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Notch signalling in carcinogenesis:

With special emphasis on T-cell lymphoma and colorectal cancer

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"What we have done for ourselves alone dies with us;
what we have done for others and the world
remains and is immortal"

Albert Pike, 1809-1891

TO WHOM IT MAY CONCERN!

Abstract

The Notch signalling pathway is an evolutionary conserved pathway, named after the Notch receptors, Notch1-4 in mammals, which upon cell-cell contact and ligand binding releases the intracellular domain (NICD). NICD translocates into the nucleus where it binds the transcriptional repressor RBP-Jκ, which together with co-activators belonging to the Mastermind-like family of proteins form a transcriptional activation complex. This complex activates genes controlling cell fate decision, embryonic development, proliferation, differentiation, adult homeostasis and stem cell maintenance. On the other hand, disrupted Notch signalling may result in pathological conditions like cancer, although the mechanisms behind the disruption are often complex and in many cases largely unknown.

Notch1 drives the lymphocyte differentiation towards a T-cell fate and activating mutations in the gene have been suggested to be involved in T-cell lymphoma. In *paper I*, genetic alterations in *Notch1* and the Notch1 regulating gene *CDC4* were investigated in tumours from murine T-cell lymphoma induced with phenolphthalein, 1,3-butadiene or 2',3'-dideoxycytidine. We identified activating *Notch1* mutations in 39% of the lymphomas, suggesting that *Notch1* is an important target gene for mutations in chemically induced lymphomas.

While it is known that constitutively activated Notch signalling has a clear oncogenic function in several solid malignancies as well, the molecular mechanisms are less known in this context. Unpublished data of our lab, together with other recent studies, suggest that mutations of Notch and Notch-related genes *per se* are uncommon in solid malignancies including colorectal cancer, while a growing body of evidence indicates that aberrant Wnt/β-catenin signalling may result in pro-tumoural Notch activation in these contexts. In *paper II*, we therefore investigated potential transcriptional interactions between the Notch and Wnt signalling pathways in colorectal cancer cell lines. The proximal Notch and Wnt pathway gene promoters were bioinformatically identified and screened for putative TCF/LEF1 and RBP-Jκ sites. In canonical Wnt signalling, Apc negatively regulates β-catenin leading to repression of TCF/LEF1 target genes. Upon repression of the Wnt pathway we observed that several genes in the Notch pathway, including *Notch2*, were transcriptionally downregulated. We also confirmed binding of Lef1 to *Notch2* as well as other Notch pathway gene promoters and luciferase assays showed an increased activity for at least one LEF1/TCF-site in the *Notch2* promoter upon co-transfection of HT29 or

HCT116 cells with mutated β-catenin. HT29 cell lines were also treated with the γ-secretase inhibitor DAPT, leading to inactivation of the Notch pathway by preventing release of NICD. However, results showed no effects on Apc, β-catenin or their target *cyclin D1*. Taken together, these results indicate that the Wnt pathway may function as a regulator of the Notch pathway through the TCF/LEF1 target gene program in colon cancer cell lines.

In summary, Notch pathway deregulation is of importance in both murine T-cell lymphoma and human colorectal cancer, although the mechanisms differ. The current results give new insights in Notch pathway alterations as well as the signalling networks in which the Notch pathway interacts, and thus increase the understanding of Notch's involvement in malignant diseases.

Table of contents

List of papers

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

I. Anneli Karlsson, **Jonas Ungerbäck**, Anna Rasmussen, John E. French and Peter Söderkvist. *Notch1* is a frequent mutational target in chemically induced lymphoma in mouse. Int. J. Cancer (2008) 123: 2720-2724.

II. **Jonas Ungerbäck**, Nils Elander, John Grünberg, Mikael Sigvardsson and Peter Söderkvist. *Notch-2* and the Notch signaling pathway are regulated by Wnt signaling in colorectal cancer, Manuscript.

List of abbreviations

Apc – Adenomatous polyposis coli
ANK – Ankyrin repeats
BCR – B-cell receptor
bHLH – Basic Helix-Loop-Helix
BLF – Butadiene-induced lymphoma in C57Bl/6×C3H/Hej F_1 mice
CDK – Cycline dependent kinase
ChIP – Chromatin immunoprecipitation
CRC – colorectal cancer
CLP – Common lymphoid progenitor
DLF – Dideoxycytidine-induce lymphoma in C57Bl/6×C3H/Hej F_1 mice
Dll – Delta-like
DLS – Dideoxycytidine-induce lymphoma in NIH Swiss mice
DN – Double negative thymocyte
DP – Double positive thymocyte
DSL – Delta/Serrate/Lag-2
EGF – Epidermal growth repeat
EMSA – Electophoretic Mobility-Shift Assay
GI – Gastrointestinal
HD – Heterodimerisation domain
Hes – Hairy enhancer of split
HL – Hodgkin's lymphoma
HSC – Haematopoetic stem cell
LEF – Lymphoid enhancer factor
Lfng – Lunatic fringe
LMPP – Lymphoid-primed multipotent progenitor
LNR – Lin12/Notch-related region
MAML – Mastermind-like
Mfng – Maniac fringe
MPP – Multipotent progenitor
MZB – Marginal zone B-cell
NECD – Notch extracellular domain
NHL – Non-Hodgkin's lymphoma
NICD – Notch intracellular domain
NK – Natural killer cell
NUMBL – Numb-like
PEST – Polypeptide rich in proline, glutamate, serine and threonine
PL – Phenolphtalein-induced lymphoma in TSG-p53™ mice
Rfng – Radical fringe
RAM – RBP-Jκ associated molecule
RNAi – RNA interference
RSS – Recombination signalling sequence
SD – Standard deviation
SEM - Standard error of the mean
siRNA – Silencing RNA
SSCA – Single stranded conformation analysis
TAD – Transactivation domain
T-ALL – T-cell acute lymphoblastic leukemia/lymphoma
TCF – T-cell factor
TCR – T-cell receptor
TD – Transmembrane domain
TF – Transcription factor
V(D)J – Variable-diversity-joining

Introduction

The oldest description of cancer (although the term 'cancer' was not used at that time) comes from Egypt and dates back to approximately 1600 B.C. Evidence for cancer has been found among fossilised bone and the removal of breast tumours has been described in papyrus scrolls. The Greek physician Hippocrates (460-370 B.C) was the first to use the word *karkinos*, the greek word for 'crab', which was later translated to the Latin word 'cancer' [10, 11]. Cancer is one of the most common diseases in the Western world and 2020 20 million new cases and 12 million deaths are predicted world wide. The increased human lifespan in Western countries, in combination with life style and environmental factors, constitute plausible reasons for the slight increase of incidence rate that is observed for many tumours. On the other hand, many cancers may be preventable by reducing common risk factors including cigarette smoking, high fat- and alcohol intake [12]. Cancer is a complex genetic disorder rising from a series of genetic changes in the DNA of a cell, leading to a neoplastic transformation and uncontrolled cell growth [13]. Additional mutations will accumulate due to the increased growth rate and, often, defective DNA repair machinery. The disorder also often involves disturbances in important embryonic cell signalling pathways like Notch and Wnt, which regulates processes such as development, proliferation and differentiation. These pathways are not only of importance during embryonic development but also play a major role in tissues that has a high self-renewal rate *e.g.* the intestinal epithelium or the haematopoietic organs, making them common sites for cancer.

In this thesis, we have analysed the Notch signalling pathway in relation to murine T-cell lymphoma and human colorectal cancer (CRC), and our results indicate that distinct molecular mechanisms activate Notch signalling in these malignancies. While T-cell lymphomas commonly display mutations of Notch and Notch related genes *per se*, colorectal tumour cells may present hyperactive Notch signalling as a result of aberrant Wnt signalling and transcriptional activation of β-catenin/Lef1/Tcf target genes.

The Notch signalling pathway

The canonical Notch signalling pathway was first identified in the context of lateral inhibition of the peripheral nervous system of insects [14]. It is an evolutionary conserved pathway, being crucially involved in cell fate decision, proliferation, development, adult homeostasis and stem cell maintenance [15-19]. The pathway is named after its core

components, the Notch receptors, which are transcribed and translated as 210-300 kDa large precursor molecules. A series of post-translational modifications are required in order for the precursors to acquire their active forms. The intact precursor molecules are first glycosylated in the endoplasmic reticulum (ER) by *O*-fucosyletransferase (Pofut-1 in mammals) (figure 1, #1) [20, 21], which adds fucose to serine or threonine sites on specific epidermal growth factor (EGF)-repeats [22-24]. The glycosylated precursors are then cleaved in the trans-Golgi network into two subunits by furin-like convertases (S1-cleavage) (figure 1, #2). This cleavage converts the precursor molecule into the noncovalently linked Notch extracellular domain (NECD) and Notch transmembrane-Notch intracellular domain (TM–NICD) complex, which is then further glycosylated by enzymes of the Fringe family. In mammals three *Fringe* genes have been identified: *Lunatic fringe* (*Lfng*), *Radical fringe* (*Rfng*) and *Maniac fringe* (*Mfng*) [25]. Fringe proteins add N-acetylglucosamines moieties to already existing O-fucose molecules on the EGF-repeats [21, 22, 26-28]. This modification in the Notch ligand-binding domain seems to alter the responsiveness of the receptor to different ligand interactions or enhance S2 mediated cleavage of the receptor [29]. The effects of Fringe dependent modification of Notch are complex and the outcome of the signalling largely seems dependent on the combination of receptor, fringe family member and ligand (reviewed in [4, 6] and [30]).

The mature Notch receptor is then translocated to the cell surface and is, via its EGF-repeats, activated upon binding to one of its ligands, which are expressed on neighbouring cells (figure 1, #3) [31]. In mammals, five different Notch ligands have been identified, three belonging to the Delta-like family (Delta1,3-4) and two to the Jagged family (Jagged1-2).

The receptor-ligand binding results in a conformational change of the receptor and the exposure of an extracellular metalloprotease site (S2). S2 cleavage of the NECD is controlled by the ADAM/TACE (a desintegrin and metallopeptidase/tumour necrosis factor α converting enzyme) family of transmembrane proteases [32-34] resulting in an active membrane anchored Notch. This Notch form is subsequently cleaved within the TD close to the cytoplasmic border by the γ-secretase complex (S3), a four-protein complex consisting of the catalytic component presenilin and the three co-factors, nicastrin, Aph-1 and Pen-2 (figure 1, #4) [35-39].

Following the multi-step cleavage of Notch and liberation of NICD from the inner membrane, activated NICD is translocated into the nucleus via endocytosis and endosomal trafficking (figure 1, #5) (reviewed in [6] and [30]). In the nucleus NICD normally binds to the transcriptional repressor RBP-Jκ, which together with co-activators belonging to the

Mastermind-like family (MAML1-3) of proteins forms a transcriptional activation complex (figure 1, #6) [40, 41]. The complex further recruits different co-regulators *e.g.* the histone acetyletransferase p300 and other chromatin remodelling factors together with the cyclin-dependent kinase (CDK) 8 [42, 43]. Recruitment of CDK8 leads to phosphorylation of NICD and thereby subsequent proteasomal degradation of the complex through E3-ligase FBW7 (Cdc4) mediated ubiquitination of the transactivation domain (TAD) and PEST domains (polypeptide rich in proline, glutamate, serine and threonine) (figure 1, #7) [44-46], thereby terminating active Notch signalling.

Other important regulators of Notch signalling are the Numb and Numb-like proteins, which act upstream of S3 cleavage to antagonise Notch signalling through direct interaction via Notch ankyrin (ANK)-repeats [47-49]. It is also likely that NICD can act in a RBP-Jκ-independent non-canonical manner and interact with several other components in the nucleus *e.g.* Hif-1α, NfκB and β-catenin (reviewed in [4, 43] and [50]). Some of the best-known target genes belong to the *Hes/Hey* (Hairy Enhancer of Split/Hairy Enhancer of Split related) family, which are basic helix-loop-helix (bHLH) transcriptional repressors important for development, proliferation, differentiation and cell fate decision (reviewed in [51, 52] and [53]). Besides bHLH transcription factors (TFs) several other genes like the protooncogene *c-myc* [54] and the cell cycle regulators $p27^{KIP1}$ and *cyclin D1* have been identified as Notch targets [55, 56].

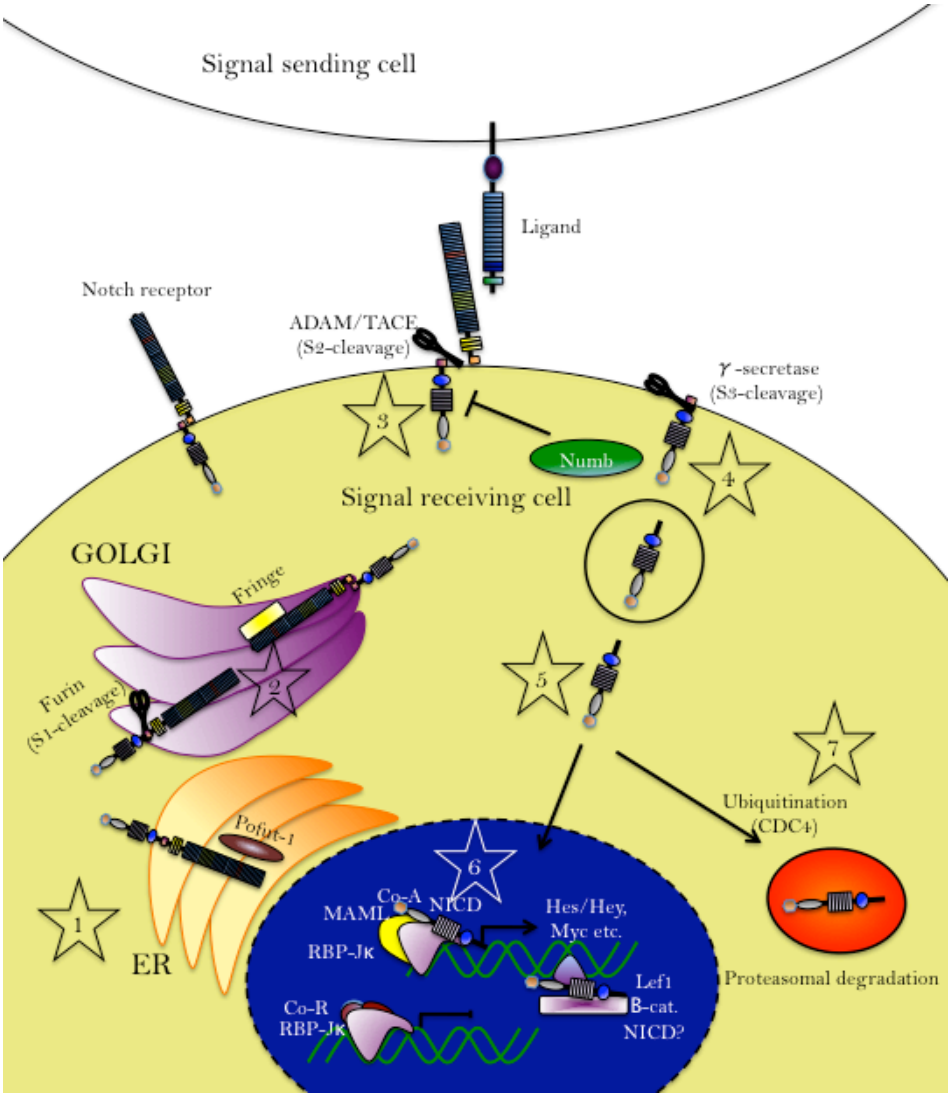

Figure 1: Canonical Notch signalling pathway (for details, see text) (adapted from [1] and [6]).

The structure of the Notch genes

The Notch receptors (Notch1-4 in mammals) [57] are large single transmembrane proteins, which also function as nuclear TFs. The different Notch genes are found on different chromosomes; *Notch1* on 9q34, *Notch2* on 1p13, *Notch3* on 19p13, and *Notch4* on 6p21. Structurally the proteins encoded by these genes are similar to the *Drosophila Notch* gene [58-60] but the Notch1 protein is the largest and most extensively studied of the Notch receptors, both in normal development and in cancer.

The Notch proteins can be divided into three different parts; NECD, TD and NICD, which

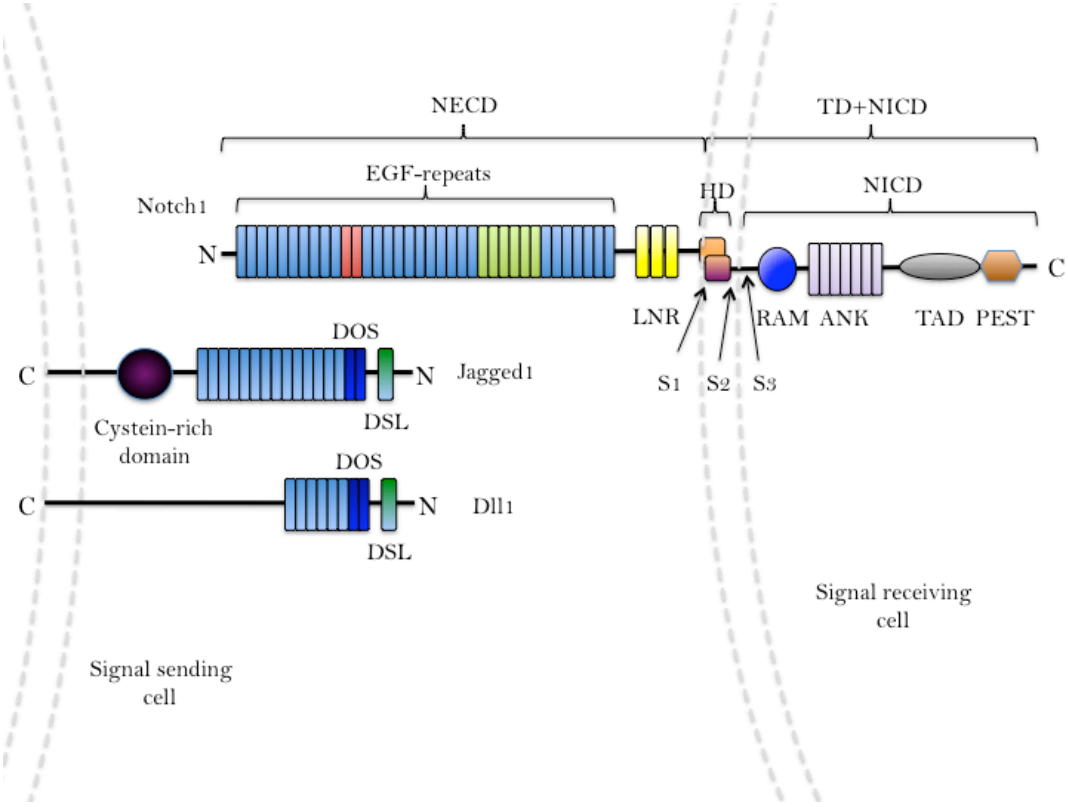

Figure 2: Structure of the Notch1 receptor and its ligands Jagged1 and Delta-like1 (Dll1). N1 – and 2 ECD contain 36 EGF-repeat of which 11-12 (red) and 24-29 (green) are important for ligand binding. The repeats are followed by the Lin12-Notch-repeats and the HD-domain. NICD contains a RAM-domain followed by a nuclear localisation signal (not shown), seven ANK-repeats, TAD and PEST-domains. In order for Notch to become activated three cleavages need to take place within the receptor at positions S1-3. Jagged1 and Dll1 as representative ligands in canonical Notch signalling. They both contain DSL (Delta/Serrate/Lag-2), DOS (Delta and OSM-11-like proteins) domains and a number of EGF-repeats. Jagged ligands also contain a conserved cystein-rich domain downstream of the EGF-repeats that possibly modulates the ligand/receptor interaction (modified from [4] and [7]).

in turn can be further subdivided into smaller structures (figure 2). The extracellular domain of the Notch proteins contains 29-36 EGF repeats that are involved in ligand binding (repeat 11-12 and 24-29, respectively, for Notch1) and signalling specificity through glycosylation of specific repeats [20, 21]. Notch1 and Notch2 both contain 36 repeats while Notch3 and Notch4 contain 34 and 29, respectively. Downstream of the extracellular repeats lies the LIN-12/Notch-related region (LNR), which together with the EGF-repeats prevents ligand-independent signalling [61, 62]. The NECD and Notch transmembrane-NICD are held together by strong noncovalent interactions between the N- and C-terminal halves of the heterodimerisation domain (HD) located C-terminal of the LNR [63, 64]. The NICD (the cytoplasmic domain of the Notch proteins) consists of a RAM (RBP-Jκ associated molecules) domain, ANK repeats flanked by nuclear localisation signals, TAD and a PEST region. NICD interacts with the DNA-binding repressor protein RBP-Jκ via its RAM domain and possible also via the ANK repeats [65, 66], but these are more importantly involved in formation of the NICD-transcriptional activation complex [67, 68]. The PEST domain is important for degradation of NICD and is, together with the HD domain, frequently mutated in human and murine T-cells neoplasms, leading to constitutive Notch signalling (reviewed in [69]).

Role of Notch signalling in the development of lymphocytes

Lymphocyte development

Lymphocytes are cells that mediate specific, inducible and generally long-lasting immune responses following an infection. They originate in the bone marrow from a common lymphoid progenitor (CLP), which have been derived from pluripotent haematopoetic stem cells (HSCs) via multipotent progenitor (MPPs) and lymphoid-primed multipotent progenitors (LMPPs) [70, 71]. The MPPs, and possibly LMPPs, can also differentiate into myeoloid progenitors, which give rise to karyocytes and granulocytes [72].

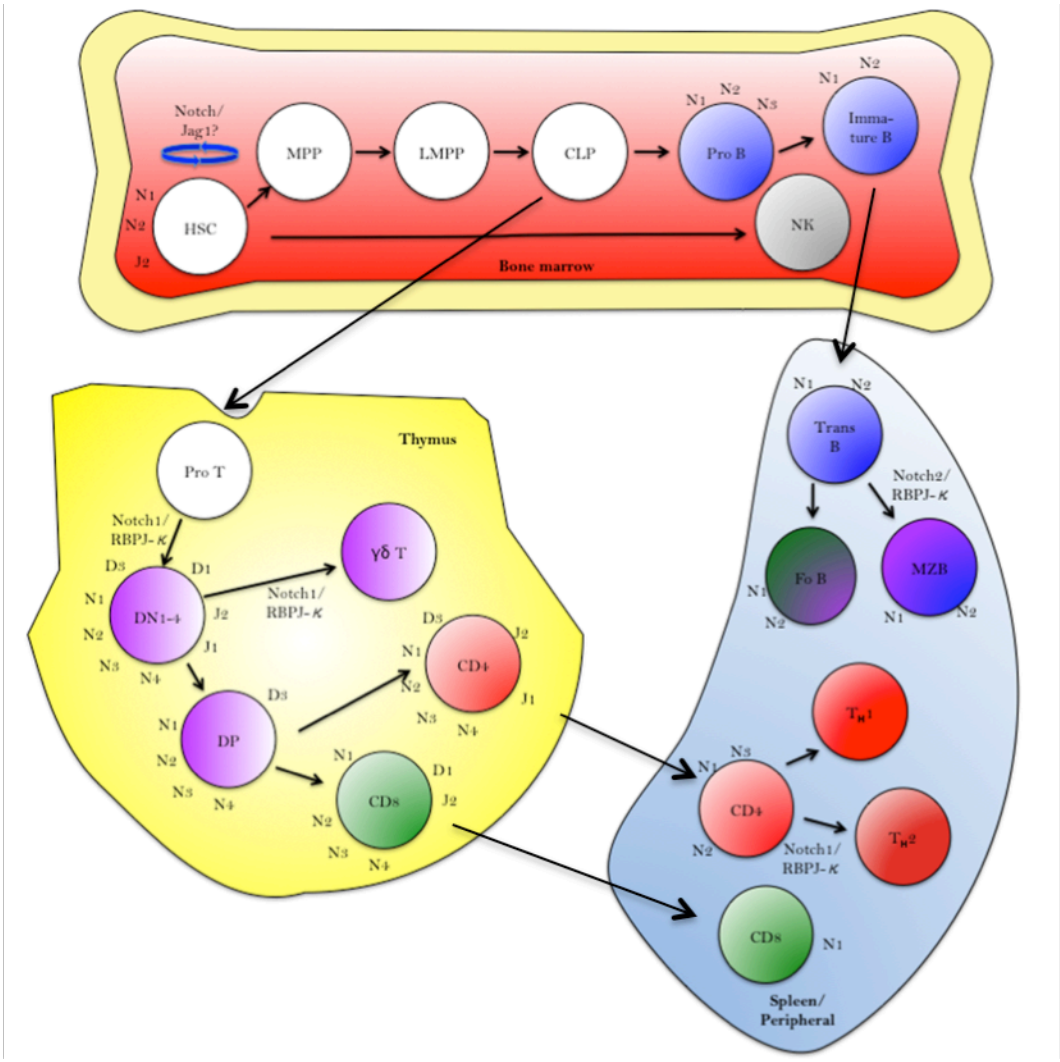

Figure 3: Notch expression in lymphocyte development. HSC, haematopoetic stem cells; MPP, multipotent progenitor; LMPP, lymphoid-primed MPP; CLP, common lymphoid progenitor; MZB, marginal zone B-cell; DN, double negative; DP, double positive; Fo B, follicular B-cell; T_H, T helper (modified from [3, 8] and [9]).

The lymphocyte differentiation is a strictly regulated process, where proteins like Ikaros and PU.1 play important roles in early development [73-75], GATA-3, Notch1 and Ikaros in the T-cell lineage development [76]; and Pax5, E2A, EBF and Notch2 in the B-cell lineage development [8, 77]. B-lymphocytes are primarily developed in the bone marrow and their main function is to produce antibodies while T-lymphocytes develop in the thymus and execute cell-mediated immune responses. Although the developmental paths are separated early during development, T- and B-cells share the common feature of using recombinase activating genes (*Rag1* and *2*) to recombine distinct genes and build clonally specific antigen receptors and antibodies, respectively. Upon commitment to the B-cell lineage pro-B and small pre-B cells start to rearrange their heavy immunoglobulin (IgH) chain genes into a pre-BCR, carried out by the variable-diversity-joining (V(D)J) recombination machinery. Signalling through the pre-BCR induces rearrangement of the light immunoglobulin (IgL) chain genes. Following the expression of a BCR, a few immature B-cells emigrates from the bone marrow and mature to long-lived B-cells that circulate the peripheral lymph organs (reviewed in [78]).

On the other hand, the maturation of T-cell lineage is characterised by the expression of several specific surface proteins including the TCR, that is also a product of V(D)J-recombination, and surface markers like CD3, CD4 and CD8. In the first stage, called double negative (DN) stage, the cells lack expression of TCR and several other surface markers. At the pre-T cell stage, the cells express the TCR-β, CD3 and pre-Tα. At the double positive (DP) stage of maturation, both CD4 and CD8 are expressed, which have the ability to recognise antigens encountered by the TCR. The mature TCR-α is expressed at the DP stage, thereby completing the TCR. Furthermore, TCR downstream signalling promotes the maturation of pro-T-cells into αβ and γδ, rather than NK- and T-cell lineages. One model suggests that the nature of the first successful TCR gene rearrangement dictates the lineage decision, although it cannot be excluded that events occurring earlier may at least partly control this process [79]. Before the mature T-lymphocytes leave the thymus, they differentiate into single positive (SP) $CD4^+$ or $CD8^+$ cells, respectively (reviewed in [78]).

Role of Notch in lymphocyte development

Notch signalling plays a major role in haematopoiesis and lymphocyte development and the Notch receptors and ligands are widely expressed in the haematopoetic system. Ligand-mediated Notch signalling has been suggested to participate in the forming of a putative stem cell niche [80] where it promotes HSC self-renewal [81-83], although Jagged1 may be of minor importance to this process [84]. This indicates that the different Notch

receptors and their specific ligand interactions may have distinct roles in HSC self-renewal and differentiation [8].

Accumulating evidence suggests that Notch1 plays a significant role in T-cell versus B-cell fate determination, and results by Radtke *et al.* implicate additional roles during further T-lineage differentiation and B-cell development (reviewed in [8] and [85]). Inhibition of RBP-J dependent Notch1 signalling completely blocks T-cell development and causes incremental development of B-lymphocytes in thymus [86-89] and vice versa, activation of Notch signalling increases the frequency of multipotent progenitor cells and drives T-cell differentiation in a dose dependent manner. High doses are required to increase the frequency of T-clones while a lower signal dose favours a NK-cell fate [90]. On the other hand, inactivation of *Notch2* does not affect T-cell development [91] indicating no redundancy for Notch1 and Notch2 in T:B lineage commitment. However, the Notch2 receptor is predominantly expressed in B-cells [91] and a Dll1/Notch2 interaction seems necessary for marginal zone B (MZB) cell differentiation [91, 92].

Notch signalling and T-lineage commitment is complex and Notch signalling may influence both αβ/γδ and CD4/CD8 T-lineage commitment. In the second lineage decision, where T-cells adopt to either αβ or γδ T-lymphocytes, Notch1 is important for β-selection. Mice with heterozygous loss of the *Notch1* locus (*Notch1*$^{+/-}$) present reduced proportion of αβ T-cells from the bone marrow progenitors [93] but just modestly affected number of γδ T-cells [94, 95]. However, the outcome of Notch signalling may be ligand-dependent [96] since *Jagged2* deficient mice showed a normal αβ T-lineage development, but produced a reduced number of γδ T-cells [97]. Furthermore, Notch signalling also plays an important role in peripheral T-cells, where Notch1, Notch2 and several of the Notch ligands are expressed [98-102]. Notch signalling has been linked to processes such as TCR-mediated T-cell activation as well as helper T-cell differentiation (reviewed in [3] and [8]) further establishing the importance of Notch signalling in lymphocyte development and function.

Notch signalling in the gastrointestinal tract

Cellular structure of the gastrointestinal tract

The gastrointestinal (GI) tract consists of the small intestine, further subdivided into duodenum, jejunum and ileum; and colon, and is continuously self-renewed with a turnover rate of two to seven days. The GI tract is a complex organ system, where specialised epithelial cells carry out functions such as absorption and secretion of mucus or digestive enzymes. A majority of the digested nutrients are absorbed in the small intestine via the

epithelium that is organised into finger-like villi and adjacent crypts of Lieberkühn, giving it its immense absorptive area. Colon contains crypt invaginations but mostly consists of a flat surface epithelium where water and salts are absorbed. In the small intestine, the crypt compartment contains the undifferentiated and partly differentiated stem cells while the villus is made up of differentiated cells. The pluripotent stem cells, which are hypothesised to be found close to the crypt bottom give rise to so called transit-amplifying stage, which are rapidly dividing into intermediate cells that differentiate into one of four cell types: enterocytes, goblet cells, enteroendocrine cells and Paneth cells (small intestine only) (reviewed by [103]).

Role of Notch in normal gut

The essential role of the canonical Wnt/Apc/β-catenin pathway in intestinal development and cell renewal is undisputed and strongly supported by numerous studies (reviewed in [104] and [105]). Furthermore, during recent years incremental results ([56, 106-108]) enlighten the importance of Notch signalling in the GI tract, and together the literature now supports an intimate and finely tuned crosstalk between both Wnt and Notch pathways which controls the proliferation and differentiation of intestinal cells (figure 4). Indeed, several Notch pathway components are expressed in the crypt compartments or adjacent structures; *Jagged1*, *Jagged2* and *Notch1*, *Notch2*, *Hes1*, *Rfng* have all been detected in the proliferating cells in the crypts while *Jagged1*, *Jagged2*, *Dll1*, *Notch1*, *Notch2*, *Notch3*, *Notch4*, *Hes1*, *Hes5*, *Hes6*, *Hes7*, *Mfng*, *Rfng and Lfng* are expressed in the villus, mesenchyme, endothelial cells and adjacent vasculature [56, 107, 109, 110]. Further, activation of canonical Notch signalling leads to upregulation of the *Hes/Hey* family of genes, including *Hes1*, which is a known repressor of the bHLH TF Hath1 (human homolog of mouse Math1 and *Drosophila* Atonal) [111]. Targeted deletion of *Math1* in the mouse intestine leads to depletion of the three secretory cell lineages (Goblet, Paneth and enteroendocrine cells) and gives a dominating enterocyte phenotype [112], whereas activation of Notch upregulates Hes1 and represses transcription of *Math1*, leading to an expansion of the population of proliferating intestinal progenitors [106]. This is in line with the results from *Hes1* or *RBP-Jκ* deleted mice where *Hes1*$^{-/-}$ mutants die from neurological abnormalities but analysis of the developing foetal intestine revealed increased proportion of mucus producing cells [113]. Within days from the conditional *RBP-Jκ* knockdown, increased levels of Math1 were detected followed by a dramatic change in crypt phenotype, where the transit-amplifying cells were completely converted into post-mitotic goblet cells [86, 108]. The results were verified with Notch signalling inhibition through γ-secretase inhibitor

treatment [108]. γ-secretase inhibitor treatment blocks all Notch signalling and later Riccio *et al.* [56] showed that knockdown of either *Notch1* or *Notch2* is not sufficient for a crypt progenitor cell differentiation into post-mitotic goblet cells, *i.e.* they have redundant roles in Notch signalling in the intestinal crypts making them essential for maintaining the crypts in an undifferentiated proliferative state. Taken together, the intestine is a complex organ system and the mechanisms controlling Notch expression in intestinal cells are poorly understood, but it is likely that a crosstalk between several of the conserved pathways is required to determine the cellular fate in the intestine.

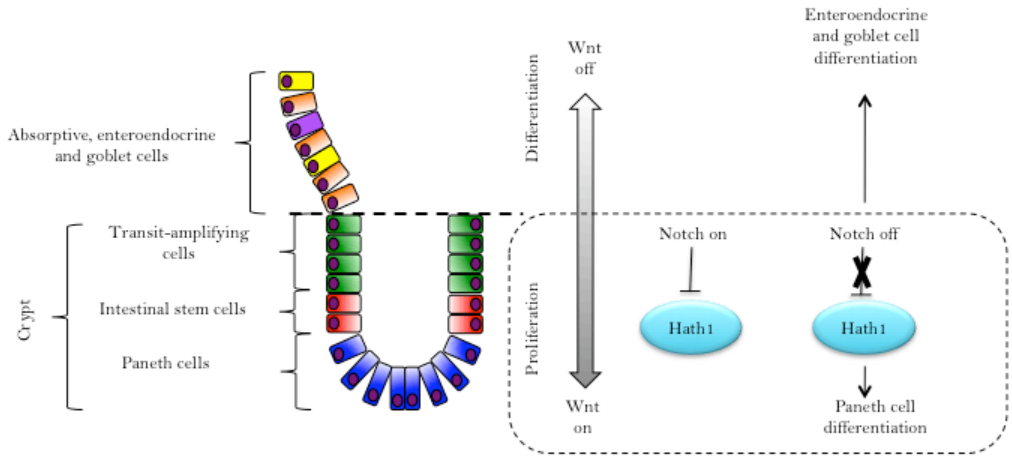

Figure 4: The role of Notch signalling in intestinal epithelial proliferation and differentiation. Active Notch and Wnt signalling keep the crypt cells in a proliferative state. Inactivation of Notch signalling results in upregulation of Hath1 and renders in a secreatory cell fate (for details, see text) (modified from [2]).

The role of Notch in carcinogenesis

Notch signalling in malignant disorders is highly complicated, often dependent on signal dose and the cellular context. While Notch signalling is crucially involved in normal regulation of cell differentiation, proliferation, development, dysregulation of the signalling cascade often has profound effect on the cellular fate and may lead to tumour formation. In cancer, Notch signalling can function both in an ongogenic as well as in a tumour suppressive manner and the outcome seems dependent on its normal function in a given tissue. Generally, Notch may act as an oncogene in tissues where it is involved in stem cell self-renewal or in cell fate decisions and may have a tumour suppressive role in tissues where Notch promotes terminal differentiation [114-117]. The human *Notch1* gene was first identified through its involvement in a t(7:9)(q34;q34.3) chromosomal translocation found in approximately 1% of T-cell acute lymphoblastic leukemia/lymphoma (T-ALL), an

aggressive neoplasm of immature T-cells [118]. Later on, activating mutations in the HD and PEST domains were discovered in 55-60% of human T-ALLs [119], indicating a broader role for Notch1 in cancer formation. An oncogenic role for Notch signalling has also been discovered in Hodgkin's lymphoma (HL), anaplastic large-cell non-Hodgin's lymphoma (NHL), some acute myeloid leukemias, B-cell chronic lymphoid leukemias as well as several epithelial malignancies of the breast, cervix, lung, colon, prostate, head and neck, kidney, pancreas, as well as gliomas, medulloblastomas, and sarcomas [115, 116, 120-122]. However, several of the mechanisms underlying the deregulation in these malignancies are unclear but altered expression of the Notch receptors or other Notch signalling pathway components are often associated with poor prognosis or tumour metastasis [123]. Overactive Notch signalling in solid malignancies may lead to overexpression of genes important for proliferation *e.g CDK2* and *cyclin D1* and repression of CDK inhibitors $p27^{Kip1}$ and $p57^{Kip2}$ [56, 124]. Increased Notch signalling may also lead to increased expression of antiapoptotic genes like *Bcl2* [125], as well as increased signalling through both the PI3K [126] and NFκB pathways [127]. Furthermore, the typical proto-oncogenes *Ras* and *c-myc* are linked to Notch signalling in tumourigenesis. Notch may function as a downstream target of Ras and in a positive feed-back loop act as an activator of Ras signalling [128]. *c-myc* is directly regulated by Notch1 in T-ALLs [129-131], further promoting cell growth and proliferation upon Notch1 overactivation. It is therefore not unlikely that a similar mechanism is important for promoting tumourigenesis in solid malignancies. By contrast, in a few tumour types, including human hepatocellular carcinoma, skin and small lung cancer, expression of *Notch1*, *Notch2*, *Jagged1* and *Hes1* are reduced [132, 133] and speculatively activated Notch signalling may function in a tumour suppressive manner [116, 120, 121]. In mouse skin, Notch signalling is hypothesised to block proliferation through increasing $p21^{Cip1}$ expression and repressing β-catenin mediated Wnt signalling [117].

Another aspect of Notch signalling in carcinogenesis is its role in tumour angiogenesis, one of the hallmarks of cancer, and a prerequisite for tumour cell invasion and metastasis [134]. Sustained angiogenesis is crucial for tumour growth and Notch1 as well as the ligand Dll4 have been shown to interact with vascular endothelial growth factor (VEGF) and Hif-1α (reviewed in [116]) which are key controllers of both normal and tumour related angiogenesis. Furthermore, Notch signalling seems important for stabilisation of the vasculature, especially in arteries and microvasculature where Notch receptor and ligand expression is significant (reviewed in [50] and [135]).

Role of Notch in lymphoma

Lymphomas are tumours originating from the lymphocytes, and can broadly be divided into HL and NHL. NHL may be further classified into a number of lymphomas based on cell type and/or differential stage but a common subclassification is B- or T/NK-cell neoplasms. T-cell malignancies account for less than 10% of the lymphoid neoplasms and the reason for this imbalance is unclear. In 2002, there were roughly 300.000 new cases of NHL (2.8% of all cancers) and about 30.000 HLs [136].

Genomic translocations are common features of lymphoma and other blood malignancies (reviewed in [137]). For example, translocation between chromosome 8 and 14 is commonly found in patients with Burkitt's lymphoma. The translocation involves the *Myc* gene on chromosome 8 and *IgH* on chromosome 14 [138, 139], leading to a constitutive expression of a normal Myc protein, as the *IgH* is actively transcribed in B-cells [140]. This drives B-cell proliferation by overactivating genes involved in cell cycle control *e.g. cyclin D1* and suppression of genes involved in growth arrest [141]. However, in most chromosomal translocations associated with lymphoid malignancies, the involved genes rearrange to form a fusion protein. A well-known example is the Philadelphia chromosome, which is the result of a 9;22 chromosomal translocation involving the *Abl* gene on chromosome 9 and the *BCR* gene on chromosome 22 [142, 143].

In haematopoetic malignancies, genetic alterations such as chromosomal translocations, chromosomal amplifications, and point mutations in the Notch receptors, are all common mechanisms for constitutive Notch activation [118, 119, 144]. As mentioned, Notch1 was first discovered from its involvement in the t(7;9) chromosomal translocation in T-ALL patients. The translocation results in a deletion of most of the N1ECD, and putting the HD and N1ICD under transcriptional control of the TCRβ locus, leading to a ligand-independent, constitutive release of N1ICD [118]. The *Notch1* gene contains sequences similar to the recombination signalling sequences (RSS) found in the *TCR* encoding genes, and the translocations are most likely therefore a result of abnormal V(D)J-recombination [145].

Notch1 is a typical proto-oncogene in T-cells and mutational activation of the gene is common in both human and murine T-cell malignancies. Mutations are commonly localised in the HD and PEST domains [5, 119, 146, 147, 148-152], but deletion of parts of the ligand binding region, due to aberrant V(D)J-recombination has also been detected in mouse lymphoma [62]. The deletion following the recombination creates a cryptic transcription start site (TSS) halfway through the gene, leading to a protein that lacks most of its EC domain and thereby ligand-independent release of N1CD [153]. Furthermore, mutations in

CDC4, where arginine residues in the target binding region is commonly altered in T-ALL, also leads to stabilisation of NICD [154, 155]. In addition, overexpression of wild type *Notch1* is frequently observed in T-cell-derived anaplastic large cell lymphoma (ALCL) together with its ligand Jagged1 [156]. The reason for this overexpression is unknown but genomic amplification of 9q34, encompassing *Notch1* and *c-Abl*, is a frequent genomic aberration in enteropathy-type T-cell lymphoma (ETL) [157-159] and could possibly explain Notch1 upregulation in other neoplasms as well.

Even though B-cell malignancies are more common than T-cell malignancies, the role of Notch signalling in the development of B-cell lymphoma is poorly understood. Due to its involvement in MZB generation, it is possible that Notch2 plays a more significant role in B-cell lymphomas than in T-cell neoplasms In line with this, gain-of-function mutations have been discovered in diffuse large B-cell lymphoma (DLBCL) [160]. Notch signalling also seems overactivated in B-cell derived HL [156] and B-chronic lymphocytic leukemia (B-CLL) [161], further indicating an oncogenic role of Notch in these malignancies. However, the results are controversial and Notch activity has also been suggested to have suppressive effect in B-cells (reviewed in [162]).

Role of Notch in CRC

CRC is one of the most common malignancies world wide with about one million new cases in the year 2002 [163, 164]. It is predominantly a disease of industrialised countries and epidemiological studies suggest several risk factors connected to the Western lifestyle, *e.g.* high intake of fat, red meat, alcohol and cigarette smoking [164, 165].

About 10-20% of the CRC cases arise in families, which carry highly penetrant mutations in single genes, giving rise to hereditary syndromes like familial adenomatous polyposis (FAP) or hereditary non-polyposis colorectal cancer (HNPCC) [166]. Concerning both hereditary and sporadic cancers of colorectum, it has been suggested that in total 4-6 genetic defects of tumour suppressor genes and/or proto-oncogenes are required during the development of the neoplasm [167]. In 1990, Fearon and Vogelstein [167] proposed this as the adenoma-carcinoma sequence. CRC springs from epithelial cells in the large bowel and rectum and the first "hit" in the sequence is often inactivation of the tumour suppressor gene *Apc* (adenomatous polyposis coli) [168, 169], which leads to the formation of benign polyps in the epithelium. These polyps can in turn acquire more genetic defects and end up in the formation of malign tumours.

In CRC, and especially in colorectal adenomas, several Notch pathway components are overexpressed [107, 170] indicating increased Notch signalling in the development of

colorectal malignancies. The reasons for this overactivation in CRC are poorly elucidated, although mutations in human CDC4 (*hCDC4*), leading to stabilisation of NICD, have been observed in a minority of hereditary and sporadic CRCs [171, 172]. Upregulated Notch signalling in colon adenocarcinomas leads to increased expression of Hes1 and thereby repression of Hath1 levels [111]. Hath1 is important for the terminal differentiation, including goblet-cell differentiation, of the intestinal epithelium and downregulation leads to increased proliferation and contributes to tumourigenesis [111]. Although goblet cells normally constitute the major secretory cell lineage in the intestinal tract, only a few goblet cells are present in intestinal adenomas [173-176]. Inhibition of Notch signalling through γ-secretase inhibitor treatment does not only have profound effects on proliferative crypt cells [108] but also significantly reduces the number of intestinal adenomas in $Apc^{Min/+}$ mice. The treatment turns the proliferative cells to post-mitotic goblet cells [177] and increases Math1 induction [108], making the Notch pathway a possible therapeutic target in CRC.
Furthermore, mutational inactivation of *Apc* renders active canonical Wnt signalling through stabilisation and nuclear translocation of β-catenin (further described in the next section), which together with members of the Lef/Tcf (Lymphoid enhancer factor/T-cell factor) family activates downstream target genes [178]. Very recently, Rodilla *et al.* [179] discovered *Jagged1* as a direct transcriptional target of canonical Wnt signalling. High levels of Jagged1 correlates with high levels of activated Notch1 and -2 [179], implicating that active Notch signalling plays a significant role in development of Apc deficient tumours. However, to further increase the complexity of Notch signalling in CRC, *Notch2* has been hypothesised to function as a tumour suppressor in this context, since the mRNA and protein levels have been found to be decreased in CRC compared to adjacent mucosa and that increased Notch2 levels have been correlated with colon cancer cell differentiation [180]. Notch signalling also interacts with other pathways or proteins important for colorectal carcinogenesis like Hedgehog [181-184], Ras [128], p53 [185], c-myc [54, 129, 131] and NFκB [186].

Molecular interactions between the Notch and Wnt signalling pathways

Processes important for embryonic development such as stem cell self-renewal, proliferation, differentiation, migration and cell death are regulated by a few but highly conserved signalling pathways, including Notch, Wnt, JAK/STAT, Transforming Growth Factor (TGF)-β, PI3K, Ras and Sonic Hedgehog (SH); and an imbalance in the network can result

in pathological conditions, *e.g.* congenital heart diseases or cancer (reviewed in [178, 187-191] and [192]).

The canonical Wnt signalling pathway is a critical regulator of embryonic development, and the expression of pathway components in progenitor cells of the growth zone and proliferating tissues, implicates that Wnt signalling is important for the body plan in vertebrates and several invertebrates [193-199]. Until now, about 20 Wnt signalling proteins have been identified in human [200], initiating Wnt signalling upon their binding to a receptor complex consisting of proteins from the Frizzled family and a member of the LDL receptor family, Lrp5/6. This activates the proteins from the Dishevelled family, which inhibits the axin/GSK-3/Apc destruction complex. This complex regulates the stability of the cytoplasmic protein β-catenin by phosphorylation and ubiquitination thereby targeting it for proteasomal degradation [201]. Active Wnt signalling renders in stabilised β-catenin, which translocates to the nucleus and turns transcriptional repressor proteins, belonging to the Lef/Tcf family, into transcriptional activators [105]. These proteins bind the DNA consensus sequence *5'*-(A/T)(A/T)CAA(A/T)G-*3'* [202] and activates canonical Wnt target genes. However, there is a great diversity in Wnt signalling due to the existence of a β-catenin-independent signalling [203].

Notch and Wnt signalling, and their interactions, are intimately linked to cell fate decisions, proliferation and differentiation [189, 204], and a skewed crosstalk may results in severe developmental or medical conditions. Wnt signalling is often placed upstream Notch signalling and may activate the expression of Notch ligands [111, 170, 179, 205-210] but an opposite mechanism has also been proposed where Notch signalling negatively regulates the canonical Wnt pathway through upregulation of known Wnt pathway inhibitors or by promoting degradation of β-catenin [117, 211-214]. Furthermore, NICD may also directly interact with β-catenin [215-217] or function as a co-activator for Lef1 [218] leading to increased transcription of Notch- or Wnt target genes.

The impact of these mechanisms in carcinogenesis is not well-known, but hypothetically Wnt and Notch related signals may either act synergistically or counteractive depending on the local of the neoplasm, cell and tissue context, as well as the the genetic profile of the respective tumour. In the skin, Wnt signalling promotes stem cell renewal and proliferation and has clearly oncogenic properties [105] while Notch1 functions as a tumour suppressor and promotes differentiation [117]. In haematopoesis and thymocyte development their functions are similar to that in skin. However, gain-of-function mutations in both pathways lead to cancer, indicating that overactivation of both Wnt and Notch pathways could be of importance for proliferation. In colonic epithelium, the pathways separately promote

differentiation but stem cell renewal and proliferation is thought to be a result of mutual activation of the two pathways [170, 188]. There are implications that a crosstalk between the two pathways also could be of importance in breast cancer [206] and hepatocellular carcinoma [219]. It is likely that these pathways and the networks they form will play a pivotal role in several cancer forms where their role remains to be discovered.

Aims

The overall aim of this licentiate thesis was to investigate the role and regulation of genes related to the Notch signalling pathway in carcinogenesis. The specific aims were:

I. To study genetic alterations in *Notch1* and *CDC4*, and to establish their role in the development of chemically induced murine T-cell lymphoma (*paper I*).

II. To identify potential genetic alterations in *Notch1* and *CDC4*, and elucidate their role in sporadic CRC.

III. To investigate the transcriptional interactions between the Notch and Wnt signalling pathways in CRC cells (*paper II*).

Materials and methods

Tissue specimen and cell lines

Murine T-Cell lymphomas

In *paper I*, we studied chemically induced lymphoma from three different mouse strains. Tumour induction was performed at the National Institute of Environmental Health Sciences, Triangle Park, North Carolina, USA, by using the chemicals phenolphthalein, 1,3-butadiene or 2',3'-dideoxycytidine, which are all known to induce lymphoma in mice [220-222]. Phenolphtalein has been used as an ingredient in laxatives and has also been found to be a carcinogen in animal models [223], whereas butadiene is a gas extensively used in the plastic industry [224] and dideoxycytidine is a drug that has been approved for treatment of HIV-positive patients [225]. The mouse strains that were exposed to the carcinogens were C57Bl/6×C3H/Hej F_1 mice, NIH Swiss mice and heterozygous *p53*-deficient C57Bl/6 (TSG-p53^{TM}) mice. A total of 104 lymphomas were received from the carcinogen exposure groups; 31 butadiene-induced lymphomas (BLF) and 16 dideoxycytidine-induced lymphomas in C57Bl/6×C3H/Hej F_1 mice (DLF); 47 dideoxycytidine-induced lymphomas in NIH Swiss mice (DLS); and 10 phenolphtalein-induced lymphomas in TSG-p53^{TM} mice (PL). All lymphomas were of T-cell origin, and collected mainly from thymus and spleen (for details, see [226]).

Murine intestinal adenomas

In 1990, Moser *et al.* [227] identified a mouse carrying multiple intestinal neoplasias (Min) in a colony of animals treated with the mutagen ethylnitrosurea (ENU). Two years later, the responsible genetic event was identified as a germline truncating mutation at codon 850 in one of the *Apc* alleles and the mutant was named Apc$^{Min/+}$ [228]. Ever since, the Apc$^{Min/+}$ mouse has become one of the most widely used animal models for studies on intestinal cancer. Mice with a homozygous mutation die *in utero* while heterozygous mice are born normally. Due to a 'second-hit' in the other *Apc* allele they develop small intestinal polyps. In the current thesis, DNA from intestinal adenomas from the Apc$^{Min/+}$ mice on the C57Bl/6 background was isolated and screened for mutations in 'hot-spot regions' of the *Notch1* gene. All animal experiments were approved by the Animal Care and Use Committee at Linköping University.

Human material

Colorectal tumour biopsies and paired intestinal mucosal biopsies taken approximately 10 cm from the tumour were collected from 46 patients at the County Hospital Ryhov, Jönköping and Linköping University Hospital, Linköping, Sweden. Clinical information regarding age and gender of the patient, tumour localisation and staging as well as pathological growth pattern were obtained from clinical records. Tissue samples were immediately snap frozen and stored at -80ºC until further handling, including isolation of protein, RNA and DNA. All studies involving human material were approved by the Research Ethics Committee at Linköping University.

Cell lines and cell cultivation

Human colon cancer cell lines HT29 and HCT116 were employed for studying the regulation of the Notch pathway *in vitro*. HT29 cells lack the expression of full length Apc, in contrast to HCT116 cells [229], which express full length Apc naturally but instead have activating mutations in the β-catenin gene (*CTNNB1*). Generally, the cells were cultivated in McCoy´s 5A media supplemented with 10% foetal bovine serum at 37ºC in 5% CO_2. Prior to the experiments, the cells were split with 0.05% Trypsin-EDTA. In *paper II*, HT29 cells harbouring a vector, with a metallothionin driven promoter coupled to the wild type *Apc* gene, were kindly provided by Dr. Bert Vogelstein [230]. Stimulation of these cells with zinc lead to the expression of full length Apc and β-catenin degradation. On the other hand, β-catenin was silenced in naïve HT29 cells and Notch pathway gene expression could further be studied. HT29 and HCT116 were also used for luciferase assays and DNA binding experiments.

Experimental procedures

Semi-quantitative Reverse Transcriptase (RT)-PCR

The invention of the polymerase chain reaction (PCR) in the middle of the 1980s was a huge step forward for the molecular biology research field and since then, several new research and clinical applications have been developed [231]. Semi-quantitative Reversed Transcriptase (RT)-PCR is a somewhat crude but fast and simple technique for studying gene expression. First, a RT is used for the production of complementary DNA (cDNA) copies from isolated mRNA. Subsequently, the cDNA product is subjected to PCR amplification using exon specific primers. The resulting PCR product is visualised on an agarose gel containing ethidium bromide or a dye for staining. From the band intensity, gene expression, relative to a house-keeping gene, can be calculated. A disadvantage with

this technique, compared to more modern real time PCR applications, is that it requires extensive optimisation to give reliable results. Optimisation is necessary since the PCR needs to be terminated and analysed in the linear amplification phase, which is not necessary in real time PCR experiments where the amount of PCR product is measured in real time for every cycle of the reaction. It is also less sensitive and results could be difficult to interpret. However, semi-quantitative RT-PCR is cheaper and may be sensitive enough to compare gene expression between two different samples. In *paper II*, semi-quantitative RT-PCR were used to compare the expression of genes in the Notch signalling pathway in a time-dependent manner upon alteration of the Wnt signalling pathway, through activation of full-length Apc or siRNA knockdown of β-catenin.

Mutation analysis

Paper I and parts of the *unpublished data* are based on mutational analysis of 'hot-spot' regions in the *Notch1* gene; exon 26, 27 (HD-domain) and 34 (TAD and PEST-domains). Since the publications by Orita *et al.* 1989 [232, 233] single stranded conformation analysis (SSCA) has become widely used as a rapid and sensitive method for the detection of DNA point mutations. Most often, PCR is used to amplify a DNA fragment of interest. In a secondary reaction, the fragments are radiolabelled, denatured by heat and electrophoretically separated on a gel, usually of poly-acrylamide, under non-denaturing conditions. The denatured single-stranded DNA will form specific secondary structures, dependent on the sequence *i.e.* presence of mutation or not. The secondary structures will yield distinct migration patterns on the gel making it possible to detect a mutation with as little as one nucleotide difference in a 300 bp fragment. Final detection and interpretation is performed upon the exposure of the gels to radiosensitive films.

PCR/DNA sequencing were employed in *paper I* in order to detect larger deletions in *Notch1* caused by the V(D)J recombination machinery, for which the approximate location of the expected deletions were known. PCR with Phusion™ high fidelity DNA polymerase, followed by DNA sequencing was used to pinpoint the exact breakpoint. Tumours with deletions yielded a ~300 bp PCR product while tumours with deletions yielded a ~16 kbp product.

Bioinformatics

Bioinformatics is the application of information technology to the field of molecular biology and now entails the creation and advancement of databases, algorithms, computational and statistical techniques, and theory to solve problems arising from biological data. The location of nucleotide patterns in sequence data analysis *i.e.* the identification and analysis of

gene promoters and the regulatory sequences within is one of the most widely used applications. In *paper II*, identification of potential promoter regions in Notch and Wnt pathway genes as well as the analysis of putative TF binding sequences were performed with Genomatix software (http://www.genomatix.de, Genomatix Software GmbH, Munich, Germany). The Gene2Promoter software was used to retrieve and identify promoters while the MatInspector [234] software was utilised for determination of putative TF binding sites. MatInspector utilises a large library of matrix descriptions for TF binding sites to locate matches in a DNA sequence.

RNAi

RNA interference (RNAi) is a gene silencing mechanism initiated by short double stranded RNA molecules in a cell's cytoplasm leading to degradation of the corresponding mRNAs. RNAi was first observed in transgenic plants in the middle of the 1980s [235] but the molecular mechanisms remained unknown. The term RNAi was first used in 1998 by Andrew Fire and co-workers [236] and the discovery resulted in a Nobel Prize in 2006. Today, RNAi is not only known as a natural process by which cells regulate gene expression, but also as a quick and robust method for gene silencing in molecular biology research [237]. Upon introduction of short double stranded RNA oligonucleotides, complementary host mRNA is cleaved by endonucleases in a cytoplasmic process controlled by the RNA induced silencing complex (RISC) [238]. RNAi may not completely suppress gene expression and unless expressed by a stably transfected vector the effects will diminish over time.

In *paper II*, RNAi was performed *in vitro* by transfecting the HT29 colon cancer cell line with small inhibitory RNA oligonucleotides (*siRNA*) against β-catenin. The attenuated expression of the targets was confirmed at protein level using Western blot, and through expression analysis of the downstream target gene *cyclin D1*.

Western blot

Western blot was first described in the late 1970s [239, 240, 241], and has become one of the most widley used techniques for detection and quantification of specific proteins, both from tissue and cell extracts. It utilises the ability to separate native or denatured proteins by the length of the polypeptide (denaturing conditions), 3-D structure of the protein (native conditions), isoelectric point, electric charge or a combination of these in gel electrophoresis. Following transfer to a nitrocellulose or PVDF membrane, the target protein is detected with specific antibodies. In *paper II*, denatured protein lysates from HT29 colon cancer cell line were seperated on polyacrylamide gels. Electroblotting to PVDF membranes was then

performed, with subsequent incubation in primary and secondary antibody solutions, respectively. Secondary antibodies were linked to horseradish peroxidase (HRP), enabling subsequent detection with enhanced chemiluminiscence and exposure to a digital camera.

DNA-binding assays

Electrophoretic Mobility-Shift Assay (EMSA), also known as bandshift assay, is a technique used to study protein-nucleic acid interactions *in vitro* [242]. The assay is commonly used to study TF binding to gene promoter regions. The assay utilises electrophoretic separation of the DNA- or RNA-protein mixture on a polyacrylamide- or agarose gel where an applied voltage separates the complexes according to size, charge and, to some extent, shape. Thus, a DNA strand bound to a protein will migrate more slowly through the gel than the unbound DNA fragment alone. Commonly, an antibody, that specifically recognises the bound protein, is added to the mixture, creating an even larger complex that migrates even slower through the gel. This approach is referred to as a supershift assay, and is used to unambiguously identify a protein present in the protein-nucleic acid complex. For visualisation purposes, the nucleic acid fragment is usually labelled with radioactive, fluorescent or biotin-streptavidin labelling and exposed to radio- or lightsensitive films. However, there are limitations with EMSA as a technique for studying DNA-protein interactions as there are several factors affecting the binding, including chromatin structure, that will not be accounted for *in vitro*.

Chromatin immunoprecipitation (ChIP) was discovered independently by two groups during the 1980s [243-246] and is a method for analysing protein-DNA-interactions *in vivo*. In principle, DNA-binding proteins in living cells are reversibly cross-linked to the DNA, usually with formaldehyde. Following the crosslinking, cells are lysed, the DNA, protected by DNA-binding factors, is sonicated into 0.2-1 kb fragments and the protein-DNA complexes are immunoprecipitated with a protein specific antibody. Afterwards, the cross-links are reversed with heat and the identity and quantity of the isolated DNA fragments can be determined by PCR.

In *paper II*, a variant of competitive EMSA together with ChIP was employed to study Lef1/Tcf4-DNA interactions in Notch pathway gene promoters with special emphasis on *Notch-2*. In the EMSA experiments, double stranded hybridisation of radioactive labelled gene specific probes was poor and instead the LEF1/TCF binding probe CD1TOP, containing two copies of the binding site 5'-CCTTTGATC-3' [247], was end-labelled using [γ-^{32}P] ATP. An excess of double-stranded cold gene specific- or a cold mutated consensus

oligonucleotide competed for binding of the DNA binding factor Lef1, which was *in vitro* translated from a full-length *Lef1* expression vector (kind gift from professor B.O Williams). A weaker CD1TOP/Lef-1 band on the visualised gel indicated specific competition of the cold probe. ChIP was used to study a potential interaction of Tcf4 with the Notch2 promoter in HT29 and HCT116 colon cancer cells.

Luciferase reporter assays

Luciferase is the generic term for the class of oxidative enzymes used in bioluminiscens. Naturally occurring luciferase enzymes are produced by a variety of species but the best studied, and also most commonly used in biomedicine, are found in fireflies [248] and the sea pansy *Renilla reniformis* [249]. The *luciferase* genes were cloned in the middle of the 1980s and early 1990s even though the proteins were purified and characterised 20-30 years earlier (reviewed in [250]). The property that all luciferases have in common is the ability to emit light upon the oxidation of their substrates, *i.e.* luciferins. In biomedical studies, luciferase activity is often used as a reporter to assess the transcriptional activity in cells that have been transfected with a vector containing the luciferase gene under the control of a promoter construct of interest [251]. In *paper II*, HT29 and HCT116 colon cancer cells were transfected with a TATA-box containing firefly luciferase reporter vector [252] with constructs covering three different regions of the *Notch2* promoter, each containing a putative LEF1/TCF site. To characterise whether any part of the promoter region were influenced by the current *Apc* status, the luciferase activity was assessed in cells with and without the induction of full-length Apc and normalised to the activity of the β-galactosidase control vector. Furthermore, to investigate the influence of β-catenin on the *Notch2* promoter, constructs were co-transfected with a vector expressing mutated β-catenin (kind gift from professor Avri Ben-Ze'ev (via professor Anita Sjölander)) and relative luciferase activity was measured.

Results

Paper I

In *paper I*, we analysed chemically induced mouse lymphomas for mutations in the *Notch1* and *CDC4* genes. Exon 1b-2 of the *Notch1* gene, which encodes the ligand-binding domain, were analysed by PCR and MegaBACE™ sequencing, while the HD, encoded by exons 26-27, and PEST, encoded by exon 34, domains as well as exons 8 and 9 of *CDC4* were analysed by SSCA and MegaBACE™ sequencing.

N-terminal deletion of exons 1b and 2, and the large intronic sequences surrounding these exons were detected in 16 of 103 tumours. Fifteen of these tumours also displayed small insertions of 1-8 nucleotides. We also identified point mutations in the *Notch1* HD domain. In total, eight of 103 tumours carried mutations of which five displayed the same point mutation in codon 1668 altering a leucine to a proline. We also found mutations in codon 1690 in two of the tumours. The Ala1690Pro was a novel mutation whereas Ala1690Asp had been previously described [146]. The last HD mutated tumour contained an insertion of 66 bp, of which 64 bp was a duplication of nucleotides 5071-5134 in the mRNA sequence. The alteration resulted in 22 extra amino acids in the HD domain.

Of the investigated *Notch1* regions the PEST domain was the most frequently mutated with genetic alterations in 29 of the 103 tumours. All the alterations (insertions, deletions and duplications) in exon 34 altered the reading frame and thereby deleting the WSSSSP amino acid sequence, located at residues 2495-2500 within the PEST domain. Deletion of this domain results in an accumulation of truncated NICD since the sequence targeting NICD for proteasomal degradation is missing [253]. All the mutations in exon 34 were identified between residues 2326 and 2494, thereby eliminating the WSSSSP sequence but keeping domains important for co-activator binding and transcriptional activation intact.

Several tumours also contained mutations in more than one region of the *Notch1* gene. Eight samples were found to have both deletions in the ligand-binding region and truncations in the PEST domain, five tumours had point mutations in the HD domain as well as altered exon 34 and four showed mutations in both alleles of exon 34.

In total, 40 of 103 (39%) chemically induced mouse lymphomas displayed *Notch1* mutations whereas no mutations were detected in exon 8 and 9 of the *CDC4* gene indicating that *Notch1* but not *CDC4* is a frequent target gene for mutations in chemically induced lymphomas (figure 5). However, PL displayed a relatively low frequency of *Notch1* mutations compared to DLS and DLF (52% and 44%, respectively), indicating that the

pattern of *Notch* mutations may differ depending on genetic background and mutational status of the *p53* tumour suppressor gene.

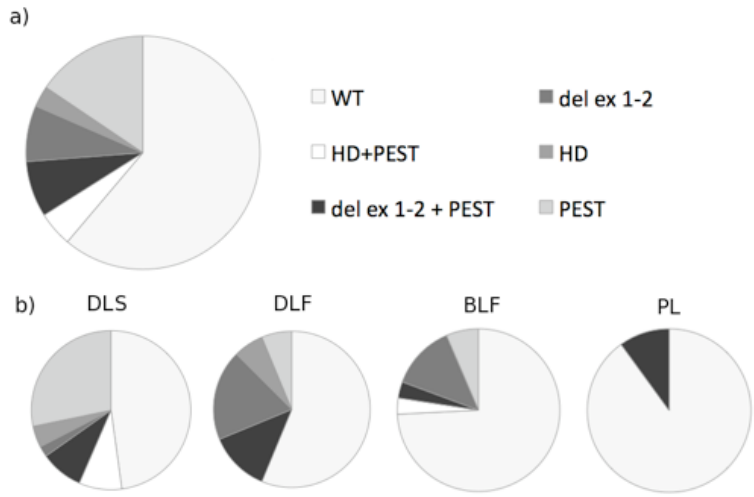

Figure 5: (*a*) Distribution of *Notch1* mutations in 103 chemically induced mice lymphomas. Overall, mutations were found in 39% of the cases. Mutations in the PEST domain were found in 28% of the cases, of which 8% also carried deletions in exon 1b and 2 and 5% had mutations in the HD domain. Mice with mutations only in HD or deletions in exon 1b and 2 were found in 3% and 8%, respectively. (*b*) The relative distributions of *Notch1* mutations in the different chemically induced tumours are as follows: 24/46 (52%) DLS (dideoxycytidine-induced lymphoma in NIH Swiss mice), 7/16 (44%) DLF (dideoxycytidine-induced lymphoma in C57Bl/6×C3H/Hej F_1 mice), 8/31 (26%) BLF (butadiene-induced lymphoma C57Bl/6×C3H/Hej F_1 mice) and 1/10 (10%) PL (phenolphtalein-induced lymphoma in TSG-p53^{TM} mice) were mutated [5].

Screening for Notch1 and CDC4 mutations in CRC (unpublished data)

In several solid malignancies like breast, cervical, lung, colon, prostate, head and neck, renal and pancreatic carcinomas, melanomas as well as in gliomas, medulloblastomas, and sarcomas [116, 120-122], Notch signalling is overactivated and seems to function oncogenically. However, the reason for this overactivation in these cancers is not known, although speculatively it may be connected to gain-of-function mutations in Notch related genes, or loss-of-function mutations in genes negatively regulating Notch signalling. In the present study, we therefore analysed both murine and human intestinal tumours with regard to mutations of the *Notch* and *CDC4* encoding genes.

Employing PCR, SSCA and MegaBACE™ sequencing, 32 intestinal tumours from $Apc^{+/-}$ mice and 32 human CRC tumours were analysed regarding mutations in the *Notch1* 'hot-spot' regions of the HD and PEST domains while 46 human CRC tumours were investigated for mutations in exons 2-10 of the *CDC4* gene. Surprisingly, no mutations in *Notch1* were detected in neither murine nor human tumours, which has been confirmed in another study [123]. Previously, mutations in *hCDC4* gene have been described in both hereditary and sporadic CRC as well as adenomas [171, 172], which we could not confirm in our material. The reasons for the conflicting data remain obscure, but potentially it may reflect different study populations with different genetic backgrounds or life styles. In conclusion, our data suggest that neither mutational inactivation of *hCDC4* or activating mutations of *Notch1* are common mechanisms for overactive Notch signalling in CRC, indicating that other upstream events play more decisive roles for Notch dysregulation in this context. While little is known about the transcriptional regulation of the Notch pathway genes or the mechanisms leading to the upregulation, it has been suggested that Notch signalling interacts with several other important pathways and cell signalling mediators that are often deregulated in malignant diseases [127, 129-131, 178, 187-192]. Thus, aberrant Notch signalling may partly be explained by these potential interactions.

Paper II

In *paper II*, we investigated the transcriptional interactions between the canonical Notch and Wnt signalling pathways at the level of transcription in HT29 and HCT116 CRC cell lines. Sixty-five genes, known to be tightly involved in Notch and Wnt signalling, were analysed and their promoter sequences were extracted using Genomatix software Gene2Promoter followed by identification of putative TF consensus sites with MatInspector. Twenty-five of the investigated gene promoters in the Notch pathways and 28 in the Wnt pathway were found to contain putative LEF1/TCF or RBP-Jκ consensus binding sites, respectively. Of these, 19 Notch pathway gene promoters containing LEF1/TCF-sites underwent functional and experimental analysis. To elucidate whether Wnt signalling could regulate the identified Notch pathway genes, a HT29 cell line carrying a Zn-inducible wt-*Apc* vector (HT29-APC) was employed. Upon activation of wt-Apc, *Notch2*, *Maml1*, *Hes1*, *Hes7*, *Rfng*, *Lfng*, *Numb* and *Numbl* all displayed a downregulation at mRNA level, while the Hes1 negatively regulated gene *Hath1* was clearly transcriptionally upregulated. To further elucidate if the genes are under direct control of β-catenin, a pool of anti-β-catenin siRNAs was used for gene silencing. Generally, downregulation was less significant compared to canonical Wnt pathway inactivation through wt-Apc induction. β-catenin was, however, not completely silenced, which could partly explain the less significant downregulation of the Notch pathway genes in this experiment. *Rfng* and *Lfng* were unaffected by β-catenin silencing, indicating that they may be regulated by Wnt signalling in a non-canonical manner. We also carried out an *in vitro* DNA-binding assay where a radiolabelled LEF1/TCF binding probe (CD1TOP) [247] competed with Notch pathway promoter probes for *in vitro* translated Lef1 binding. *Notch2*, *Jagged1*, *Maml1*, *Hes1*, *Rfng*, *Numb*, *Lfng* and *Numbl* all showed binding of at least one LEF1/TCF site in their identified promoter regions. Interestingly, two of the strongest binders were found in the *Jagged1* promoter at position -1933 and -1635 relative translation start site, even though we could not detect any downregulation of the gene upon Wnt pathway inhibition. However, we notice that Wnt signalling transcriptionally can regulate *Notch2*, *Maml1*, *Hes1*, *Hes7*, *Rfng*, *Lfng*, *Numb* and *Numbl*, and further that at least one putative LEF1/TCF-site in the gene promoters has the capability to bind *in vitro* translated Lef1. Altogether, our results support the theory that Wnt signalling may affect the Notch pathway at several levels, further implicating the importance of crosstalk between the two pathways in CRC.

Notch1 and *Notch2* are both expressed in normal intestine and CRC [107] and signalling through these genes is important for maintaining an undifferentiated proliferative crypt

compartment. However, only simultaneous inactivation of *Notch1* and *Notch2* results in the same phenotype as RBP-Jκ inactivation, indicating redundant roles in the intestine [56]. *Notch2* has been found expressed at very high levels in a small subset of cells scattered throughout the colonic crypts [107] indicating that a potential regulation of *Notch2* through increased Wnt signalling could be of importance in the early tumour formation. On the other hand, *Notch1* lacks LEF1/TCF-sites in its proximal promoter, while *Notch2* contains four, which indicates that the *Notch1* and *Notch2* encoding genes are differentially influenced by active Wnt signalling. The interactions between canonical Wnt signalling and *Notch2* was therefore subjected for a more detailed analysis.

To confirm the effects of *Notch2* mRNA downregulation on protein level, Notch2 was also analysed with Western blot, revealing attenuated protein levels 18-24 h post wt-Apc induction. Of the four LEF1/TCF sites investigated in the *in vitro* DNA-binding assays (-2261, -869, -689 and -110 relative to translational start site) at least -110 showed binding to *in vitro* translated Lef1 while -2261 and -689 displayed a potentially weak binding. A luciferase assay conducted in HCT116 and HT29 cells revealed enhanced promoter activity of an approximately 250 bp fragment of the *Notch2* promoter containing -110 LEF1/TCF site but not for -2261 or -689. Using luciferase experiments, we also examined the ability of Wnt signalling to activate the *Notch2* promoter. pGL3-reporter plasmids containing a TATA-box [252] and LEF1/TCF-sites -2261, -689 and -110 were transfected into HT29-APC vector as well as co-transfected into naïve HT29 and HCT116 cells with a vector containing mutated β-catenin (S33Y). In general, transfection into the HT29-APC was poor but results may indicate a decreased signal activity of the -110 construct upon wt-Apc induction, but not for the others. However, upon co-transfection with mutated β-catenin, we identified increased luciferase activity for the -110 construct, but not for -2261 or -689, indicating that β-catenin has the ability to activate the *Notch2* promoter via LEF1/TCF -110 *cis*-element. The other elements showed no activity and may be of less biological significance, or may require a larger part of the promoter in order to be biologically functional. Surprisingly, ChIP assays did not reveal binding of endogenous Tcf4 to the *Notch2* promoter, which indicates that the effect on the *Notch2* -110 Lef1/Tcf binding element may occur via other members of the Tcf/Lef family.

Furthermore, we conducted a DAPT treatment of HT29 cells. DAPT is known to be a robust γ-secretase inhibitor, thereby blocking release of NICD and Notch signalling. Previous studies [117, 212, 213] together with results from the bioinformatical searches indicate that Wnt signalling may act downstream of Notch signalling. Despite a clear

downregulation of *Hes1* mRNA and protein levels upon DAPT treatment, no effects on β-catenin protein levels or mRNA expression of the established β-catenin/Lef1/Tcf target gene *cyclin D1* were observed. Thus, preliminary results cannot confirm β-catenin or *cyclin D1* as transcriptional Notch targets in CRC cell lines.

General discussion

Constitutive Notch signalling stimulates proliferation and cell survival and may thereby promote carcinogenesis in many organs. In several malignancies, including CRC and T-cell lymphoma, aberrant Notch signalling is clearly oncogenic [7, 162], while in some others the function is tumour suppressive [132, 133]. The pathway is, together with Wnt and other evolutionary conserved pathways, an arbiter of cell fate decisions [178, 187-192] and forms strictly regulated signalling networks dependent on the cellular context. Disrupted Notch signals skew the balance in the networks, with different consequences depending on cell type. Gain-of-function mutations in the *Notch1* gene or loss-of-function mutations in the *CDC4* gene are hallmarks of human T-ALLs but are also commonly found in human and murine T-cell lymphoma [5, 119, 146-152]. Mutational activation of the pathway leads to a consistent transcription of Notch target genes *e.g. Hes1* and *Hey1* [51], driving progenitor proliferation and T-cell differentiation [90]. Radiation is a common source of carcinogenesis in mouse thymic lymphoma where rearrangements in the *Notch1* locus have been found in more than 50% of the cases, making it one of the most commonly observed genetic alterations in these tumours [62, 153, 254]. Phenolphthalein, 1,3-butadiene or 2',3'-dideoxycytidine are carcinogens all known to induce lymphoma in mice [220-222] and are commonly used in health care and industry [223, 224]. Although, none of the chemicals are known to induce lymphoma in humans the molecular mechanisms behind the chemically induced murine tumours are largely unknown and they might still be regarded as carcinogenic and a threat to human health. In *paper I*, activating mutations in the *Notch1* gene were observed in 39% of murine T-cell lymphoma induced by phenolphthalein, 1,3-butadiene or 2',3'-dideoxycytidine, while no mutations in *CDC4* were identified. The most common alteration of *Notch1* was mutations in the PEST domain followed by deletions of exons 1b-2 and the large intronic sequences surrounding these sequences. In 2004, Tsuji *et al.* [62] reported that deletions of early exons caused by V(D)J recombination in *Notch1* are major contributors to the tumourigenesis in radiation induced mouse lymphomas. The deletion breakpoints were located close to sequences similar to the RSS that are normally found in the gene encoding the TCR. Recombination of the receptor is important in order to maintain a high variability and thereby the ability to detect the wide variety of antigens that the receptor is exposed to. This variability is generated by the V(D)J recombination machinery that recognises the RSS, which are DNA segments consisting of a highly conserved heptamer followed by a 12

or 23 bp spacer and a highly conserved nonamer. The V(D)J recombinase introduces double strand breaks adjacent to the RSS, whereas the recombination-activated genes Rag1 and 2 creates hairpin structures at the ends before they are rejoined at the new site. The deletions detected in our study may be a result of illegitimate recombination of the *Notch1* gene by the V(D)J recombination machinery, which would also explain the insertions, which resemble the P-nucleotides normally inserted at the splice site to further increase the diversity of TCR [145]. The deleted product lacks most of the extracellular domain and this conformational change of the Notch1 receptor leads to an overexposed S3-cleavage site and thus increased NICD release and overactive Notch signalling in the radiation induced murine lymphoma [153]. It is likely that this mechanism is similar in chemically induced mouse lymphoma.

Mutations in the HD-domain were also detected, however not to the same extent. Five of the eight mutations detected in the HD-domain displayed the same point mutation in codon 1668, altering a leucine to a proline and thus potentially representing a mutational 'hot-spot'. This mutation has previously been reported in murine lymphomas [148] as well as in the corresponding codon of human *Notch1* [146, 152]. Proline is known to cause α-helix disruption in protein structures, indicating that the mutation may lead to ligand-independent cleavage of NICD. Interestingly, one third of the mice carrying *Notch1* mutations contained mutations in both the PEST and the ligand-binding domains or the PEST and HD domain. Possibly, combined mutations will have a synergistic effect on the NICD activity and Weng *et al.* [119] showed that mutations in both HD and PEST domains gave significantly higher luciferase activity than single mutations. It would be interesting to study if the same result would be obtained if combining exon 1b-2 deletions and mutations in the PEST domain and further study how compound mutations in *Notch1* would affect survival and tumour progression compared to single mutations alone. Furthermore, the chemically induced lymphomas have previously been analysed for mutations in *p53* [255] and a crosstalk between the Notch and the p53 pathways can occur at multiple levels, in a manner that is dependent on the cell types or the tissues in which they function [185]. p53 may induce *Notch1* transcription via sequence-specific p53 binding sites [256, 257], a mechanism more likely in tissues where Notch1 functions as a tumour suppressor. Accordingly, in thymocytes where *Notch1* is a typical proto-oncogene, low expression or absence of p53 is correlated with increased *Notch1* levels [258]. Our data indicate a reverse relationship between the genes, which is also supported by the low frequency of *Notch1* mutations in tumours from phenolphthalein-induced $p53^{+/-}$ mice (10%) compared to tumours induced in Swiss or C57Bl/6×C3H/Hej F_1 mice induce by

dideoxycytidine (52% and 44%, respectively) or 1,3-butadiene (26%). Both mutational inactivation of *p53* and mutational activation of *Notch1* result in cell growth, why mutations in both genes may be unnecessary for lymphomagenesis and could explain the low frequency of *Notch1* in $p53^{+/-}$ mice treated with phenolphthalein. Likely, there are also other possible explanations to overactive Notch signalling in lymphomas except for mutational activation of *Notch1 e.g.* ligand and receptor overexpression or genomic amplification of regions containing genes in the pathway [156-159]. Furthermore, constitutively activated N1ICD may affect canonical Wnt signalling through a direct interaction with β-catenin [215-217] or Lef1 [218] which could lead to uncontrolled cell proliferation, a mechanism that could possible contribute in other malignancies where the pathways function oncogenically.

Mechanisms controlling Notch pathway signalling are less well known in solid malignancies. The present results, together with other recent reports, indicate that mutations of *Notch* and *CDC4* are absent or rare in CRC since no mutations in the HD or PEST domains were detected in neither murine intestinal polyps nor human CRC. Similar results were obtained by Lee *et al.* [123], who screened 48 colorectal, 48 gastric, 48 breast and 48 lung tumours for mutation in the HD and PEST domains of *Notch1-4*. In the study by Lee *et al.* only one colorectal tumour was found to contain a missense mutation in the *Notch3* PEST domain and one breast tumour was found to contain a nonsense mutation in the *Notch2* PEST domain.

Mutations in the *hCDC4* gene have previously been reported in 4-10% of both sporadic and hereditary CRC [171, 172], a mechanism that could contribute to constitutive Notch pathway activation in solid malignancies. Surprisingly, we found no *hCDC4* mutations in our material but only a relatively small number of tumours were investigated. The conflicting results may partly be explained by the use of different study populations with different genetic background or different life styles. Taken together, mutational inactivation of *CDC4* in colorectal tumours is a relatively uncommon event and likely not the major contributor to Notch pathway overactivation in CRC. Furthermore, the ligand-binding domains of the *Notch* genes needs to be investigated for deletions or other genetic alterations that could possibly affect receptor-ligand binding before any final conclusions can be drawn. Nonetheless, results indicate that mechanisms separate from mutational activation of the *Notch* genes are more likely to explain the overactive Notch signalling in CRC, even though stabilisation of NICD from mutational inactivation of *hCDC4* may contribute in a minority of cases.

Thus, the question to why the Notch pathway is overactivated in many solid malignancies [115, 116, 120-122] largely remains unanswered. Although the Notch pathway often is

upregulated in cancer, transcriptional regulation of the included genes, and especially of the Notch receptors, is poorly understood. Also, transcription may change during development or disease stage [170] making the issue even more complicated. *Notch1* is positively regulated by the tumour suppressor p53 [256, 257], a mechanism that probably is of great importance in tissues where *Notch1* acts in a tumour suppressive manner but might be of minor importance where *Notch1* has an oncogenic role. Recently, it was shown that *Notch1* is autoregulated via several RBP-Jκ elements in the promoter in the pre-β-stage of mouse thymocyte development as well as via canonical E-box TF binding E2A sites but is rapidly downregulated in the thymocytes after the cell fate selection. If not downregulated at this stage, Notch signalling in thymocytes may be oncogenic [259], a mechanism that could also be of importance in human malignancies where overactive Notch signalling could lead to higher levels of *Notch1* mRNA and further increased proliferation. However, increased *Notch1* transcription does not *per se* means incremental Notch signalling in terms of induced target genes, since the pathway is regulated at several levels before N1ICD exerts its biological active role in the nucleus, but it is likely that it contributes. Notch signalling influences many intracellular pathways important for tumour development and progression *e.g.* those related with NFκB and c-myc signalling [127, 129-131]. C-myc is a typical proto-oncogene in CRC, directly regulated via canonical Wnt signalling [260]. The human *Notch1* promoter contains putative RBP-Jκ, c-myc and NFκB elements (MatInspector) and it would be of interest to functionally study these sites and see whether auto or feed-back regulatory mechanisms are of importance for *Notch1* regulation in carcinogenesis. It would also be of interest to study if other *Notch* genes have the potential to activate *Notch1* transcription via the RBP-Jκ-sites.

The Notch pathway interacts with a few but highly conserved pathways during embryonic development as well as in cancer [178, 187-192]. One of the most important for intestinal homeostasis and CRC is the canonical Wnt signalling pathway. Although incremental evidence suggests a crosstalk between the Notch and the Wnt pathways [205-209, 211, 215-217], information about their interactions in the intestinal tract has hitherto been scarce. This kind of crosstalk would be of no less importance in colonic epithelium and CRC, where Notch and Wnt signalling can function in synergy and together promote stem cell renewal and drive proliferation [170, 188]. Thus, dysregulation and overactivation of one of the two pathways could potentially lead to simultaneous activation of the other giving rise to increased proliferation and tumour formation. In CRC, canonical Wnt signalling is most often aberrantly activated through inactivating mutations in the *Apc* gene [105], leading to stabilisation and nuclear translocation of β-catenin [261], which transcriptionally activates

the LEF1/TCF target gene program [178]. In *paper II*, we bioinformatically identified putative LEF1/TCF and RBP-Jκ consensus site in Notch and Wnt pathway gene promoters, respectively. Consensus sequences could randomly occur in the genome and it is therefore important to functionally investigate the putative sites. Twenty-five genes in the Notch pathway and 28 genes in the Wnt pathway, known to be important for Notch and Wnt signalling, respectively, were found to contain LEF1/TCF and RBP-Jκ sites (*paper II*, Table 2). To study the effects of Wnt signalling on Notch pathway genes a HT29 cell line carrying a Zn-inducible wt-*Apc* vector was used as well as silencing of β-catenin with siRNA. In general, downregulation was less significant when siRNA was used compared to wt-Apc induction, and *Rfng* and *Lfng* were not affected by β-catenin silencing, indicating that β-catenin independent Wnt signalling might influence transcription or post-transcriptional processes like mRNA-stability of the studied genes. Furthermore, β-catenin was not completely silenced, possibly contributing to the less significant downregulation of the Notch pathway genes in this experiment. It would therefore be of interest to study the effects on these genes upon complete inhibition of β-catenin dependent Wnt signalling, using a dominant negative form of Tcf or Lef that lacks their β-catenin binding function. Upon wt-Apc induction *Notch2*, *Maml1*, *Hes1*, *Hes7*, *Rfng*, *Lfng*, *Numb* and *Numbl* were found to be transcriptionally downregulated while *Hath1* was upregulated, indicating effects downstream of the Notch pathway. Whether this is a direct effect of Apc activation or a Notch dependent mechanism remains to be elucidated. We also carried out an *in vitro* DNA-binding assay against identified LEF1/TCF-sites in several of the semiquantitatively investigated genes. *Notch2*, *Jagged1*, *Maml1*, *Hes1*, *Rfng*, *Numb*, *Lfng* and *Numbl*, all showed binding of at least one LEF1/TCF to *in vitro* translated Lef1. Interestingly, two of the strongest binders were found in the *Jagged1* promoter at position -1933 and -1635 relative translation start site, even though we could not detect any downregulation of the gene upon Wnt pathway inhibition by Apc-wt induction.

Very recently Rodilla *et al.* [179] presented data, which partly contrast our findings, suggesting that Jagged1 is upregulated upon activation of Wnt signalling pathway, and being the molecular link between the Wnt and Notch signalling pathways. The reason for the unconsistent results is obscure but different models have indeed been used. They blocked β-catenin in Ls17T CRC cells using a dominant negative Tcf4 inducible vector, and moreover, that stabilisation of β-catenin in the nontumourigenic cell line NIH 3T3 by treatment with the GSK-3β inhibitor LiCl, which led to increased *Jagged1* mRNA and protein levels. Albeit speculatively, it is therefore possible that the cellular context, and the

mechanism by which Wnt signalling pathway is activated, may be crucial for the functional outcome with regard to activation of Jagged1 and downstream Notch signalling.

Notch1 and *Notch2* are both expressed in CRC [107] and signalling through these genes is important for maintaining an undifferentiated crypt compartment. However, only simultaneous inactivation of *Notch1* and *Notch2* results in the same phenotype as RBP-Jκ inactivation, indicating redundant roles in the intestine [56]. To further increase the complexity of Notch signalling in a CRC context, recently Chu *et al.* [180] demonstrated that *Notch2* mRNA as well as protein levels are decreased in CRC compared to adjacent normal mucosa and that high levels correlates with differentiation of colon cancer cells. The results somewhat contrasts Riccio *et al.* [56] further indicating that we need a deeper understanding of Notch signalling and the Notch receptors in CRC. *Notch1* was not found to contain any LEF1/TCF consensus sites in the proximal promoter, indicating that it is not directly regulated by β-catenin/Lef1/Tcf complex. *Notch2* contained four putative sites at positions -2261, -869, -689 and -110 relative translation start site; and -110 showed strong binding to *in vitro* translated Lef1. Luciferase assays indicated an increased activity for site -110 upon co-transfection with mutated β-catenin and a slightly suppressed activity upon activation of wt-Apc, which was however not significant. This, together with the result from Rodilla *et al.* implies that disrupted Wnt signalling may affect Notch signalling by several mechanisms, resulting in increased Notch signalling in CRC via both Jagged1 and Notch2.

Surprisingly, we could not detect binding of Tcf4 to the LEF1/TCF-sites in the *Notch2* promoter in neither HT29 nor HCT116 CRC cell lines, using ChIP. The reason for this is obscure but one possible explanation could be that the signalling is mediated via Lef1 rather than Tcf4 in CRC. *Lef1* is normally not expressed in colon but expression has been detected in CRC and several CRC cell lines [262]. A hypothesis is that in CRC, Lef1 binds to the *Notch2* promoter and via β-catenin activates the gene expression, a mechanism that would especially be of significance in *Apc*- or β-catenin mutated tumours. However, this needs to be further investigated with ChIP assays against Lef1 in CRC or CRC cell lines. Since, *Notch1* also often is upregulated in CRC and directly could interact with β-catenin [215-217] or Lef1 [218] this could, together with the autorregulatory function of *Notch1*, further stimulate overactivation of Notch signalling and cell proliferation in CRC.

Some previous studies also suggest that Notch signalling may be placed upstream of the canonical Wnt pathway [117, 212, 213] and indeed we find putative RBP-Jκ sites in several promoters of Wnt pathway genes. However, upon Notch pathway inhibition with the γ-secretase inhibitor, DAPT, no effects on β-catenin or *cyclin D1* gene expression were observed. However, none of the earlier studies have been carried out on human CRC cells,

and the interactions described in *Drosophila* or in mouse are not necessarily of significance in human CRC. Our results are in close agreement with a study by Fre *et al.* [106] where the expression of Tcf4 and Lef1 were found to be unaffected by Notch activation in mouse intestine. Except for the Wnt pathway, Notch signalling is likely to interact with other embryonically important signalling pathways, *e.g.* SH, in intestinal tumour formation and it would be of interest to further elucidate the importance of these interactions and their implication in cancer biology.

Regardless of the responsible molecular mechanism, constitutive or overactive Notch signalling affects proliferation in lymphocytes and colonic epithelium and thereby promotes development and progression of T-cell lymphoma and CRC. The mechanisms and the role of Notch signalling in carcinogenesis is far from fully understood, but modulation of the Notch pathway and its related signalling networks may provide future strategies for improved diagnosis, classification, and treatment of neoplasms.

Conclusions

Incremental evidence suggests that Notch signalling plays a major role in the development and progression of several malignancies In the present thesis, we reveal that distinct molecular events contribute to hyperactive Notch signalling in haematological and intestinal cancers, respectively. While mutations of genes encoding Notch, and the Notch regulating protein Cdc4, are commonly observed in murine T-cell-lymphomas, colorectal cancer cells are rather characterised by dysregulated Notch signalling due to aberrant Wnt signalling.

- We identified activating mutations in *Notch1* in 39% of the chemically induced murine T-cell lymphoma,
- Mutation of the *Notch1* gene is not a common event in colorectal cancer, suggesting that other events, like upregulation of upstream genes or pathways, more significantly contributes to aberrant Notch signalling in colon cancer cells.
- Several potential target genes of Wnt/β-catenin signalling among genes traditionally classified as belonging to the Notch pathway were identified. More specifically, we suggest that *Notch2* is a novel target, activated by β-catenin and Wnt signalling in colon cancer cells.

Populärvetenskaplig sammanfattning

När ett foster utvecklas och växer regleras en mängd viktiga processer av ett fåtal evolutionärt bevarade biologiska signalvägar. Dessa processer involverar celldelning men också cellmognad och cellspecialisering. Även i den vuxna individen spelar dessa signalvägar en viktig roll, framför allt i vävnader som ständigt förnyas, till exempel tarmslemhinnan och blodbildande organ. Det är viktigt att dessa mekanismer är i balans eftersom en överaktivering av signalvägarna kan leda till okontrollerad celltillväxt och så småningom utveckling av cancer. Mekanismerna som ligger till grund för överaktiveringen kan variera men vanliga bakomliggande orsaker är genetiska förändringar, *s.k.* mutationer, i, eller en felaktig reglering av de gener som ingår i signalvägen. I denna avhandling har en av de embryonalt viktiga signalvägarna, Notchsignaleringsvägen, och genetiska förändringar i denna, studerats i muslymfom samt human tjock- och ändtarmscancer.

Notchsignaleringsvägen har fått sitt namn från Notchreceptorerna, vilka kan aktiveras vid kontakt mellan två celler. Vid aktivering klyvs receptorns inre celldel loss och tar sig in till cellkärnan där den aktiverar gener viktiga för celldelning och/eller cellspecialisering. Effekterna av denna signalering beror till stor del på hur stark signal cellen får men också i vilken vävnad signaleringen sker. Mutationer i receptorn kan leda till en felaktig receptoraktivering som är oberoende av cell-cell kontakt eller till att receptors inre celldel inte bryts ner på ett korrekt sätt. I båda fall fås en överaktivering av de gener som Notchsignalvägen reglerar, vilket kan bidra till okontrollerad celldelning. Även en felaktig avskrivning av de gener som kodar för ingående proteiner i signalvägen kan leda till överaktivering och ökad celldelnng. Dessutom kan en felaktig samverkan med andra signalvägar eller mekanismer i cellen få motsvarande effekter.

I den första studien studerades mutationer i den största och mest kända av Notchreceptorerna, Notch1, i kemiskt orsakade muslymfom. Lymfom är en tumörsjukdom som orsakas av en okontrollerad celldelning av en av immunförsvarets celler, lymfocyterna. Sjukdomen är närbesläktad med en annan typ av blodcancer kallad akut lymfoblastisk leukemi (ALL), i vilken mutationer i *Notch1*-genen är vanligt förekommande. I studien behandlades möss med kemikalier vilka används dagligen inom industri och sjukvård. Dessa kemikalier gav upphov till en rad genetiska förändringar hos mössen och vi upptäckte aktiverande mutationer i *Notch1* genen i 40 av de 103 (39%) kemiskt inducerade lymfomen.

Dessa mutationer leder till en konstant aktivering av signalvägen och därmed en okontrollerad celldelning av lymfocyterna. Utifrån dessa resultat drar vi slutsatsen att *Notch1* är en av de viktigaste och också vanligast muterade generna i kemiskt inducerade muslymfom.

Förändrad Notchsignalering är också ett vanligt fenomen i flertalet solida cancerformer, till exempel i tjock- och ändtarmscancer även om orsakerna tycks vara andra än aktiverande mutationer i någon av *Notch*-generna. Notchsignaleringsvägen samverkar med ett flertal andra embryonalt viktiga signalvägar och intracellulära processer. En sådan är Wntsignaleringsvägen där inaktiverande mutationer i en av signalvägens gener, *Apc*, är en av de vanligaste händelserna i tjock- och ändtarmscancer. Inaktivering av *Apc* leder till att proteinet β-catenin inte regleras och bryts ner korrekt utan kan ta sig in i cellkärnan och aktivera ett flertal gener som är viktiga för celldelning. Resultat från tidigare studier tyder på att det finns en möjlig samverkan mellan Notch- och Wntsignaleringsvägarna och att detta skulle kunna vara av betydelse vid uppkomst av cancer.

I den andra studien undersökte vi om Wntsignalering skulle kunna styra gener i Notchsignaleringsvägen och vice versa samt om de två signalvägarnas samspel påverkar uppkomst och utveckling av tjock- och ändtarmscancer. I stora drag visar resultaten från studien att Wntsignalering kan binda till och reglera flera gener i Notchvägen, bland annat *Notch2*. Notch2 har, tillsammans med Notch1, visat sig ha stor betydelse för hur tarmens celler delar sig och utvecklas. Därför skulle en störning i någon av dessa två gener kunna ha stor betydelse för tumörutveckling i tarmen. Resultaten från vår studie tyder på att det finns ett samspel mellan Notch- och Wntsignaleringsvägarna i tarmceller och att detta samspel skulle kunna vara viktig för uppkomsten och utvecklingen av tumörer i tarmen.

Sammantaget spelar Notchsignalering en stor roll vid uppkomsten av muslymfom så väl som tjock- och ändtarmscancer, två vitt skilda tumörsjukdomar. Orsakerna till den störda Notchsignaleringen i de båda tumörsjukdomarna tycks vara av olika ursprung vilket ytterligare stärker betydelsen av Notch roll för uppkomst och utveckling av flera tumörsjukdomar. Förhoppningsvis bidrar dessa resultat till en ökad förståelse för de signalnätverk Notch verkar i samt för den roll Notch spelar i cancer.

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