken good care of a sick dog makes the above picture start falling apart. Sven is actually a damn good professor and a genuine, honest, caring and straightforward person. Sven, I'm grateful for that you are always enthusiastic and supportive and that you would never compromise with the visions of the lab. You are also Top-3 in the world on neuronal differentiation and Top-400000 on computers in Skåne. Thanks for all support and knowledge and for cheering up Håkan about the JCI e-mails.

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Bass guitars and drums: Elisabet (can I send you my food orders to you, in the near future?), Siv (can you take care of our dishwasher at home if it breaks down?), Kristin (can you take care of my tax return form, from now on?), Inger, Christina, and Elise six lovely girls, that makes everything go smoothly, with a warm-hearted kindness. Big cheers!

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Alla vänner boendes i Malmö, i Lund, i övriga Skåne, på Frösön, på Öland, i Göteborg, i Stockholm, i Östersund, i övriga Sverige, i Basel, i USA, , eller på övriga platser i vår stora vida värld (en så kallad helgartering), tack för att ni finns. Ni är fullständigt, oåterkalleligt livsnödvändiga!

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När jag tänker på oss två nu
För du var solskenet
Och jag var ensam

Avslutningsvis måste jag tacka alla jag glömt.

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