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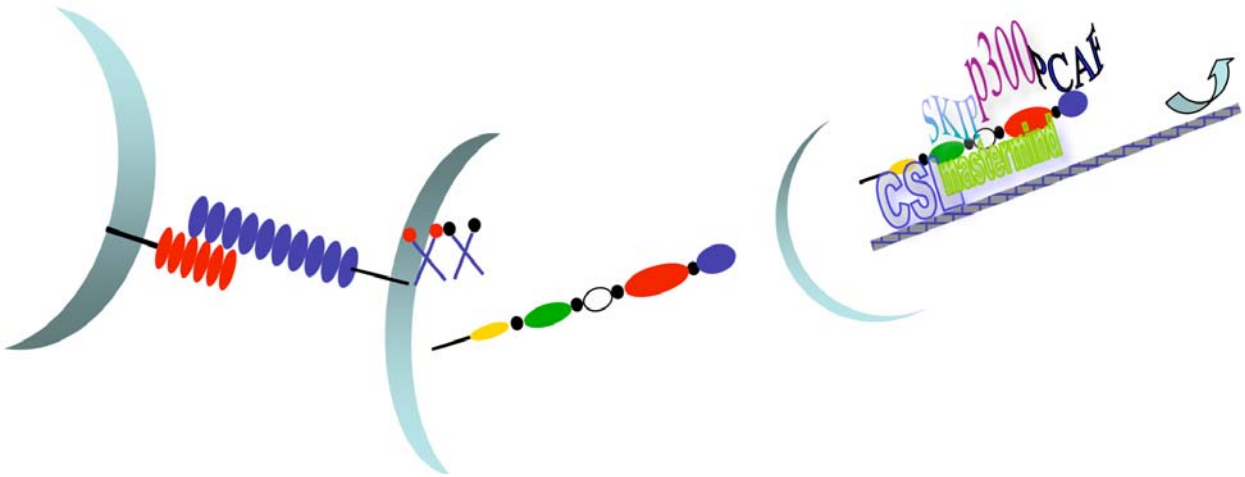
Lining Liu

Stockholm 2008



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ABSTRACT

The development of mutlicellualr organisms relies on a highly conserved signaling network, of which one of the key components is the Notch signaling pathway. The core Notch signaling pathway consists of receptors, ligands and an intracellular mediator. Activation of Notch signaling involves the interaction of the receptor to membrane-bound ligand, leading to the consecutive proteolytic cleavages of the receptor and the release of the Notch intracellular domain (NICD) from the cell membrane. The NICD then translocates to the nucleus and interacts with the DNA-binding transcription factor CSL (CBF-1/Su(H)/Lag-1), resulting in transcriptional activation of E(spl)/Hes family and other target genes.

The work presented in this thesis is focused on two specific aspects of Notch signaling: i) the interplay between Notch and its intrinsic negative regulator Numb/Numblike and the role of Numblike in the early differentiation of embryonic stem (ES) cells; ii) the mechanism on how aberrantly high Notch signaling in T-cell acute lymphoblastic leukemia (T-ALL) translates into deregulated cell cycle control and the transformed cell type.

Numb was originally identified as an inhibitor of Notch signaling pathway in *Drosophila* melanogaster and acts as a cell-fate determinant during asymmetric cell division. In vertebrates there are two mammalian homologs: Numb and Numblike. In paper I we suggested a model in which Numb/Numblike and Notch had reciprocal antagonistic activities. We found that Numb promoted differentiation in the mouse moyogenic cell line C2C12 at low but not high levels of Notch signaling. In keeping with a differentiation-promoting role, we observed that a C2C12 cell line stably expressing Numblike showed accelerated myogenic differentiation. Unexpectedly we also observed high levels of Notch signaling was accompanied with a reduction of Numb/Numblike at protein levels in C2C12 stable cells. We extended our study and reached the same conclusion in human ovarian carcinoma cell (SKOV-3 cells) and in the developing chick central nervous system. We also found that the Notch mediated reduction of Numblike required the PEST domain in the Numblike protein and was blocked by the proteasome inhibitor MG132. Next, we try to address the role of Numblike in the early differentiation processes as little is known about it. In paper III a mouse ES cell line with inducible expression of Numblike protein was generated. We found that Numblike expression impaired differentiation towards neural and mesodermal differentiation of ES cells. However myocardial differentiation was not affected upon induction of Numblike protein later at Flk-1 positive stage of mesodermal differentiation.

In more than 50% of all T-ALL cases activating mutations of Notch1 have been identified. Thus a better understanding of the effects of Notch signaling strength and timing on the pre-T cell transformation would provide more therapeutic bases. In paper II, we analyzed downstream responses resulting from the high level of Notch1 signaling in T-ALL. Our microarray study revealed that besides Hes1 and Hey1, the canonical downstream targets of Notch signaling, other immediate Notch downstream genes were also activated. Specifically we observed that Notch signaling increased the expression level of the E3 ubiquitin ligase SKP2, thus leading to the reduction of its target protein p27Kip1. Blocking Notch signaling resulted in G0/G1 cell cycle arrest, which could be mimicked by transfection of p27Kip1 or, to a smaller extent, a dominant negative SKP2 allele. In sum our data suggest that the aberrantly high Notch signaling in T-ALL maintains SKP2 at a high level and reduces p27Kip1, leading to more rapid cell cycle progression.

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. Chapman G*, **Liu L***, Sahlgren C, Dahlqvist C, Lendahl U.
High levels of Notch signaling down-regulate Numb and Numblike.
J Cell Biol. 2006 Nov 20;175(4):535-40.
Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, SE-171 77 Stockholm, Sweden.

II. Dohda T, Maljukova A, **Liu L**, Heyman M, Grander D, Brodin D, Sangfelt O, Lendahl U.
Notch signaling induces SKP2 expression and promotes reduction of p27Kip1 in T-cell acute lymphoblastic leukemia cell lines.
Exp Cell Res. 2007 Aug 15;313(14):3141-52. Epub 2007 May 5.
Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, SE-171 77 Stockholm, Sweden.

III. **Liu L**, Lanner F, Lendahl U.
Expression of Numblike impairs early mesodermal and neural differentiation in embryonic stem cell.
Manuscript.
Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, SE-171 77 Stockholm, Sweden.

*Equal contribution

TABLE OF CONTENTS

ABBREVIATIONS

aPKC	atypical protein kinase C
APP	amyloid pharynx phenotype
CIR	CBF1-interacting corepressor
CSL	CBF1/Suppressor of Hairless/Lag-1
DNA	deoxyribonucleic acid
DSL	Delta/Serrate/Lag-2
EC	endothelial cell
EGF	Epidermal Growth Factor
EMT	endothelial-mesenchymal transition
ES	embryonic stem
GCP	granule cell progenitor
GFAP	glial fibrillary acidic protein
GSI	γ-secretase inhibitor
HDAC	histone deacetylase
Hes	Hairy/Enhancer of split
Hey	Hairy/Enhancer of split with YRPW
HIF	hypoxia-inducible factor
Hprt	hypoxanthine phosphoribosyl transferase
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
Lgl	lethal giant larvae
LNR	Lin Notch Repeat
MAML	mastermind like
Mash1	mammalian achaete-scute complex homolog-like 1
N-CoR	nuclear hormone co-repressor
NICD	Notch intracellular domain
NΔE	membrane tethered form of Notch receptor
PCAF	p300/CBP associated factor
PEST	proline, glutamic acids, serines, threonines
Pon	partner of Numb
PRR	proline rich region
PTB	phosphotyrosin binding
PTEN	a putative protein tyrosin phosphatase
RAM	RBP-Jκ associated molecule
RE/AC	repression/activation
RBP-Jκ	recombination signal binding protein of the Jκ immunoglobulin gene
rtTA	reverse tetracycline transactivator
S1,2,3	Site 1, 2, 3
SKIP	Ski-interacting protein
SMRT	silencing mediator of retinoid and thyroid hormone receptor
SOP	sensory organ prescursor
Su(H)	Supressor of hairless
TACE	TNF-α converting enzyme
TAD	transcriptional activation domain
T-ALL	T cell acute lymphoblastic leukemia
TM	trans-membrane

BACKGROUND

Introduction

The building of an organism from a fertilized egg to the mature adult structure is a very complex and carefully regulated process, which involves the coordination of cell proliferation, pattern formation, morphogenesis, cell differentiation, cell migration, cell death and growth. Indeed it is in multicellular organisms that cell-cell communication reaches its highest level of sophistication to meet the needs of the organism as a whole. But the means of cell-cell communication does seem limited and relies on a relatively small number of signaling pathways, which are highly evolutionarily conserved and used repeatedly in different contexts and combinations to achieve unique developmental goals. Key among these signaling pathways important for a broad range of cell fate decisions are wingless/wnt, hedgehog, transforming growth factor-β, tyrosine kinase receptors, nuclear factor receptors, JAK/STAT and Notch. The focus of the thesis is on the Notch signaling pathway, which represents a distinct mechanism for how cells perceive and interpret environmental cues. I will begin with describing the molecular mechanism and regulation of Notch signaling pathway before presenting the role of Notch signaling pathway in cancer. I will conclude the thesis by presenting and discussing the papers and our contributions to the understanding of the Notch signaling pathway.

A short history of Notch biology

The Notch signaling pathway was originally identified and studied in the fruit fly, *Drosophila melanogaster*. The name 'Notch' derives from the characteristic notches at the ends of the wing blades found in the mutant flies (Dexter, 1914; Mohr, 1919; Morgan and Bridges, 1916). This particular phenotype is the result of haploinsufficiency (a partial loss of function) of the *Notch* locus. What really brings this locus to prominence is the classical analyses of the homozygous Notch mutations in flies, which result in an embryonic lethal phenotype (Poulson, 1940). The complete deletion of *Notch* leads to failure of the early neurogenic ectoderm to segregate neural and epidermal cell lineages (Poulson, 1940). Cells destined to become epidermis switch fate, which results in a hypertrophy of the neural tissue at the expense of

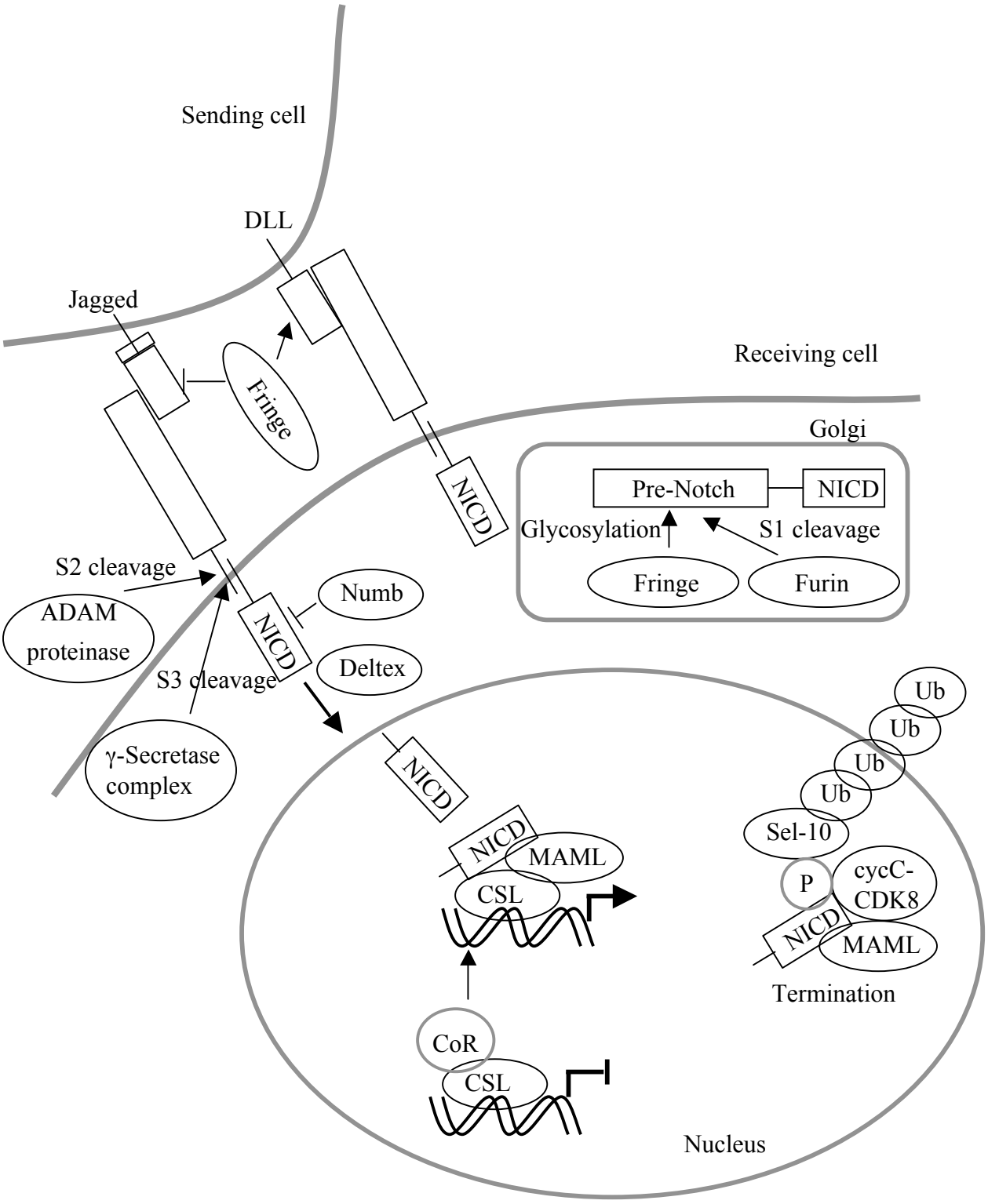

Figure 3: Diagram of the activation and regulation of Notch signaling

epidermal structures. The cloning of the *Notch* locus by Artavanis-Tsakonas and co-workers uncovers the existence of a novel cell interaction mechanism as well as becomes the starting point of the molecular study of Notch signaling. The *Notch* gene, first cloned in *Drosophila melanogaster*, encodes a 300kDa single–pass transmembrane receptor (Artavanis-Tsakonas, Muskavitch et al. 1983). The neurogenic phenotype of embryos lacking Notch function indicates that Notch-dependent cellular communications are critical for cell fate decisions. Over the years, extensive genetic and molecular interaction studies have revealed the mechanistic basis of the core Notch pathway and the pleiotropic action of Notch signaling in numerous aspects of development and cancer.

Distinct modes of Notch signaling

Notch signaling is used widely for cell fate decisions and to control the formation of biological patterns, while at a cellular level the roles of Notch signaling can be divided into lateral inhibition and inductive signaling (Figure 1) (Lewis 1996; Haines and Irvine 2003).

In lateral inhibition, Notch signaling inhibits all but one among a group of equipotent cells from adopting a particular fate during development. Initially, cells with equal developmental potential both send and receive Notch signals. Later, a small variation of Notch ligand expression occurs in some cells through a stochastic event or by other unknown mechanisms, which results in a rapid amplification of the difference by a transcriptional feedback mechanism. The cell with higher levels of Notch ligand on its surface leads to increased activation of Notch signaling in the neighboring cells, thus inhibits these neighboring cells from choosing the same cell fate (Figure 1A). The selection of *Drosophila* neural precursors and the *C.elegans* vulva development are classical examples of lateral inhibition (Parks, Huppert et al. 1997; Greenwald 1998).

Inductive Notch signaling typically occurs between non-equipotent cell populations. In this case, one group of cells signals to a distinct adjacent group of cells to create a new cell fate along the interface between these two distinct cell populations (Figure 1B). In contrast to lateral inhibition where Notch signaling represses a given cell fate among equipotent cells, inductive Notch signaling instructs cells to adopt a particular

cell fate as a result of cell-cell interactions at the boundary between distinct cell populations. Well-understood examples include *Drosophila* wing development and vertebrate somitogenesis (Conlon, Reaume et al. 1995; Couso, Knust et al. 1995).

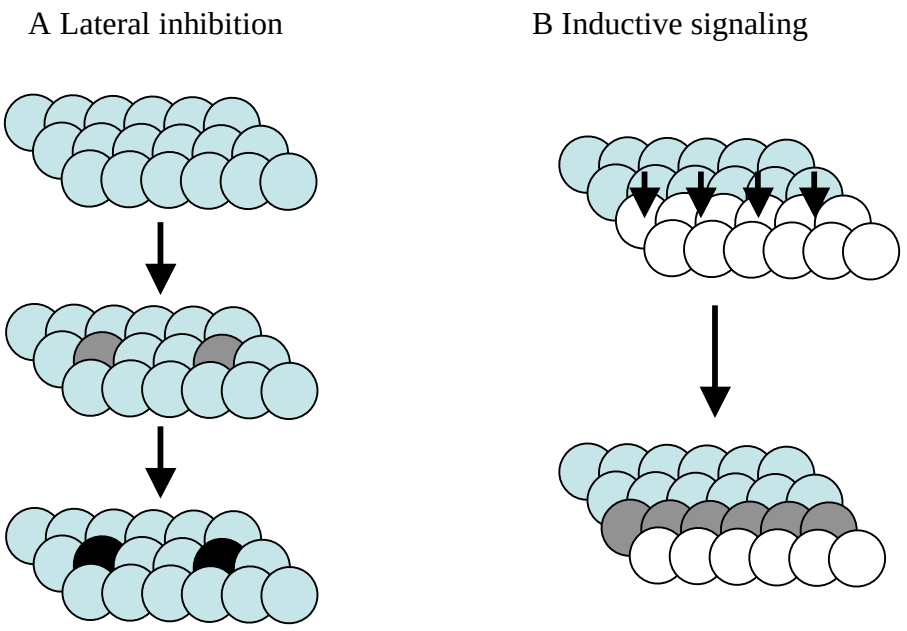

Figure 1: A schematic representation of distinct modes of Notch signaling: later inhibition (A) and inductive signaling (B).

Molecular mechanism of Notch signaling

Unlike many other signaling pathways, which involve a complicated sequence of proteins as second messengers and amplification by a signaling cascade within the cell, the core Notch signaling pathway relies on relatively few components to convey the signal from the cell surface to the transcriptional machinery. Binding of ligands to Notch receptors in neighboring cells triggers proteolytic cleavage of Notch receptor, which results in the release of the intracellular domain of Notch (NICD) from the plasma membrane. NICD then travels to the nucleus and associates with a CSL (CBF-1/Suppressor of Hairless/Lag-1) DNA-binding protein. This association converts CSL-containing complexes from transcriptional repressors to transcriptional activators, and thereby changes the transcriptional program of these cells. Superimposed on this relatively direct core Notch signaling pathway are a wide array of modulators of Notch signaling, most of which are specific for only a subset of Notch modes of action.

Structure of the Notch receptors and ligands

The Notch receptor family encodes large single-pass transmembrane proteins that are present at the plasma membranes as heterodimers (Figure 2). They consist of an extracellular domain and a membrane-tethered intracellular domain, which share some common characteristic features. On the extracellular side, the receptors contain a variable number of tandemly-arranged Epidermal Growth Factor (EGF)-like repeats and a family-specific LNR (Lin Notch Repeat) region (Figure 2) (Wharton, Johansen et al. 1985). The LNR region, which is a highly conserved cysteine-rich region, has been shown to depend on calcium for the proper folding and maintenance of the structural integrity and to protect the association of the extracellular and the transmembrane part of the receptor in the absence of ligand (Greenwald and Seydoux 1990; Lieber, Kidd et al. 1993; Aster, Simms et al. 1999). EGF-like repeats 11 and 12 are believed to be involved in ligand binding (Rebay, Fleming et al. 1991). In the intracellular domain of Notch four main domains are characterized: the RAM, ankyrin repeat, RE/AC and C-terminal region. The main function of the RAM domain is believed to mediate direct interaction with CSL, perhaps together with the ankyrin repeats (Tamura, Taniguchi et al. 1995; Roehl, Bosenberg et al. 1996). The ankyrin

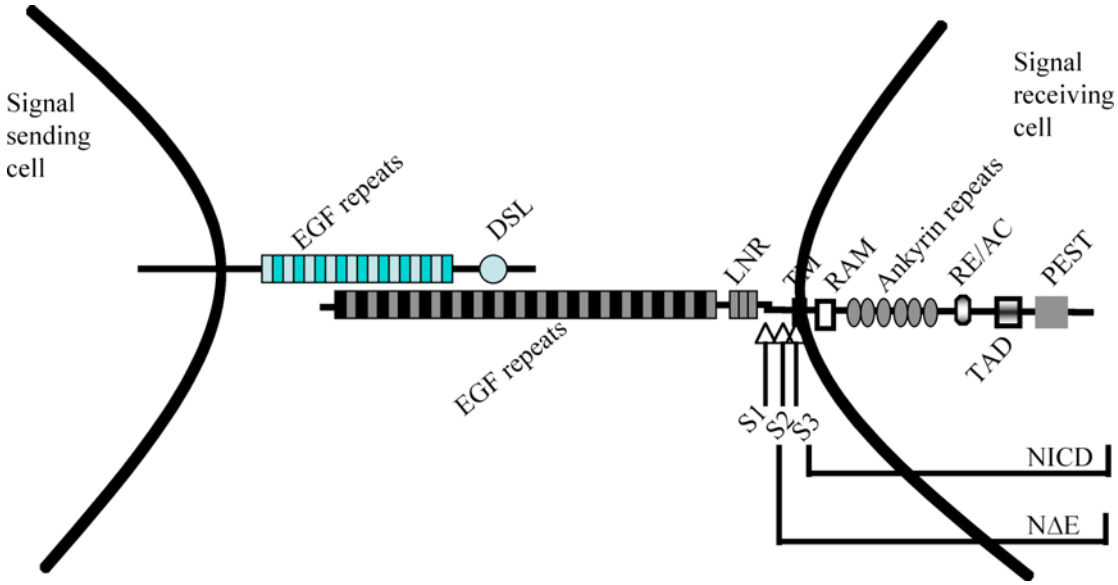

Figure 2: Schematic depiction of Notch receptor and ligand: structural and functional domain.

repeats, also called CDC10 repeats, were first reported in cell cycle proteins CDC10, SW14 and SW16 (Breeden and Nasmyth 1987) and they mediate protein-protein interactions (Kodoyianni, Maine et al. 1992; Rebay, Fehon et al. 1993; Kopan, Nye et al. 1994). The RE/AC domain is important for the transactivating capacity and specificity of the Notch receptor (Bigas, Martin et al. 1998; Beatus, Lundkvist et al. 2001). Within the most C-terminal part is the PEST domain, which is rich in Prolines (P), Glutamic acids (E), Serines (S), Threonines (T). PEST sequences are thought to be important for the ubiquitination and stability of the protein (Rogers, Wells et al. 1986; Greenwald 1994). The NICD contains two nuclear localization signals, located on opposite sides of the ankyrin repeats region (Lieber, Kidd et al. 1993; Rebay, Fehon et al. 1993; Roehl, Bosenberg et al. 1996).

There are two types of Notch ligands, Delta and Serrate. Similar to the Notch receptors, the ligands are also single-pass transmembrane proteins, with a large EC domain containing tandemly arranged EGF-like repeats (Figure 2). In addition the EC domain also contains a region specific for both classes of the ligands, the DSL domain, which consists of about 45 amino acids and resembles a partial or modified EFG-like repeat. While the EGF-like repeats can be deleted with no apparent

reduction in receptor activation capacity, the N-terminal region of the EC domain as well as the DSL domain are both required. The Serrate family of ligands shares a unique cystein-rich region (Fleming, Scottgale et al. 1990; Thomas, Speicher et al. 1991). Following the transmembrane domain is a relatively short IC region of 70-215 amino acids, showing little structural conservation. For a schematic depiction of the Notch ligands and their functional domains, see Figure2.

Table 1 Core components of the Notch signaling pathway

Component	*Drosophila*	*C. elegans*	Mammals
Receptor	Notch (Artavanis-Tsakonas et al., 1983; Kidd et al., 1983; Wharton et al., 1985)	Lin-12 (Yochem et al., 1988) Glp-1 (Yochem and Greenwald, 1988)	Notch 1-4 (Ellisen, Bird et al. 1991; Weinmaster, Roberts et al. 1992; Lardelli, Dahlstrand et al. 1994; Uyttendaele, Marazzi et al. 1996)
Ligand	Delta (Kopczynski et al., 1988) Serrate (Fleming et al., 1990; Thomas et al., 1991)	Apx-1 (Mello et al., 1988) Lag-2 (Tax et al., 1994)	Delta-like1,3,4 (Bettenhausen, Hrabe de Angelis et al. 1995) (Dunwoodie, Henrique et al. 1997) (Shutter, Scully et al. 2000) Jagged1, 2 (Lindsell, Shawber et al. 1995) (Shawber, Boulter et al. 1996)
DNA-binding mediator	Suppressor of Hairless (Fortini and Artavanis-Tsakonas, et al., 1994)	Lag-1 (Christensen et al., 1996)	CSL (Matsunami et al., 1989)
Target gene	Hairy (Ohsako et al., 1994) Enhancer of Split (Bailey and Posakony, et al., 1995; Lecourtois and Schweiguth, et al., 1995)	Lin-22 (Wrischnik and Kenyon, 1997) REF family (Neves and Priess, 2005)	Hes1, 5, 7 (Bessho et al., 2001; Jarriault et al., 1995; Ohtsuka et al., 1999) Hey1, 2, L (Leimeister et al., 1999; Steidl et al., 2000)

There are five characterized mammalian ligand homologs: Deltalike1 (Bettenhausen, Hrabe de Angelis et al. 1995), 3 (Dunwoodie, Henrique et al. 1997), 4 (Shutter, Scully et al. 2000) and Jagged 1 (Lindsell, Shawber et al. 1995) and 2 (Shawber, Boulter et al. 1996) and four mammalian Notch receptors: Notch 1 to Notch 4 (Table 1) (Ellisen, Bird et al. 1991; Weinmaster, Roberts et al. 1992; Lardelli, Dahlstrand et al. 1994; Uyttendaele, Marazzi et al. 1996). The Jagged ligands correspond to *Drosophila* Serrate because of the presence of the characteristic cystein-rich region. There does not seem to be any definite consensus in the expression pattern between a particular ligand and receptor and indeed all ligands except Deltalike3 (Ladi, Nichols et al. 2005) have the ability to activate all the available receptors in vitro. To date the only established specificity regarding to ligand-receptor binding and activation of the Notch pathway lies in the Fringe-mediated modifications described below.

The activation of Notch receptor

The Notch signaling mechanism is characterized by a series of proteolytic events referred to as S1, S2, and S3 cleavages. After its synthesis as an approximately 300 kD precursor molecule in the ER, the Notch receptor is transported through the secretory pathway to the trans-Golgi network, where it is constitutively cleaved by a furin-like convertase (S1 cleavage) (Aster, Pear et al. 1994; Blaumueller, Qi et al. 1997; Logeat, Bessia et al. 1998). Mapping of the cleavage site revealed that the processing occurs on the carboxyl site of amino acids 1651-1654 (RQRR in Notch 1), yielding a 180 kDa fragment containing the majority of the EC part of the receptor, and a 120 kDa fragment consisting of a membrane tethered intracellular domain with a short EC region (Logeat, Bessia et al. 1998). The two fragments are held together non-covalently and on the continuous transportation through the trans-Golgi network the bipartite form of Notch receptor is further modified for glycosylation. After the addition of the first fucose by the enzyme O-fucosyl transferase (Ofut), the Notch receptor can be further glycosylated at the multiple EGF-like repeats in the EC part by the N-glycosyl transferase Fringe family (Bruckner, Perez et al. 2000; Moloney, Shair et al. 2000; Munro and Freeman 2000; Wang, Shao et al. 2001; Panin, Shao et al. 2002). Thus a matured Notch receptor appears as a heterodimeric molecule at the cell surface with glycosylation imprints.

S2 cleavage occurs following the ligand binding to the Notch extracellular domain and releases the majority of the extracellular domain. This cleavage is thought to be mediated by the Tumor Necrosis Factor-α Converting Enzyme (TACE), a disintegrin and metalloprotease domain (ADAM) protein in vertebrates (Brou, Logeat et al. 2000). The EGF-like repeats 11 and 12 of the Notch receptor are necessary and sufficient for binding to both Delta and Serrate ligands (Rebay, Fleming et al. 1991). Modification of the Notch receptor by Fringe potentiates activation by Delta and renders Notch resistant to activation by Serrate (Fleming, Gu et al. 1997; Pan and Rubin 1997). The underlying biochemical mechanism is not well understood, but it has been suggested that glycosylation at the EGF-like repeats alters the capability of ligands to bind Notch receptor differentially.

Although the DSL domain is required to produce NICD from full-length Notch receptor, it is still unclear how ligand-binding promotes S2 cleavage by ADAM, a necessary step for γ-secretase mediated S3 cleavage. It has been proposed that ligand endocytosis is required for receptor activation by promoting heterodimer dissociation (Klueg and Muskavitch 1999; Parks, Klueg et al. 2000; Ahimou, Mok et al. 2004), which is discussed in detail as follows. On the other hand, ligand-independent activation of Notch receptor points to that allosteric conformation change of the intact Notch receptor is critical for ADAM mediated S2 cleavage (Nichols, Miyamoto et al. 2007). The addition of the chelating agent EDTA results in activation of the Notch receptor, probably through the removal of calcium ions from the LNR-region of the receptor and thus making the protein conformation susceptible to S2 cleavage (Rand, Grimm et al. 2000). Missense mutations in the heterodimerization domain of the Notch receptor characterized in T-ALL alters the heterodimer stability and results in constitutive activation independent of ligand (Weng, Ferrando et al. 2004; Malecki, Sanchez-Irizarry et al. 2006). More interestingly, amino acid insertions near the S2 cleavage site do not alter heterodimer stability but nonetheless generate constitutively active Notch signaling (Weng, Ferrando et al. 2004; Malecki, Sanchez-Irizarry et al. 2006). The inserted residues are suggested to allosterically expose the S2 cleavage site within the intact heterodimer of Notch receptor. In *C. elegans* secreted soluble ligands lacking the transmembrane domain retain the signaling potential (Chen and Greenwald 2004). In keeping with this, when clustered soluble ligands have been

shown to activate Notch signaling in cultured mammalian cells (Varnum-Finney, Wu et al. 2000). S2 cleavage remains an important aspect for investigation, particularly because ADAM metalloprotease is revealed for the potential regulation by external factors, membrane environment and intracellular signaling pathways.

Rapidly following S2 cleavage, the membrane anchored Notch receptor is constitutively cleaved at a third site (S3) and the signal mediator NICD is released from the membrane. S3 cleavage is executed in the transmembrane region of Notch receptor by a large enzymatic complex known as γ-secretase complex. The mechanism of this particular cleavage has attracted considerable interest as the amyloid precursor protein (APP) is also processed by γ-secretase complex (De Strooper, Saftig et al. 1998). Aberrant APP cleavage may underlie neoplague formation in Alzheimer's disease, and the prospect of making γ-secretase inhibitors (GSI) has been a central goal for the pharmaceutical industry. Currently GSI have too many side effects, because of the block of Notch signaling, to be used clinically. A functional γ-secretase complex consists of membrane spanning protein presenilin, nicastrin, Pen-2 and Aph-1, which are all required for catalytic activity (Francis, McGrath et al. 2002; Goutte, Tsunozaki et al. 2002). Besides Notch receptor other substrates of γ-secretase complex include p75, ErbB4, E-cadherin, N-cadherin, APP, CD44 etc (Haass and Steiner 2002).

Endocytosis and Notch activation

Endocytosis is an essential cell-surface trafficking event that delivers soluble molecules, membrane components or receptors from the cell surface to membrane bound intracellular vesicles. It has recently become clear that endocytosis can not

only downregulate the signal by sending the receptor and /or ligand to a degradation compartment, but also contribute to the activation of the signal, modulate interactions between signaling molecules and their inhibitors, regulate receptor presentation at the cell surface and provide a localized environment where signaling takes place (Piddini and Vincent 2003). Following endocytosis the internalized cargoes are delivered to early endosomes, which function as sorting stations to rapidly segregate the recycling molecules and downregulated molecules away from each other. Whereas the recycling molecules return to the cell surface at least partly through recycling endosomes, downregulated molecules are collected in multivesicular bodies and then destined to late endosomes or lysosomes for resorting or degradation (Gruenberg and Stenmark 2004). In the case of Notch signaling, endocytosis provides a powerful means of fine-tuning Notch activity.

Genetic loss of function study of shibire (the *Drosophila* homolog of dynamin) in *Drosophila* gave the first evidence that endocytosis is necessary both in signal sending cells, to allow acquirement of signal-sending capability, and in signal-receiving cells, to promote ligand-dependent Notch activation (Chen, Obar et al. 1991; van der Bliek and Meyerowitz 1991). Since then more endocytic components have been identified to be essencial for the activation of Notch signaling. Genetic screens in *Drosophila* and zebrafish suggest epsin, mind bomb and neuralized (RING-finger-containing E3 ubiquitin ligases) as key regulators of DSL ligand endocytosis and required for signal sending capability (Deblandre, Lai et al. 2001; Lai, Deblandre et al. 2001; Pavlopoulos, Pitsouli et al. 2001; Yeh, Dermer et al. 2001). Ubiquitylation of DSL ligands by neuralized or mind bomb is proposed to serve as a signal for DSL ligand internalization by promoting ligand interaction with the endocytic adaptor protein epsin and subsequent endocytosis. To be noted, ubiquitylated DSL ligands are only in the presence of epsin competent to activate Notch receptor on neighboring cells. Although ubiquitylation and endocytosis are clearly required for Notch signaling, the exact mechanism of the modifications is still under debate. One model has been proposed, due to the detection of the extracellular part of the Notch receptor in the signaling cell, that ligand endocytosis is needed for generation of mechanical forces to remove the EC part of the receptor and expose membrane tethered receptor for proteolysis (Klueg and Muskavitch 1999; Parks, Klueg et al. 2000; Ahimou, Mok et al. 2004). In the receptor-expressing cell, monoubiquitylation and clathrin-

dependent endocytosis of the S2 cleaved receptor has been reported to be prerequisites for processing by the γ-secretase complex (Gupta-Rossi, Six et al. 2004). The physiological location of γ-secretase cleavage of Notch is controversial and data to support cleavage at the cell surface as well as within endosome has been reported. Endosomes may be attractive sites for γ-secretase proteolysis as the low pH environment, like in endosome, can enhance the catalytic activity of γ-secretase (Pasternak, Bagshaw et al. 2003). Mutations in vps25 and vps23/tsg101, components of ESCRT (endosomal sorting complex required for transport) in *Drosophila* result in overactivation of Notch signaling in the mutant cells, perhaps in a ligand independent way (Thompson, Mathieu et al. 2005; Vaccari and Bilder 2005; Herz, Chen et al. 2006).

Transcriptional switch

Following Notch activation and S3 cleavage (see above), the intracellular domain of Notch translocates to the nucleus and directly induces transcription of downstream target genes. Although it is the primary effector of the canonical Notch signaling pathway, NICD cannot bind to cognate DNA by itself, but interacts with the protein CSL (CBF-1/Suppressor of Hairless/Lag-1, RBP-Jk) (Tamura, Taniguchi et al. 1995), which is a highly conserved DNA-binding protein through evolution. In the absence of Notch ICD, CSL represses transcription (Zeng, Dou et al. 1994) by interacting with ubiquitous corepressor proteins to form multiprotein transcriptional repressor complexes, which in turn recruits histone deacetylase complexes (HDACs) to the site and convert the local chromatin into a transcriptionally silent state. Different corepressors are utilized for specific interaction with CSL. In *Drosophila*, Su(H) uses Hairless to recruit both CtBP (C-terminal binding protein) and Groucho corepressor complexes. In vertebrates, CSL associates with SMRT (silencing mediator of retinoid and thyroid receptors)/N-CoR (nuclear receptor corepressor) (Zhou, Fujimuro et al. 2000), SHARP (also known as MINT/SPEN) (Oswald, Kostezka et al. 2002) and CIR (CBF1-interacting corepressor) (Hsieh, Zhou et al. 1999). In the presence of NICD, a group of transcriptional coactivators are recruited in association with CSL, leading to the switch from transcriptional repression to activation of genes with CSL binding sites. The transcriptional regulator SKIP (Ski-interacting protein) bridges interactions between CSL and either NICD or corepressors, but not both simultaneously, and SKIP is thought to be present during both transcriptional activation and repression (Zhou,

Fujimuro et al. 2000). Activation of transcription occurs by NICD displacement of corepressors, formation of the CSL–NICD–MAML ternary complex (Wu, Sun et al. 2002) and recruitment of the general transcription factors PCAF/GCN5 (Kurooka and Honjo 2000) and CBP/p300 (Kurooka and Honjo 2000) to the transcriptional activator complex. Identification of the NICD binding sites on CSL was greatly aided by initial deletion mutagenesis studies and more recently by several crystal structures of DNA-bound CSL proteins (Nam, Sliz et al. 2006; Wilson and Kovall 2006). CSL is composed of three integrated domains: a N-terminal Rel homology domain (NTD), a central beta-trefoil domain (BTD) and a C-terminal Rel. The RAM domain of NICD binds to the BTD of CSL, which induces conformation change for corepressors displacement (Wilson and Kovall 2006). The interaction between CSL and the ankyrin repeats of NICD creates a groove for binding to the N-terminal part of MAML (Nam, Sliz et al. 2006). Recently a new dynamic model has been proposed for the interaction of CSL on cognate DNA. A fast exchange and low occupancy is detected at target DNA sites when CSL complexes with corepressors (Krejci and Bray 2007). Activation of Notch receptor significantly facilitates CSL occupancy at target DNA sites, therefore making it competent to make cooperative interactions with transcription complex (Krejci and Bray 2007).

As CSL is the focal point of transcriptional switch, it is not surprising that the arrangement and spacing of CSL-binding sites are important determinants in the selectivity and sensitivity of target genes to variation in Notch signal strength and developmental context (Ong, Cheng et al. 2006). The core CSL-binding sites (TGGGAA) are highly conserved through evolution, but the flanking sequence is varied dependent on species and target genes, which might contribute to the difference of binding affinity for CSL. Recent studies have revealed paired CSL-binding site in a number of Notch target genes, including genes of the hairy/enhancer-of-split family in organism ranging from *Drosophila* to humans (Ong, Cheng et al. 2006; Nam, Sliz et al. 2007). The so-called paired CSL-binding site is characterized in a head-to-head orientation of two CSL-binding sites that typically are separated by 16 or 17 nucleotides (Nam, Sliz et al. 2007). CSL itself does not dimerize on cognate DNA sites, but the assembled CSL-NICD-MAML cooperatively dimerize on paired CSL-binding site dependent on the ankyrin repeats of NICD (Nam, Sliz et al. 2007). The study suggests that cooperative formation of dimeric Notch transcription

complexes on promoters with paired CSL-binding sites ensure tight transcriptional regulation of target genes.

Downstream target genes

The effects downstream of canonical Notch signaling pathway are not completely understood, but two families of basic helix-loop-helix transcription factors, Hes and Hey (the latter also known as Herp, Hesr, HRT, CHF and gridlock) have been first well established to be primary downstream targets following Notch activation (Sasai, Kageyama et al. 1992; Jennings, Preiss et al. 1994; Jarriault, Brou et al. 1995; Kokubo, Lun et al. 1999; Leimeister, Externbrink et al. 1999; Nakagawa, Nakagawa et al. 1999; Troulis, Kearns et al. 2000; Zhong, Rosenberg et al. 2000). Hes and Hey genes are evolutionarily closely related and their proteins differ basically in that Hes proteins contain a conserved proline residue in the basic region, while Hey proteins do not. Both Hes and Hey function as transcriptional repressors and in turn reduce expression or block the function of tissue-specific genes promoting differentiation, such as Neurogenin (Cau, Gradwohl et al. 1997), Mash (Van Doren, Bailey et al. 1994; Chen, Thiagalingam et al. 1997) and MyoD (Kopan, Nye et al. 1994). Thus the role of Notch signaling activation through Hes and Hey is to keep the signal-receiving cells in an undifferentiated state. Two mechanisms have been proposed for the biological outcome through Hes and Hey. It has been shown that Hes and Hey proteins form homo- or heterodimer to bind to E-box motifs on promoters and actively repress the transcription of downstream effectors by recruitment of corepressor Groucho/TLE (Paroush, Finley et al. 1994; Grbavec and Stifani 1996). Additionally, Hes and Hey can directly sequester important transcriptional cofactors without DNA binding (Sasai, Kageyama et al. 1992; Hirata, Ohtsuka et al. 2000). For example, Hes and Hey proteins heterodimerize with other bHLH factors such as E12/E47, thereby disrupting functional interaction with MyoD or Mash1.

Given the pleiotropic function of Notch signaling in development, it is evident that there are more primary targets downstream of canonical Notch signaling. Microarray is a powerful tool for global analysis of gene expression and to date, a few independent microarray studies have been performed to identify potential Notch target genes in different contexts, such as in T-ALL cells, keratinocytes or myoblast C2C12 cells (Nguyen, Lefort et al. 2006; Weng, Millholland et al. 2006). These

results have extended our understanding of the downstream effectors, which Notch signaling can exert especially in tissue-specific manner. Based on the presence of CSL-binding sites in the promoter regions and experimental data from chromatin immunoprecipitation, a few new Notch target genes have been generally accepted, including pre-Tα (Reizis and Leder 2002), interleukin-6, glial fibrillary acidic protein (GFAP) (Ge, Martinowich et al. 2002), Cyclin D1 (Ronchini and Capobianco 2001), p21 (Rangarajan, Talora et al. 2001), SKP2 (Sarmento, Huang et al. 2005; Dohda, Maljukova et al. 2007), c-Myc (Weng, Millholland et al. 2006; Sharma, Draheim et al. 2007) and smooth muscle α-actin (Noseda, Fu et al. 2006). It should be noted that expression of these target genes induced by Notch activation is strictly dependent on the cell context, suggesting that additional specific proteins may be involved in regulating their expression.

Besides the canonical CSL-dependent mechanism, some evidence suggests that Notch signaling can also function in a CSL-indpendent way. Observations from myogenic differentiation argue that Notch receptors that have not undergone S1 cleavage (and hence no other cleavages) still block myogenesis, and that NICD constructs lacking the CSL-interacting domain exert effects in blocking myognesis (Bush, diSibio et al. 2001). Furthermore, certain *Drosophila* Notch alleles (mcd alleles) seem to function in a Su(H)-independent but Deltex-dependent manner (Ramain, Khechumian et al. 2001). In line with this, activation of Notch signaling promotes Bergmann glia differentiation through Deltex, but not Su(H). Possible CSL-independent mechanisms are proposed that Notch can exert action simply by interacting with Deltex in the cytoplasm or transcriptional factors other than CSL in the nucleus, such as Mef2 or LEF1 (Wilson-Rawls, Molkentin et al. 1999; Ross and Kadesch 2001).

Turning off the signal

To keep balance and stay responsive to new input, cells have developed effective means to tightly control the signaling strength. For Notch signaling, MAML acts as both coactivator and terminator for NICD transcriptional activity. It has been shown that MAML directly recruits cyclin C:Cdk8 complex to the CSL-NICD-MAML transcriptional coactivator complex in the nucleus, followed by phosphorylation of NICD within the TAD and PEST region (Fryer, White et al. 2004). Three groups have

demonstrated that Sel-10/cdc4/Fbw7 is an E3 ubiquitin ligase, inducing polyubiquitylation of phosphorylated NICD and triggering the rapid degradation by proteasome (Gupta-Rossi, Le Bail et al. 2001; Oberg, Li et al. 2001; Wu, Lyapina et al. 2001). The proteosome-dependent turnover of NICD depends on its C-terminal PEST region.

Regulation of Notch signaling activity

Over the past few years, it has become increasingly apparent that signal transduction pathways are coordinated into complex signaling networks to generate precise and unique cell responses. In this section regulation of Notch signaling activity will be described at two levels: integration with various signaling pathways and interaction with intrinsic proteins that do not belong to a particular signaling mechanism but play a role in regulating Notch signaling.

Crosstalk with other signaling pathways

Notch and TGFβ/BMP

Both Notch and TGFβ/BMP play critical roles in cell fate determination and exert similar functions in a number of developmental processes, including myogenic, endothelial and neuronal differentiation. A direct link between Notch and TGFβ/BMP pathway was originally described in several recent papers. Activation of the TGFβ/BMP pathway, via SMADs (known as TGFβ/BMP effectors), is shown to relay the signaling information into the Notch pathway and enhance expression of Notch primary downstream genes (Hes1 and Hey1) (Blokzijl, Dahlqvist et al. 2003). An interaction between the intracellular mediators in the two pathways, SMADs and NICD, is also observed. A synergistic effect between Notch and BMP signals is reported to block myogenic differentiation (Oberg, Li et al. 2001), whereas a more interesting functional interaction between these two pathways is observed during migration of endothelial cells (EC) (Itoh, Itoh et al. 2004). When EC cells without cell-cell contact are exposed to secreted BMP ligands, BMP downstream target gene Id1 is induced and promotes EC migration. Upon contact with Notch ligand expressing cells, Notch signaling is activated, leading to a synergistic activation of

Hey1 expression, which subsequently promotes degradation of Id1 and blocks migration. However, upregulation of Hey1 induced by TGFβ is recently reported to be necessary for epithelial-mesenchymal transitions (EMT) in kidney and mammary gland epithelial cells (Zavadil, Cermak et al. 2004). Another novel finding indicates that Notch signaling is required for the epithelial cell cycle arrest induced by TGFβ. Their data suggests that induction of Jagged1 by TGFβ and subsequent activation of Notch signaling contribute to p21 gene upregulation and epithelial cytostasis (Niimi, Pardali et al. 2007).

Notch and Ras/MAP kinase

The interactions between Notch and Ras/MAP kinase are complex, which can be cooperative or antagonistic, depending on the cellular context (Sundaram 2005). Initial insights into the crosstalk between the two pathways stem from the C. elegans vulva development. The adoption of primary cell fate (the 1^0 cell) in one of the six vulva precursor cells requires inductive signal of Ras-EGFR-MAP kinase, which results in the degradation and endocytosis of Notch in the 1^0 cell (Shaye and Greenwald 2002). In contrast, in the two neighboring vulva precursor cells, which adopt a second cell fate (the 2^0 cell), Notch signaling is activated and subsequently Ras/MAP kinase signaling is negatively regulated by Notch target gene expression of *lst* (Yoo, Bais et al. 2004). A different interaction mode is recently found in tumor angiogenesis. Ras/MAP kinase signaling induces upregulation of Notch ligand Jagged1 expression in head and neck squamous cell carcinoma, which in turn activate Notch signaling in the neighboring endothelial cells, promoting angiogenesis and tumor development (Zeng, Li et al. 2005). Regarding tumor formation, active Notch signaling has been reported to be required for neoplastic phenotype in Ras-transformed cells (Weijzen, Rizzo et al. 2002), whereas in mouse mammary gland tumors Notch mediated tumor transformation requires active Erk/MAP kinase signaling (Fitzgerald, Harrington et al. 2000). A novel mode of antagonistic interaction between Notch and Ras/MAP kinase has been proposed in *Drosophila* vein formation and bristle patterning, where MAP kinase induced phosphorylaion of Groucho reduced its repressor activity and assistance for Hes mediated transcriptional repression (Hasson, Egoz et al. 2005).

Notch and Wnt

The interplay between Notch and Wnt (Wingless in *Drosophila*) signaling pathway occurs at several molecular levels in the pathways. First direct interaction between the two pathways is observed during the eye patterning in *Drosophila*, where Wingless signaling acts upstream and negatively regulate Notch signaling, probably through interaction between Dishevelled, a cytoplasmic adaptor protein in the Wnt pathway, and NICD (Strutt, Johnson et al. 2002). Secondly, NICD interacts with LEF1 in the nucleus, thus potentiating the transcriptional activity of LEF1, which is a transcriptional activator in the Wnt pathway (Ross and Kadesch 2001). The third convergence point is GSK3, a Wnt intracellular mediator, which can phosphorylate and stabilize NICD (Foltz, Santiago et al. 2002). A recent link between Wnt and Notch signaling is demonstrated in keratinocytes where Notch acts as tumor suppressor in association with downregulation of Wnt signaling. This is achieved, at least in part, by suppression of Wnt4 gene expression by the Notch target p21 (Devgan, Mammucari et al. 2005). Nrarp, a Notch downstream gene negatively regulating Notch (Lamar, Deblandre et al. 2001), is recently shown to stabilize the LEF1 protein in neural crest cells (Ishitani, Matsumoto et al. 2005). Ablation of the Notch 1 gene in the mouse epidermis led to derepression of β-cantenin signaling (Nicolas, Wolfer et al. 2003). Finally, Notch signaling has been shown to be necessary for Wnt-mediated maintenace of hematopoietic stem cells in the undifferentiated state (Duncan, Rattis et al. 2005).

Notch and Hypoxia

Hypoxia is a physiological process that regulates cell differentiation during development as well as plays a crucial role in cardiovascular disease and cancer. Many of the intracellular aspects of the hypoxic response are principally similar to the intracellular routing of signal pathways. Reduced oxygen levels lead to the stabilization of hypoxia inducible factor (HIF) 1α, which is a target of the Von Hippel-Lindau (VHL) tumor suppressor for ubiquitination and proteosomal degradation at normoxia (Bruick 2003). Subsequently stabilized HIF-1α is relocated to the nucleus and activates transcription of genes through dimerization with HIF-1β. The initial observation that hypoxia upregulates Notch downstream target genes in a microarray study provokes an important investigation on the relationship between

Notch signaling and hypoxia (Gustafsson, Zheng et al. 2005). It is found that HIF-1α interacts with NICD and is recruited to the Notch target genes in a Notch-dependent manner. Upregulation of Notch signaling is required to maintain undifferentiated stem/progenitor cells in hypoxia state. In addition, induction of Notch ligand expression could be another mechanism for upregulation of Notch signaling in hypoxia. For example, hypoxia induces the expression of Notch ligand Deltalike4 in endothelial cell, thus potentiating Notch signaling (Mailhos, Modlich et al. 2001; Patel, Li et al. 2005).

Notch-interacting proteins

The adaptor protein Numb was originally identified as an inhibitor of Notch signaling in *Drosophila melanogaster* (Uemura, Shepherd et al. 1989). It plays a key role in establishing the asymmetric cell divisions during the proper development of *Drosophila* sensory neurons. Numb interacts directly with the cytoplasmic domain of Notch, but the mechanism by which Numb negatively regulates Notch signaling is still an open issue. The involvement of E3 ubiquitin ligase Itch (McGill and McGlade 2003), the transmembrane protein Sanpodo (Skeath and Doe 1998; Cayouette and Raff 2002; O'Connor-Giles and Skeath 2003) and the endocytic machinery (Berdnik, Torok et al. 2002) has been suggested to inhibit Notch signaling in different models, which are described in detail in the next section.

Deltex is another intrinsic regulator of Notch signaling. Direct interaction has been reported between Deltex and the ankyrin repeats of NICD (Diederich, Matsuno et al. 1994; Matsuno, Diederich et al. 1995). However, the role of Deltex in tuning Notch signaling is complicated. Originally Deltex was identified to be a positive regulator of Notch signaling in a *Drosophila* genetic study (Xu and Artavanis-Tsakonas 1990). Intriguingly mammalian Deltex homologue 1 (mDTX1) antagonizes Notch-dependent effects on neurite outgrowth (Sestan, Artavanis-Tsakonas et al. 1999) and overexpression of mDTX1 favors the differentiation of hematopoietic stem cell towards the lineage of B cells at the expense of T cells (Izon, Aster et al. 2002), consistent with inhibition of Notch 1 function. At the molecular level *Drosophila* Deltex is able to form trimeric protein complex with Notch and Kurtz, a homologue of mammalian β-arrestin (Mukherjee, Veraksa et al. 2005). Thus by coupling Notch

with β-arrestin, Deltex promotes the internalization of Notch receptors at the cell surface. In agreement with this notion, loss of Kurtz results in elevated membrane levels of Notch receptor and produces phenotypes of elevated Notch signaling in the wing. However, Deltex mutants show the opposite phenotype, so the precise relationship between Deltex and Notch remains to be elucidated.

Endocytosis has been reported to be required for Notch activation in the receptor expressing cells. However there is no evidence for a direct link between Notch receptor and components of the endocytic machinery. My unpublished data on the study of the localization of the S2-cleaved Notch receptor suggests that a membrane tethered Notch receptor physically interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a molecule well-known for coordinating endocytotic trafficking. Interstingly the coiled-coil domain2 but not the ubiquitin interacting motif (UIM) of Hrs is required for binding to Notch (unpublished data). Another adaptor protein intersectin, which is involved in clathrin-dependent endocytosis, is under investigation for interaction with Notch as its coiled-coil domain shares relatively high homology to that of Hrs.

Numb and Numblike – negative regulators of Notch signaling

Genetic studies in *Drosophila* have shown that Numb acts upstream of Notch and antagonizes its activity to determine cell fate during the development of external sensory organs and specific neurons of the peripheral and central nervous system (Uemura, Shepherd et al. 1989; Frise, Knoblich et al. 1996). The sensory organ precursor (SOP) cells divide asymmetrically, giving rise to the IIA and IIB cells; the IIA cell subsequently divides again to yield two outer (hair and socket) cells, while the IIB cell also undergoes a further asymmetric division to generate two inner (sheath and neuron) cells (Jan and Jan 1998). As the precursor prepares to divide, Numb protein is asymmetrically localized and preferentially distributed into only one of the two daughter cells (Uemura, Shepherd et al. 1989; Frise, Knoblich et al. 1996).

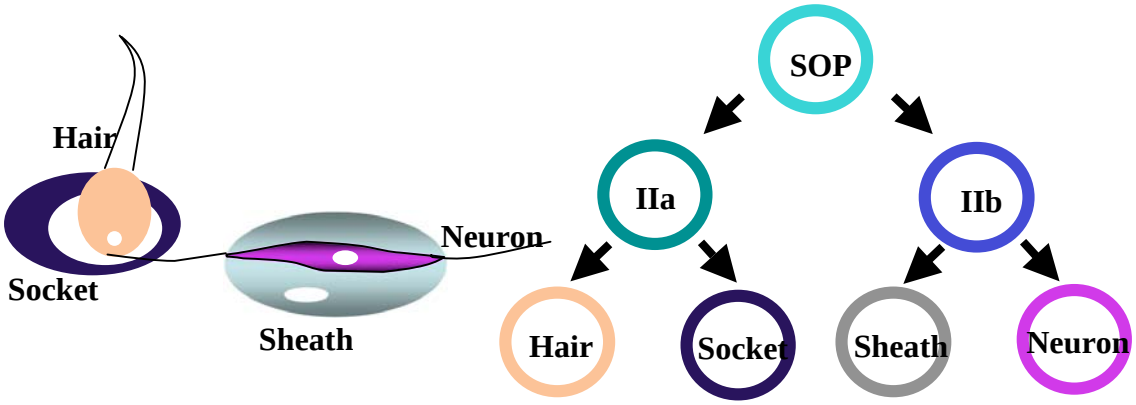

Figure 4: Diagram of the cell fate specification in the external sensory organ lineage.

Drosophila Numb is a membrane associated protein and has two mammalian homologues, Numb and Numblike (Verdi, Schmandt et al. 1996; Zhong, Feder et al. 1996; Zhong, Jiang et al. 1997). Structurally, Numb resembles an adaptor protein that possesses an N-terminal phosphotyrosin-binding (PTB) domain and a C-terminal proline rich region (PRR). Numb also has two tripeptide sequence motifs associated with endocytic proteins: an Eps 15 homology binding motif (Asn-Pro-Phe) (Salcini, Confalonieri et al. 1997) and two binding sites for α-adaptin (Asp-Pro-Phe), a component of the AP-2 clathrin adaptor complex (Berdnik, Torok et al. 2002). *Drosophila* and mammalian Numb interact with the NICD through the PTB domain

(Guo, Jan et al. 1996; Zhong, Feder et al. 1996; Wakamatsu, Maynard et al. 1999), but the mechanism by which Numb negatively regulates NICD is not well established. According to one model, mammalian Numb interacts with Itch, a member of Nedd4 family of HECT domain E3-ubiquitin ligases, and together they act to ubiquitylate membrane-tethered forms of Notch 1 leading to decreased stability of NICD (McGill and McGlade 2003). A second model proposes a role for the *Drosophila* Sanpodo gene in Numb's ability to suppress Notch during asymmetric cell divisions. Sanpodo encodes a unique four-transmembrane spanning protein that functions at the cell membrane to promote Notch signaling by an unknown mechanism. Numb interferes with Sanpodo function by preventing membrane localization of Sanpodo and thereby antagonizing Notch signaling (Skeath and Doe 1998; Cayouette and Raff 2002; O'Connor-Giles and Skeath 2003). A third possible mechanism is related to Numb's ability to interact with components of the endocytic machinery such as α-adaptin and Eps15. The interaction between a-adaptin and Numb is required for asymmetric segregation of α-adaptin and appropriate cell lineage decision following division of *Drosophila* sensory organ precursors (Berdnik, Torok et al. 2002). Thus, by coupling with the endocytic machinery Numb causes endocytosis and sequestration of full-length, membrane-spanning S2-cleaved Notch receptor or NICD.

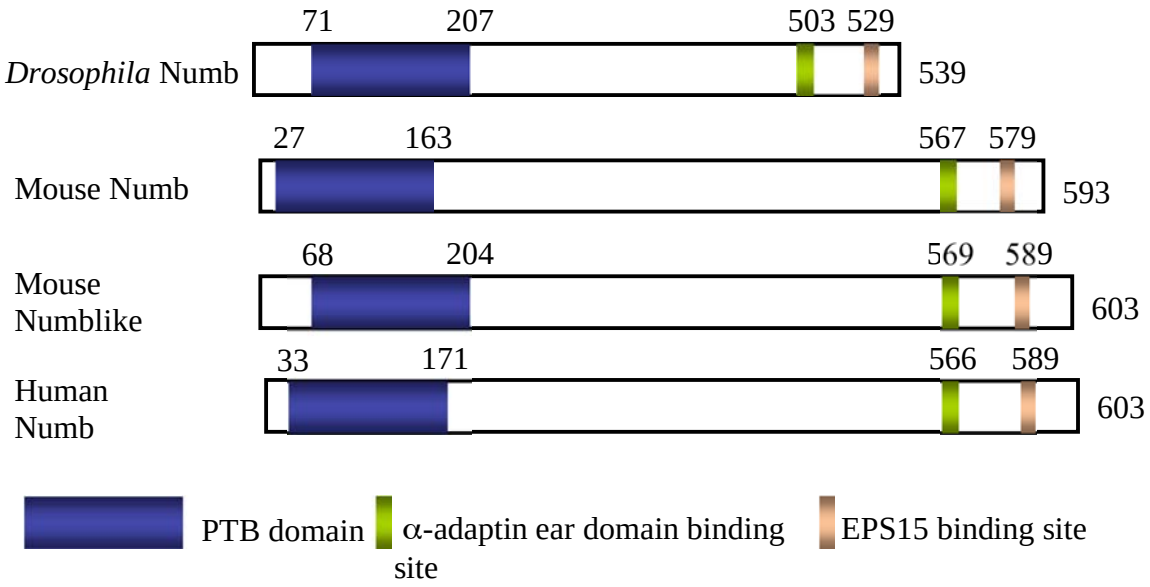

Figure 5: Schematic depiction of Numb protein of different species.

Mammalian Numb has four isoforms processed through alternative splicing, which differ by small insertions of 11 amino acids in the PTB domain and 48 amino acids in the PRR region (Dho, French et al. 1999; Verdi, Bashirullah et al. 1999). The PTB domain is important for the intracellular localization of Numb proteins. Numb isoforms with an insertion in the PTB domain are membrane associated protein, whereas Numb isoforms with shorter PTB domain are cytoplasmic protein (Dho, French et al. 1999). It is still debated in different studies whether Numb is localized in the endosome compartment and trans-Golgi network (Santolini, Puri et al. 2000; Nishimura and Kaibuchi 2007). Distinct cellular functions are also observed in different Numb isoforms when they are ovexepressed in the P19 embryonal carcinoma cell line. Differentiation but not proliferation is promoted by Numb isoform with short PRR region (Verdi, Bashirullah et al. 1999). In contrast, proliferation but not differentiation is directed by Numb isoform with long PRR region (Verdi, Bashirullah et al. 1999).

The asymmetric segregation of Numb between daughter cells is important for cell fate decision. The underlying mechanism was poorly understood until a recent study, which shows that phosphorylation of Numb by atypical protein kinase C (aPKC) is necessary to maintain its asymmetric localization in both mammalian epithelial cells and mitotic *Drosophila* SOP cells (Smith, Lau et al. 2007). It should be noted that localization of Numb is also dependent on its interaction with lethal giant larvae (Lgl) through the adaptor protein Partner of Numb (Pon) (Betschinger, Mechtler et al. 2003). Polo, a key cell cycle regulator, plays an important role in Numb localization by directly phosphorylating Pon (Wang, Ouyang et al. 2007).

In vertebrates, Numb and Numblike have redundant functions and are expressed widely in tissues and organs. Although genetic studies in *Drosophila* have clearly demonstrated that Numb inhibits Notch signaling, all mouse mutant analyses do not support such a function. Numblike mutant mice were viable, fertile and indistinguishable from wild type, whereas Numb mutants died around E11.5 (Zhong, Jiang et al. 2000; Zilian, Saner et al. 2001). Studies on conditional knockout of both genes in the developing nervous system show controversial results. One group found widespread precocious neuronal differentiation (Petersen, Zou et al. 2002; Petersen, Zou et al. 2004), while the other group found that the neural progenitor pool was

expanded at the expense of neuronal differentiation (Li, Wang et al. 2003). The difference of the observed phenotype could be due to the timing of the deletion by driving Cre at different stages of neurogenesis. An alternative explanation is that Numb may also act in a Notch-independent way. Numb and Numblike can interact with cadherins to maintain cell-cell adhesion of radial glial cells, or to regulate the integrity of the adult CNS stem cell niche (Kuo, Mirzadeh et al. 2006; Rasin, Gazula et al. 2007). Furthermore, Numb has been demonstrated to direct endocytosis of integrins at the leading edge of migrating cells (Nishimura and Kaibuchi 2007).

Although Numb is an evolutionarily conserved developmental protein, the downregulation of Numb has been found in correlation with human breast cancer and medulloblastoma. Loss of Numb control over Notch signaling contributes to ~50% of human mammary carcinomas, in which specific Numb ubiquitination and proteasomal degradation is enhanced (Pece, Serresi et al. 2004). Mechanistically, Numb acts as tumor suppressor as its enforced expression in Numb-negative, but not Numb-positive tumor cells reverts Notch signaling to basal level and inhibits proliferation. In the developing cerebellum Numb antagonizes Hedgehog signal and promotes growth arrest and differentiation of cerebellar granule cell progenitor (GCP), by targeting the Hedgehog transcription factor Gli1 for Itch-dependent ubiquitination and degradation (Di Marcotullio, Ferretti et al. 2006). Therefore, decreased expression of Numb emerges to be relevant to medulloblastoma, which is the result of an interruption in the process of GCP maturation.

Notch signaling in cancer

Given the broad range of processes that require normal Notch signaling, it is not surprising to find that a number of human diseases and cancers are caused by mutations in components of Notch pathway and/or dysregulation of Notch signaling. The mounting evidence suggests two faces of Notch signaling in cancer: oncogene and tumor suppressor. Again the varied and complex roles of Notch in cancer depend on the dose and context.

Notch as an Oncogene

The first compelling evidence for a Notch oncogenic function was found in patients suffering from T-cell lymphoblastic leukemia (T-ALL). About 1% of the cases possess a specific chromosomal translocation, t(7;9)(q34;q34.3), resulting in the fusion of the carboxy-terminal region from within the EGF-repeat 34 of Notch 1 to the enhancer sequences of the T cell antigen receptor β subunit (TCR-β) (Reynolds, Smith et al. 1987; Ellisen, Bird et al. 1991). Importantly, the t(7;9) translocation in all T-ALLs drives the expression of a truncated Notch 1 receptor that corresponds to N1ICD, which behaves in a constitutively active fashion. More recently two types of activating mutations within Notch 1 were found in 55-60% of human T-ALL cases (Weng, Ferrando et al. 2004). The first type occurs in the heterodimerization region and leads to ligand-independent activation of Notch receptor. The second type involves the C-terminal PEST region of Notch receptor, resulting in the stabilization of NICD. Synergistic mutations of these two types are found together in *cis* in 10-20% of human T-ALL (Weng, Ferrando et al. 2004). Although Notch activation seems to be dysregulated in human T-ALL, the receptor is still dependent on γ-secretase cleavage for activation. Therefore γ-secretase inhibitors (GSI) offer an attractive targeted therapy for human T-ALL dependent on deregulated Notch activity. However, GSI resistance is observed in a large fraction of human T-ALL lines and primary leukemias with aberrant activation of Notch signaling. Recently several groups have found independently that inactivating mutations or homozygous deletion of the gene FBW7 (human Cdc4, Sel-10, Ago) in human T-ALL contributes to the GSI resistance (Malyukova, Dohda et al. 2007; O'Neil, Grim et al. 2007; Thompson, Buonamici et al. 2007). FBW7 is a ubiquitin ligase implicated in NICD turnover (Gupta-Rossi, Le Bail et al. 2001; Oberg, Li et al. 2001; Wu, Lyapina et al.

2001) and these mutations abrogate the binding of FBW7 not only to NOTCH1 but also to another two characterized targets, c-Myc (Yada, Hatakeyama et al. 2004) and cyclin E (Minella, Welcker et al. 2005). Stabilization of both NICD and its principle downstream target, Myc, may contribute to transformation in leukemias with FBW7 mutations. More interestingly a recent study shows that loss of PTEN is another critical determinant of GSI resistance in T-ALL dependent on Notch signaling (Calzavara, Chiaramonte et al. 2007; Palomero, Sulis et al. 2007). In the absence of PTEN, the uninhibited Akt activation leads to aberrant prosurvival and proliferative events independent of Notch signaling activity (Calzavara, Chiaramonte et al. 2007). Moreover, many of the same GSI-resistant T-ALL cells are found to harbor both dominant-negative FBW7 mutations and mutations or deletions of PTEN (Palomero, Sulis et al. 2007). These data suggest that FBW7 and PTEN are novel tumor suppressors in T cell leukemia, and implicate the loss of FBW7 and PTEN function as a potential mechanism of drug resistance in T-ALL. A breakthrough on the crucial targets for the transformation by Notch signaling is the identification of c-Myc as direct target of Notch1 in T-ALL and in a critical stage of normal pre-T cell development (Weng, Millholland et al. 2006). The study shows that c-Myc inhibitors interfere with progrowth effects of activated Notch1 and that forced c-Myc expression rescues Notch1-dependent T-ALL cell lines from Notch withdrawal (Weng, Millholland et al. 2006).

During lymphopoiesis, T cell precursors form in bone marrow and migrate to the thymus to become CD4-CD8- double negative (DN) progenitors, which can be subdivided into three consecutive developmental stages based on the expression of CD117 (c-kit), CD44, and CD25 (Godfrey, Kennedy et al. 1993). Following β-selection, the DN progenitors further differentiate into CD4+CD8+ double positive (DP) progenitors. Mouse models have provided insight into the effects of Notch signaling strength and timing on the normal T-cell development and T-ALL pathogenesis. Notch 1 has a non-redundant role for the initial commitment to T-cell fate and subsequent maturation of uncommitted DN T cell progenitors to DP pre-T cells (Huang, Gallegos et al. 2003). Presumably Notch leukemogenic activity stems from the exaggeration of a normal developmental function. In mouse T-ALL models, Notch signaling is maintained at high levels in post-β-selection thymocytes at DN3

stage (CD117+CD44-CD25+) in contrast to the normal situation (Ciofani, Schmitt et al. 2004; Ciofani and Zuniga-Pflucker 2005; Ciofani, Knowles et al. 2006).

In addition to T-ALL, the most compelling evidence for Notch oncogenic effect in other context stems from breast cancer and melanoma. Tissue microarray studies have shown that high expression levels of Jagged1 and/or Notch 1 in human breast cancer are associated with a more aggressive disease course (Santagata, Demichelis et al. 2004; Reedijk, Odorcic et al. 2005; Bismar, Demichelis et al. 2006). As in T-ALL, c-Myc appears to be an important growth-promoting downstream target of Notch 1 in breast cancer (Klinakis, Szabolcs et al. 2006). Other work in breast cancer cell lines suggests that Notch 1 confers chemoresistance by inhibiting p53-mediated apoptosis (Beverly, Felsher et al. 2005). Melanoma is a skin cancer that originates from melanocytes, which are positioned at the epidermal-dermal junction and interspersed among the basal keratinocytes. The expression of Notch receptors and their downstream target genes is upregulated in primary human melanomas (Hoek, Rimm et al. 2004; Balint, Xiao et al. 2005). Notch1 activation is insufficient to transform melanocytes but enables primary melanoma cells to gain metastatic capability (Balint, Xiao et al. 2005; Liu, Xiao et al. 2006). The oncogenic effect of Notch signaling correlates with the activation of Wnt signaling in melanoma cells, which promotes the expression of adhesion molecule N-cadherin (Balint, Xiao et al. 2005).

Notch as a tumor suppressor

Evidence that Notch signaling is not exclusively oncogenic but is also tumor suppressive comes from studies on skin. In human primary keratinocytes increased Notch 1 activity promotes commitment of self-renewing stem cells to transit-amplifying populations that continue to proliferate, although only for a limited time (Lowell, Jones et al. 2000; Rangarajan, Talora et al. 2001). In primary mouse keratinocytes Notch induces cell cycle arrest and entry into differentiation. Conditional ablation of Notch 1 in murine epidermis leads to epidermal hyperplasia within 25 days and skin carcinoma (basal cell carcinoma-like tumors) over time (Nicolas, Wolfer et al. 2003). Furthermore, Notch 1 deficiency in the skin facilitates chemical-induced carcinogenesis (Philipp, Vo et al. 1999; Topley, Okuyama et al. 1999; Weinberg, Fernandez-Salas et al. 1999). More recently conditional transgenic mice with a dominant negative form of MAML1, a pan Notch inhibitor, have a

hyperplastic epidermis and develop cutaneous squamous cell carcinomas that are associated with Wnt pathway activation (Proweller, Tu et al. 2006).

The tumor suppressive effect of Notch 1 in the epidermis appears to be mediated by induction of p21, an important inhibitor of cell cycle progression (Rangarajan, Talora et al. 2001). Notch 1 activation in differentiating keratinocytes functions directly to target RBP-Jκ to the p21 promoter (Rangarajan, Talora et al. 2001) as well as indirectly to act on the p21 TATA box proximal region through the interplay with Calcineurin-NFAT (nuclear factor of activated T cells) pathway (Mammucari, Tommasi di Vignano et al. 2005). In normal epithelium Notch 1 activation suppresses Wnt signaling, which is associated with maintenance of keratinocytes in their stem cell compartment. The mechanism for the negative interaction between Notch and Wnt has recently been established that in this context p21 represses Wnt4a expression through association with E2F1 transcription factors at the Wnt4a promoter (Devgan, Mammucari et al. 2005). Thus, the tumor suppressive function of Notch signaling in epidermis and in keratinocytes is to induce terminal differentiation processes and to withdraw proliferating cells from the cell cycle.

Notch in EMT and tumor progression

Epithelial–mesenchymal transition (EMT) is a fundamental process that involves the switch from polarized epithelial cells to contractile and motile mesenchymal cells. EMT takes place at critical phases of embryonic development such as gastrulation, formation of the neural crest cells from the neural tube, formation of the cardiac valve primordium during heart development etc (Thiery and Sleeman 2006). EMT is also involved in tumor progression when cells from a primary epithelia tumor lose cell polarity and intercellular adhesion, become mesenchymal, thus facilitating tumor metastasis. The molecular mechanisms underlying EMT are under intense study and it is found that EMT during embryonic development and tumor progression is controlled by the same signaling pathways, including transforming growth factor-β, Wnt, Notch, hypoxia and growth factors acting through tyrosine kinase receptors (Thiery 2002; Thiery and Sleeman 2006).

One of the first events that initiate EMT is the loss of the E-cadherin mediated cell-cell adhesion. Key transcriptional repressors of E-cadherin expression have been

identified to be the Snail superfamily of zinc-finger transcription factors (Batlle, Sancho et al. 2000; Cano, Perez-Moreno et al. 2000). Recent studies suggest that Notch induces EMT during cardiac valve development and acts upstream of Snail (also referred to as Snail1) in human endothelial cells to promote mesenchymal transformation (Timmerman, Grego-Bessa et al. 2004). However, in primary human breast tumor cells Jagged1 activation of Notch signaling upregulates the transcriptional repressor Slug (also referred to as Snail2) to initiate EMT and facilitate cancer cell metastasis (Leong, Niessen et al. 2007). Notch signaling has also been suggested to be required in the hypoxia-induced EMT and cell migration in tumor cells by controlling two key components of EMT, Snail1 and lysyl oxidase (LOX) (Sahlgren, submitted). Another mechanism of Notch-mediated tumor progression may involve the interplay with TGFβ signaling pathway, which is a well-known inducer of EMT during embryonic development and later stages of tumor progression. Evidence from the Zavadil's work shows that TGFβ induces Hey1 and Jagged1 expression at the onset of EMT in epithelial cells and the subsequent activation of Notch signaling following Jagged1 accumulation is necessary for the sustained induction of EMT (Zavadil, Cermak et al. 2004).

PRESENT INVESTIGATION

Aims

The work presented in this thesis aims to investigate the role of Notch signaling and Numb/Numblike in cell fate decision and the mechanism of Notch signaling in cancer. In particular, we focus on:

- The reciprocal regulation between Notch and its inhibitor Numb/Numblike protein
- The mechanism underlying the effect of aberrantly high Notch signaling on dysregulated cell cycle and transformation in T-ALL
- The role of Numblike in the early differentiation of mouse embryonic stem cells, with focus on early neural and mesodermal differentiation

Results and Discussion of papers

Reciprocal regulation between Notch and Numb/Numblike (Paper I)

Numb, originally identified in genetic studies in *Drosophila*, acts upstream of Notch signaling and antagonizes its activity to determine cell fate during development (Uemura, Shepherd et al. 1989; Frise, Knoblich et al. 1996). In vertebrates there are two homologues: Numb and Numblike. When we started our study, the biochemical mechanism on how Numb/Numblike negatively regulated Notch signaling was largely unknown and various hypotheses had been proposed. Briefly, and as discussed earlier, Numb could exclude NICD from translocation to the nucleus, thus inhibiting Notch signaling; Numb has no effect on NICD but membrane tethered form of Notch, etc. As some of these models could be assumed to operate only on the cleaved NICD, whereas others could predict an action by Numb/Numblike on the membrane-tethered form of Notch, we first studied if Numb regulated NICD and/or a membrane-tethered form of Notch.

First, we studied to what extent Numb/Numblike downregulated Notch signaling. Both a truncated membrane bound form and an intracellular form of Notch was reduced at protein levels by Numb in a dose-dependent manner. Consistently Notch downstream target genes, Hes1 and Hey1, were decreased at mRNA level. We also found that NICD, cleaved from full-length Notch, was predominantly localized to the nucleus even in the presence of Numb protein.

Next, we investigated the effect of the interplay between Notch and Numb/Numblike in the myogenic differentiation. C2C12 cells can be differentiated after serum starvation and then form myotubes and express terminal differentiation markers such as myosin heavy chain (MHC). In our study we had setup co-culture system, in which different levels of Notch signaling could be achieved by growing mouse myoblast C2C12 cells, or an engineered C2C12 cells stably expressing full-length Notch1 receptor (C2C12-N1), with Notch ligand expressing cells (3T3-J1) or the control cells (3T3). We found that Numb could override the differentiation-inhibiting effects of Notch only at low levels of Notch signaling and promote myogenic differentiation, but not at high levels of Notch signaling. The differentiation role of Numb/Numblike

is also supported from other's study of adult mouse muscle progenitors (Conboy and Rando 2002).

In the experiments described above we noticed a strong decrease in Numb protein level at high Notch signaling strength, which suggested to us that high Notch signaling may in fact downregulate Numb/Numblike. We generated Numb or Numblike stable C2C12 cells in co-culture with control cells or ligand expressing cells for investigation of Notch effect on Numb/Numblike. Robust downregulation of Numb/Numblike was observed by Notch signaling, but to a much lesser extent by Hes1 or Hey1, which suggested the downregulation of Numb/Numblike was a direct but not secondary effect by Notch signaling.

PEST domains, which are rich in Prolines, Glutamic acids, Serines and Threonines, have been implicated in proteasome-mediated protein degradation (Reverte, Ahearn et al. 2001). We found two PEST domains in Numb and one in Numblike. Our observations indicated that the PEST domain was required for the downregulation of Numblike as mutant Numblike lacking PEST domain could not be reduced by high levels of Notch signaling. Addition of proteasome inhibitor MG132 abrogated the Notch mediated repression of Numblike, but had no effect on the Numblike mutant lacking PEST domain.

Finally, the reciprocal negative regulation between Notch and Numb/Numblike may play a role in stabilizing the cell fate switch by an asymmetric cell division, especially downregulation of Numb/Numblike by high levels of Notch may assure that Numb/Numbe like levels are kept low and, thus, safeguard the outcome of the cell fate resulting from Numb segregation. To test the in vivo biological relevance of our finding, both loss- and gain-of-function studies were performed. GSI treatment in human ovarian carcinoma cell line SKOV-3 resulted in reduction of NICD in accompany with elevated levels of endogenous Numb expression. Overexpression of NICD in the developing chick central nervous system reduced the endogenous Numb expression.

Downstream event of Notch signaling in T-ALL (Paper II)

T-ALL correlates with aberrations at different stages of thymocyte differentiation. Dysregulated Notch signaling severely compromises T cell development and promotes T cell leukemia as described earlier. Recent studies have demonstrated that mutations in the Notch signaling pathway are the most frequent genetic component and observed in over 50% of T-ALL cases (Weng, Ferrando et al. 2004). All these mutations result in aberrantly high level of Notch signaling. Although it is recently identified that c-Myc is a major target of Notch signaling and can rescue T-ALL from cell death after withdrawing Notch signaling (Weng, Millholland et al. 2006), other downstream responses are still possible as c-Myc is not activated in all the T-ALL cell lines where high Notch signaling exists.

In this report, we aimed at identifying alternative downstream outputs of the aberrantly high Notch signaling in T-ALL, and we have concluded that SKP2 is under the direct control of Notch signaling and downregulates p27Kip1, contributing to altered cell cycle progression in human T-ALL cells. This assumption receives support from several experimental observations. First, in agreement with a previous study, we observe that Notch 1 ICD binds to the *SKP2* promoter under conditions of high Notch signaling in T-ALL cells. Inhibition of Notch signaling with GSI treatment suppresses both *SKP2* mRNA and protein levels. Second, an inverse correlation between the level of Notch signaling and p27Kip1 expression is mediated by SKP2. Introduction of a dominant negative SKP2 increased p27Kip1 levels in T-ALL cells with high Notch signaling. We also note that not only p27Kip1 protein but also mRNA levels are altered, which suggests that other mechanisms, in addition to SKP2-mediated degradation of p27Kip1, exist to control p27Kip1 level in T-ALL cells. Hes protein, another Notch target, could play a role in it as a recent finding shows that Hes proteins can act as transcriptional repressors of the p27Kip1 gene (Murata, Hattori et al. 2005). Finally, the Notch/SKP2/p27Kip1 axis has an effect on aberrant cell cycle progression in T-ALL cells. For example, G0/G1 cell cycle arrest is observed in the “Notch off” situation, i.e. after GSI treatment, as well as after introduction of p27Kip1 or, to a minor extent, the dominant negative SKP2.

Deregulated cell cycle control is a fundamental mechanism in all cancers, and several lines of evidence show that SKP2 and p27Kip1 play an important role in the pathogenesis of various malignancies. Recently, SKP2 and p27Kip1 dysregulation is

shown in chronic myelogenous leukemia (CML), where BCR-ABL is shown to regulate SKP2, resulting in altered p27Kip1 levels (Andreu, Lledo et al. 2005). It should be noted however that in most cancers, only the p27Kip1 protein, but not the mRNA, level is decreased, which is different from the situation we observed in the T-ALL cells.

The molecular output of the deregulated Notch signaling in T-ALL is likely to be more complex, as our micro array results reveal that a large number of genes are up- or down-regulated in response to Notch inhibition in the T-ALL cells. Compared with other micro array data using a different T-ALL cell line, TB6 (Nguyen, Lefort et al. 2006), we notice that the majority of the most highly up- or down-regulated genes differ between the MOLT4 and TB6 data sets, which further underscores the notion of heterogeneity between different T-ALL cell lines and divergent downstream responses.

In conclusion, we propose that the Notch/SKP2/p27Kip1 axis play an important role in rapid cell cycle progress in T-ALL, which could serve as a platform for developing future treatment of T-ALL patients.

The role of Numblike in the early differentiation of embryonic stem cells (Paper III)

Embryonic stem (ES) cells can generate all somatic cell types. They constitute a unique cellular system for basic studies of regernative medicine and for gaining the molecular insight into pluripotency and primary lineage commitment at early developmental stages. Numb and Numblike are conserved developmental proteins, which are critical for cell fate decision. Here we take advantage of a system for inducible gene expression in ES cells (Kyba, Perlingeiro et al. 2002) to study the possible role of Numblike in the early differentiation of ES cell.

In this study, we use the ROSA-TET system to generate a mouse ES cell line for inducible expression of a Numblike-EGFP fusion protein. In this system rtTA (reverse tetracycline transactivator) (Gossen, Freundlieb et al. 1995)was introduced into the ROSA26 locus (Zambrowicz, Imamoto et al. 1997) and the desired cDNA

was integrated into Hprt locus by a single loxP-based strategy. Numblike-EGFP can be expressed in our ES cell line at various differentiation stages, with no leakage in the "off state" and rapid induction within three hours after doxycyclin administration.

The cellular distribution of Numb has been shown to be important for its function as a cell fate determinator, but the exact subcellular localization of Numb is not consistently defined and thus remains to be well clarified. This is also true for Numblike and we find that Numblike expression is largely confined to intracellular vesicles, both in ES cells, and when Numblike is transfected into 293T cells. Our data indicate that Numblike does not colocalize with markers of the endocytic pathway and therefore possibly resides in another type of intracellular vesicles. We also show that Numblike does not co-localize with Notch, when Notch is trapped in early endosomes following block of S3 cleavage. Thus Numblike may act more indirectly on inhibition of Notch signaling, possibly via the E3 ubiquitin ligase Itch (see the discussion on different models for how Numb/Numblike controls Notch signaling above).

Differentiation of ES cells into cardiomyocytes is favored by inactivation of the Notch1 receptor (Nemir, Croquelois et al. 2006; Schroeder, Meier-Stiegen et al. 2006), whereas endogenous Notch signaling promotes differentiation of ES cells into the neuronal lineage (Lowell, Benchoua et al. 2006). Therefore we are interested in studying any opposite effect of Numblike on ES cell differentiation into those lineages. We find that Numblike expression impairs the early neural lineage differentiation, as judged by reduced expression of Sox-1, as compared to the non-induced cells. This is in keeping with a role for Numblike in negatively regulating Notch signaling and more easily reconciled with the findings that loss of Numb and Numblike leads to precocious neuronal differentiation in vivo (Petersen, Zou et al. 2002; Petersen, Zou et al. 2004). However, more complicated effect is observed when we induce differentiation of Numblike inducible ES cell towards mesodermal lineage. Elevated Numblike expression impaired early mesodermal differentiation, i.e. up to the Brachyury- and Flk-1-positive mesodermal progenitor stage. In contrast, a later mesodermal differentiation step, from Flk-1-positive cells to contracting cardiomyocyte colonies, was not significantly affected. It is unexpected that Numblike expression and Notch signaling have similar effect on the early

mesodermal differentiation, suggesting a role of Numblike independent of Notch signaling. Indeed, recent studies have shown that Numb or Numblike can regulate other key proteins, such as integrins and cadherins. Furthermore, Numb can negatively interfere with Sonic Hedgehog signaling by targeting Gli1 for itch-dependent ubiquitylation and degradation. To what extent such interactions are important to explain the data observed here remains an important topic for future studies.

Numblike expression induces a transient increase in the expression of the transcription factor specific for cardiomyocyte differentiation at day 8, but cannot further promote differentiation, as compared to the non-induced state. This poses the question as to whether Numblike need other cooperative factors to promote cardiomyocyte differentiation in long term.

Future Perspectives

- What are the different trafficking routes of Notch ligand and receptor? How exactly do they modulate Notch signaling activity?
- How to monitor Notch activity in real time and study the dynamics of Notch signaling in each developmental process?
- How and why are different target genes activated depending on cell type and time?

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