Department of Cell and Molecular Biology
The Medical Nobel Institute
Karolinska Institutet,
Stockholm, Sweden

Molecular Diversity in the Notch Receptor Family

by

Paul Beatus

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To Hoja

Abstract

We have studied the Notch family of transmembrane receptors, which play a crucial role in cell fate determination. The Notch signalling pathway represents a highly conserved mechanism to mediate signalling between adjacent, equivalent cells and to direct them to adopt different cell fates. This process, called lateral inhibition, is involved in many processes during development e.g. bristle development in *Drosophila* and retina and pancreas development in vertebrates. The current view is that the Notch receptor is cleaved intracellularly upon activation by Delta or Serrate ligands on neighbouring cells. The intracellular Notch domain then translocates to the nucleus, binds to the DNA-binding factor *Suppressor of Hairless* (CSL in mammals), and acts as a transactivator of *Enhancer of Split* (HES in mammals) gene expression. Several studies have demonstrated that the intracellular domain alone functions as a constitutively-active receptor. There are four known mammalian Notch receptors with overlapping patterns of expression. The role of the different receptors is poorly understood, but mutations in the receptors result in distinct genetic disorders like CADASIL and a variety of tumours. CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) is an inherited form of stroke and dementia. The histopathological hallmarks are degeneration of vascular smooth muscle cells (VSMCs) and accumulation of granular osmiophilic material (GOM) between the degenerating VSMCs. CADASIL is caused by missense mutations in the human Notch3 gene. The mutations always affect cysteine residues located in the extracellular domain of the Notch3 receptor. There is currently no biochemical data available on the mechanism by which the mutated Notch3 receptor causes CADASIL.

The aim of this study has been to understand the extent of functional diversity in the Notch receptor family. More specifically, we have investigated the molecular differences between the Notch1 and Notch3 receptors in terms of transcriptional activity and interactions with transcriptional activators and repressors. We have also extended the *in vitro* knowledge in studying the effects of Notch3 signalling during pancreas development and in CADASIL pathogenesis.

We show that the intracellular domain of Notch3 (Notch3 IC), unlike Notch1 IC, is a weak activator of HES gene expression both in cell-based systems and *in vivo* (Paper I). Furthermore, we find that the low transcriptional activity of Notch3 IC is dominant over Notch1 IC-mediated activation. Hence, Notch3 IC behaves as a functional repressor of Notch1 signalling with respect to the HES genes. To learn, in detail, which domains are responsible for the differences between the receptors, we have molecularly dissected the Notch1 and Notch3 ICs (Paper II). We make two important observations. First, we have identified a region, RE/AC, which is required for both activation and repression. Loss of this region in both ICs results in complete loss of function, i.e. Notch1 IC loses its activator function and Notch3 IC loses its ability to act as a repressor. Second, we show that the origin of the ankyrin-repeat region alone determines the transcriptional activity.

Two lines of *in vivo* experiments corroborate these results. First, HES-5 expression is reduced in transgenic mice that misexpress the Notch3 IC in the neural tube, indicating that Notch3 IC also in vivo can affect Nocth1 mediated activation of HES-5 (Paper I). Second, disruption of Notch1 signalling by loss of either the ligand Delta-like1 (Dll1) or RBP-Jk results in accelerated differentiation of pancreatic endocrine cells. A similar phenotype was observed in mice over-expressing the intracellular form of Notch3 (Paper III). These data also show that Notch3 functions to block lateral inhibition by repressing Notch1 function.

In adult humans, Notch3 is expressed in VSMCs, the degeneration of which is the key pathogenic feature of CADASIL. The role of Notch3 in CADASIL has been studied in a transgenic mouse model (Paper IV). The wildtype Notch3 allele was replaced with a CADASIL (R140C)-mutated Notch3 gene by homologous recombination in ES cells. Mice heterozygous for the CADASIL-mutated allele have a distinct vascular phenotype with disturbed vessel wall organisation. Interestingly, some ultrastructural changes observed in CADASIL patients, GOM, have not been observed in our transgenic mice, suggesting that GOM could be a secondary effect of the pathogenesis.

Publications

I **Paul Beatus**, Johan Lundkvist, Camilla Öberg and Urban Lendahl.
The Notch 3 intracellular domain represses Notch 1-mediated activation through Hairy/Enhancer of split (HES) promoters.
(1999) *Development* **126**, pp. 3925-3935

II **Paul Beatus***, Johan Lundkvist*, Camilla Öberg, Kia Pedersen and Urban Lendahl.
The origin of the ankyrin repeat region in Notch intracellular domains is critical for regulation of HES promoter activity.
Mechanisms of Development, in press

III Åsa Apelqvist, Hao Li, Lukas Sommer, **Paul Beatus**, David J. Andersonk, Tasuku Honjo, Martin Hrabe de Angelis, Urban Lendahl and Helena Edlund.
Notch signalling controls pancreatic cell differentiation.
(1999) *Nature* **400**, pp. 877-881

IV Johan Lundkvist, Shunwei Zhu, **Paul Beatus**, Petra Schweinhardt, Emil Hansson, Helena Karlström, Clas Johansson, Matti Viitanen, Anne Joutel, Christian Spenger, Abdul Mohammed, Hannu Kalimo and Urban Lendahl. Mice carrying a knock in Notch 3 CADASIL missense mutation develop extracerebral arteriopathy and alterations in vascular smooth muscle cells.
Submitted.

*these authors contributed equally to this work.

Contents

1. INTRODUCTION

1.1 General introduction

The ability of a cell to sense its surroundings is a matter life and death. To safeguard this ability, the cell is equipped with a battery of molecular instruments, represented by cellular receptors that can identify and correctly interpret different external signals. Cellular receptors are thus the nose of the cell and just as we can discern a wine's bouquet, cells sense minute differences in their environment. Notch signal transduction represents a distinct mechanism for how cells perceive and interpret environmental cues. Appreciation of Notch signalling will be much greater if viewed in the light of some other signalling pathways and therefore I would like to begin with some examples of molecular signalling, followed by a detailed description of the Notch signalling mechanisms as it stands today.

Notch signalling is deeply integral to the embryonic development of animals. Therefore, it is appropriate at this point to give a brief, general overview of the fundamental developmental processes, followed by a summary of the work that has revealed the role of Notch signalling in development and disease. I will conclude by presenting and discussing our contribution to the understanding of Notch signalling complexity.

1.2 The importance and the means of cell-cell communication

In multicellular organisms, cell-cell communication is a prerequisite for development and survival. Cell-cell communication spans from the communication between cells located far apart in the body through soluble factors, to specific contacts between cells that are juxtaposed. Several signalling systems have evolved to send and receive messages. On the receiving cell, the signal must be transmitted from the cell surface to the cell nucleus, in order to effect decision-making in the cell regarding proliferation, differentiation, migration or other responses. There is a limited number of signalling pathways that are used repeatedly in different contexts and combinations during development and adult life, which are highly evolutionarily conserved. There is also an intriguing complexity of crosstalk between these signalling pathways, which further increases the number of responses a cell can make to different stimuli. This 'embarrassment of riches' has only recently begun to be revealed in detail.

Before describing the Notch signalling pathway, I will briefly review some of the key signalling pathways important in developmental biology, in order to provide examples of the multitude of molecular mechanisms used to convey a signal from the plasma membrane to the cell nucleus.

Receptor tyrosine kinase (RTK) signalling is a widely used system for cell-cell communication. In RTK signalling (e.g. EGF, FGF, PDGF etc), the ligand is a soluble protein that induces two transmembrane receptors to dimerise (Figure 1A). Upon dimerisation, the receptors are activated by

cross-phosphorylation of specific tyrosine residues, which in turn initiates a phosphorylation cascade of intracellular mediators and eventually leads to the activation of target genes. Many of the intracellular signal mediators possess intrinsic enzymatic activities in addition to protein modules that bring about interactions with other proteins, phospholipids or nucleic acids (Alberts 1994, Schlessinger 2000). One of these intracellular mediators, Ras, mediates activation of one or more of three pathways of protein kinases. Several different RTKs mediate their response through the same set of intracellular mediators (reviewed in Tan and Kim 1999). This specificity is achieved by quantitative differences in response and by crosstalk with other signalling pathways.

Another pathway, which also utilises receptor phosphorylation, is the TGF-β signalling pathway (reviewed by Itoh *et al.* 2000). Transforming growth factor-β (TGF-β) family members include TGF-βs, activins and Bone Morphogenetic Proteins (BMPs) and are structurally related, secreted cytokines found in species ranging from insects to mammals. The TGF-β family members elicit their cellular responses through the formation of heteromeric complexes of specific type I and type II serine/threonine kinase receptors (Figure 1B). The type II receptors are constitutively-active kinases which, upon ligand-mediated heteromeric complex formation, phosphorylate particular serine and threonine residues in the type I receptors. Signalling specificity within the heteromeric complex is determined by the type I receptors. The signal is relayed through a family of intracellular mediators called Smads, which can be divided into three distinct subfamilies: receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads) and inhibitory Smads (I-Smads). Activated type I receptors phosphorylate particular R-Smads, which then recruit the CO-Smads, resulting in the accumulation of heteromeric complexes in the nucleus. These complexes regulate the transcription of target genes either through binding DNA directly or via other DNA-binding proteins.

The Wnt signal transduction pathway (Figure 1C) plays an important role in a number of developmental processes including body axis formation, central nervous system (CNS) development and limb development (reviewed in Cadigan and Nusse 1997). The Wnt family of proteins consists of more than 15 closely related, secreted glycoproteins. They bind to the *frizzled* (*Fz*) family of transmembrane receptors and the Wnt signal is transduced to a cytoplasmic protein called *Dishevelled* (*Dvl*). Upon activation by the Wnt signal, *Dvl* inhibits the activity of *glycogen synthase kinase-3*β (*GSK-3*). In the absence of a Wnt signal, *GSK-3* is thought to phosphorylate and subsequently induce the degradation of β-catenin. Therefore, the Wnt signal stabilises and causes the accumulation of β-catenin, which can then translocate to the nucleus and associate with the TCF/LEF family of transcription factors and together with them activate the target genes (reviewed in Akiyama 2000). β-catenin is turned over by the ubiquitin-dependent proteolysis system (Aberle *et al.* 1997) in which APC and Axin negatively regulates β-catenin stability by mediating ubiquitination.

Hedgehog (*Hh*) signalling in *Drosophila* is interesting from a historical perspective, since it has long been a mystery how the presumptive receptor *Smoothened* (*Smo*) could respond to a *Hh* signal without

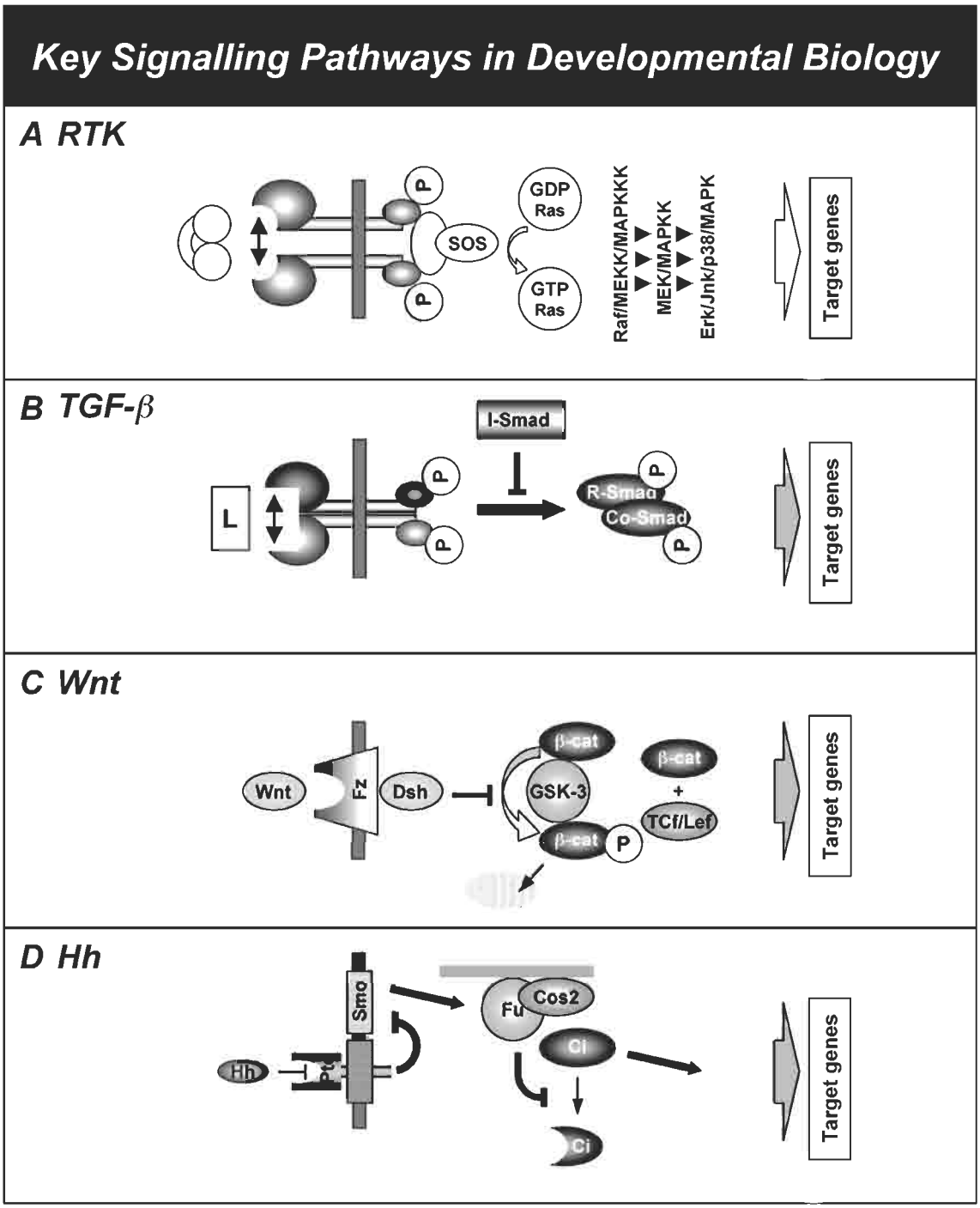


Figure 1. Schematic representation of four classical signal transduction pathways in development.

physically binding the ligand. Only recently has the true receptor been identified and shown to be encoded by *Patched* (*Pct*) (Marigo *et al.* 1996). *Smo* shows some resemblance to G-coupled transmembrane receptors and it is a constitutively-active signalling mediator of *Hh* (Figure 1D). However, in the absence of ligand, *Smo* is physically associated with *Ptc*, which represses its activity (reviewed by Ingham 1998). The ligand, *Hh*, alleviates repression by *Ptc* and consequently *Smo* becomes active (Figure 1D). Intracellular signal transduction is mainly mediated by a transcription factor of the Zn-finger family, called *cubitus interruptus* (*Ci*). In the absence of *Hh*, *Ci* is proteolytically processed so that the transcriptional activation domain is removed, thus converting *Ci* into a transcriptional repressor (Aza-Blanc *et al.* 1997). In response to *Hh* activation, degradation of *Ci* is inhibited and a number of full-length molecules accumulate and translocate to the nucleus. In the nucleus, it associates with CBP (a histone acetyl transferase) and activates transcription of its target genes. Interestingly, *Hh* signalling is subjected to autoregulation at several levels. The *Hh* protein itself is autoproteolytically cleaved during maturation to generate distinct forms (Lee *et al.* 1994). In addition, both *Hh* and *Ptc* are transcriptionally regulated by *Ci*. The pathway is largely conserved in vertebrates where three *Hh* homologues have been identified, *Sonic*, *Indian* and *Dessert Hedgehog*.

1.3 The Notch signalling pathway

The Notch signalling pathway shows some unique characteristics compared to the signalling pathways described above. First, it appears only to function between cells in immediate contact, and the ligands appears to be strictly cell-bound. Second, the receptor undergoes a complex proteolytic cleavage, as discussed in detail below. The Notch signalling pathway represents a highly conserved mechanism to mediate signalling between adjacent, equivalent cells and to direct them to adopt different cell fates (for review see Artavanis-Tsakonas *et al.* 1995, Artavanis-Tsakonas *et al.* 1999, Greenwald 1998, Kopan and Turner 1996, Lewis 1996, Lewis 1998, Muskavitch 1994). This process, called lateral inhibition, is involved in many processes during development e.g. morphogenesis (tooth, lung, hair), boundary formation (wing, somites, limb), cell specification (CNS, PNS and pancreas) and apoptosis (in cultured neural crest cells).

The Notch signalling pathway was originally identified and studied in the fruit fly, *Drosophila melanogaster*. The name ‘Notch’ derives from the characteristic notched wing found in flies with only one functional allele of the Notch gene (Moohr 1919). Homozygous Notch mutations in flies result in lethal phenotypes. These mutations produce a ‘neurogenic’ phenotype, where cells destined to become epidermis switch fate and give rise to neural tissue (Poulson 1937, Wright 1970). Notch is also involved in many other aspects of *Drosophila* development, such as specification of cell fate in the somatic gastric nervous system (Gonzalez-Gaitan and Jackle 1995), in muscle founder cells (Bate *et al.* 1993), in bristle formation (Heitzler and Simpson 1991), in specialised follicle cells in the ovary

(Ruohola *et al.* 1991) and in most cells of the *Drosophila* eye (Baker and Zitron 1995, Cagan and Ready 1989).

Notch also plays important roles in cell specification in the nematode *Caenorhabditis elegans* (reviewed in Kimble and Simpson 1997). Interestingly, *C. elegans* has two Notch homologues, LIN-12 and GLP-1, which are more diverged than any other pair of Notch receptors, suggesting a very early gene duplication event in the nematode. Nevertheless, each receptor can substitute for the other when expressed in the appropriate tissue (Fitzgerald *et al.* 1993). GLP-1 regulates blastomere specification in the early *C.elegans* embryo (Bowerman *et al.* 1992, Hutter and Schnabel 1994, Mango *et al.* 1994) whereas LIN-12 is important for gonad development in later stages of growth (Greenwald *et al.* 1983).

Notch receptors have been identified in many vertebrate species and some examples illustrating their function in development and disease will be discussed in detail below.

The Notch family

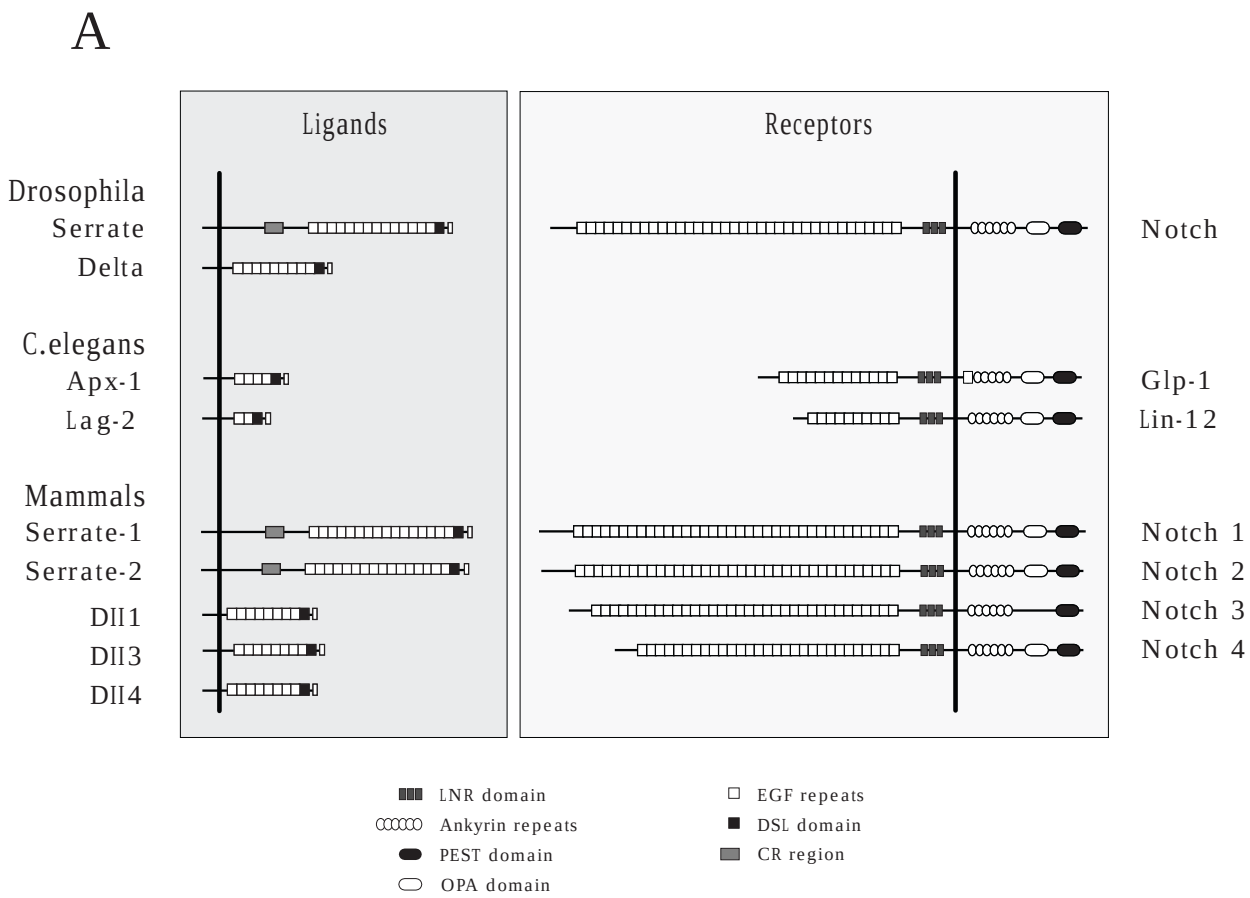
The members of the Notch signalling pathway can be found throughout the metazoa. In mammals, four Notch receptors and five ligands have been identified (Figure 2A) (reviewed in Beatus and Lendahl 1998). *Drosophila* Notch and mouse Notch1 are the best-characterised members and most of what is known about Notch signalling comes from work on these two proteins. The receptors and ligands of this signalling pathway are structurally related and encode single transmembrane proteins. Activation of the Notch receptor from cell-bound ligands on adjacent cells leads to proteolytic cleavage of the receptor and translocation of the intracellular part to the nucleus, where it controls gene expression together with a DNA-binding protein. The signalling mechanism will be described later.

The receptors

The Notch receptor family encodes large single-pass transmembrane proteins that share some common characteristic features (see Beatus and Lendahl 1998 for review). On the extracellular side, the receptors contain a large number of tandemly-arranged extracellular EGF repeats and a family-specific LNR (Lin Notch Repeat) region (Wharton *et al.* 1985) (Figure. 2B). Proper folding of the EGF-like repeats has been shown to be Ca^{2+}-dependent (Rand *et al.* 2000, Rand *et al.* 1997, Rao *et al.* 1995) and EGF-repeats 11 and 12 are believed to be involved in ligand binding (Rebay *et al.* 1991). A recent report suggests that ligand specificity is further influenced by glycosylation (see next section). The precise role of the LNRs has not yet been confirmed, but there are indications that they are important for receptor regulation both in the absence of ligands and for proper activation (see below).

Four main regions can be distinguished in the intracellular domain of Notch: the RAM, ankyrin, RE/AC and C-terminus (Figure 2B). The region immediately inside the membrane is referred to as the RAM domain. Its main function seems to be to mediate direct interaction with CBF1/Su(H)/LAG-1

Receptors and Ligands in Notch Signalling



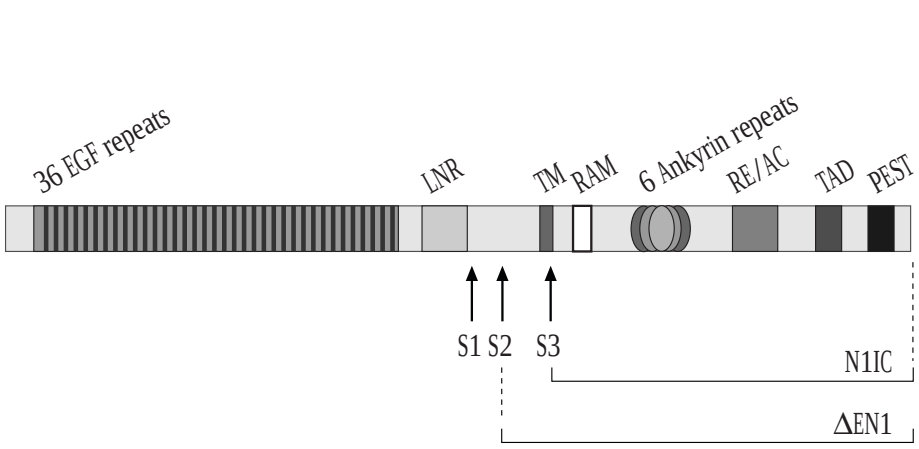


Figure 2. Schematic representation of the Notch receptor family.
A) The ligands and receptors in different species. B) Representation of the domains in the Notch1 receptor. S1-S3 indicate the crucial proteolytic sites. N1IC and ΔEN1 are constitutively active mutants of Notch1.

(CSL) (Tamura *et al.* 1995). The ankyrin repeat region is the most highly conserved portion of Notch. It consists of six ankyrin-like repeats, which can also be found in the cdc10 and SWI6 proteins, which are involved in cell cycle control (Breeden and Nasmyth 1987). They are believed to mediate interactions with factors involved in transcriptional activation, of which many are still unknown (see below). The ankyrin repeats have been shown to be very important for signalling, and several mutations in this region affect receptor function negatively (Aster *et al.* 2000, Jarriault *et al.* 1995, Kato *et al.* 1997, Kodoyianni *et al.* 1992, Kopan *et al.* 1994, Kurooka *et al.* 1998, Rebay *et al.* 1993). We and others have identified RE/AC as a region that is crucial for Notch function (Paper II and see Discussion), but the functional role of this region remains to be elucidated. Finally, the C-terminus contains two interesting regions. The OPA-domain is rich in glutamine residues and has been shown to function as a transcriptional activation domain (TAD) when fused to a GAL4 DNA-binding domain (Kurooka *et al.* 1998). It is required for Notch-mediated activation of reporters with multimerised CSL binding sites. However, it seems to be dispensable both for activation of reporters (based on the endogenous promoters HES-1 and HES-5) and for receptor function *in vivo* (Paper II). The C-terminus also contains a PEST sequence that mediates ubiquitination of Notch and is thus involved in protein stability (Öberg *et al* unpublished results). Notch also contains two nuclear localisation signals (NLS) that are located on opposite sides of the ankyrin domain (Lieber *et al.* 1993, Rebay *et al.* 1993, Roehl *et al.* 1996, Struhl *et al.* 1993).

It has been suggested that the intracellular domain of Notch (NIC) functions as a constitutively-active receptor. This is supported by several independent observations. Chromosomal translocation of the intracellular domain of the human homologue of Notch (TAN-1) induces T lymphoblastic leukaemia (Ellisen *et al.* 1991). It has subsequently been shown that artificial introduction of the intracellular domain of Notch1 (N1IC) in thymocytes can influence cell-fate switching between CD4 and CD8 T-cell lineages (Robey *et al.* 1996). As will be discussed later in detail, the N1IC alone has also been shown to affect cell fates in a number of tissues and cell lines (Coffman *et al.* 1993, Dorsky *et al.* 1995, Kopan *et al.* 1994, Nye *et al.* 1994, Rebay *et al.* 1993, Roehl and Kimble 1993, Shawber *et al.* 1996b, Struhl *et al.* 1993). Thus, N1IC can function as a constitutively-active, gain-of-function receptor. More importantly, a membrane-tethered version of the N1IC, called ΔEN1 (Figure 2B), can also function as a constitutively-active receptor and it has been shown to be correctly processed to generate N1IC (Kopan *et al.* 1996, Schroeter *et al.* 1998), see below). The extracellular and membrane-tethered intracellular domains of Notch are presented on the cell surface as heterodimers associated by non-covalent bonds. Rand *et al* show that Notch signalling can be induced by disruption of the heterodimer, thus mimicking a ΔEN1 (Rand *et al.* 2000).

Effects of genetic disruption of the receptors

The function of the mammalian Notch receptors has been analysed by gene-targeting experiments in which Notch1-4 specifically have been disrupted. Notch1 null mutations are embryonically lethal and affect proper segmentation by disrupting somite organisation. Increased apoptosis can also be detected, but is not considered to be the main cause of developmental arrest (Conlon *et al.* 1995, de la Pompa *et al.* 1997, Swiatek *et al.* 1994). Unfortunately, Notch1's role in organogenesis (e.g. neurogenesis and immune system development) cannot be studied since mutant embryos die prior to embryonic day 11.5 (E11.5). Notch2-deficient mice also die at E11.5. However, no obvious developmental retardation has been identified except for abnormal levels of apoptotic cells (Hamada *et al.* 1999). It should be noted that only the ankyrin repeat region has been removed in the mice generated here and the entire extracellular domain is still expressed as a fusion protein with β-galactosidase. The Notch2 gene has also been targeted by another group, resulting in a hypomorphic allele rather than a true null allele (McCright *et al.* 2001). The mutation results in perinatal lethality due to kidney dysfunction. Mutants exhibit defective differentiation and patterning of the glomeruli and vascular defects of the eye. Preliminary results indicate that Notch3$^{-/-}$ mice are viable and fertile and do not display any overt gross phenotype (P.B. unpublished results). This is surprising, given the specific pattern of expression for Notch3 during embryogenesis, and suggests a possible redundancy by some of the other Notch receptors. Finally, disrupting Notch 4 results in viable and fertile progeny. However, the Notch4 mutation displays genetic interactions with a targeted mutation of the Notch1 gene. Embryos homozygous for mutations of both the Notch4 and Notch1 genes display a more severe phenotype than Notch1 homozygous mutant embryos. Both Notch1 mutant and Notch1/Notch4 double mutant embryos display severe defects in angiogenic vascular morphogenesis and remodelling (Krebs *et al.* 2000). In addition, a cleavage-defective Notch1 mouse has been generated by a 'knock-in' approach. This mouse exhibits many of the phenotypes seen in the Notch1 knock-out mouse (Huppert *et al.* 2000).

The Ligands

There are two types of Notch ligands, Delta and Serrate. The ligands are similar to Notch receptors in the extracellular domains, i.e. they also carry multimerised EGF repeats (Figure 2A) (see Nye and Kopan 1995 and Beatus and Lendahl 1998). Ligands of both classes contain a common DSL (for Delta/Serrate/Lag-2) domain. In addition, the ligands of the Serrate class share a cysteine-rich (CR) region. Unlike many other signalling pathways, it is not yet established exactly which ligands activate which receptors. Ligand-receptor interactions have been studied by several different approaches such as co-expression analysis, genetic linkage in knock-out experiments, biochemically by *in vitro* interactions, interactions in co-cultures and reporter gene activation in co-cultured cells. In mammals, the ligands are expressed in almost all embryonic tissues and their expression patterns partly overlap spatiotemporally (Bettenhausen *et al.* 1995, Dunwoodie *et al.* 1997, Felli *et al.* 1999, Krebs *et al.*

2000, Kusumi *et al.* 2001, Lindsell *et al.* 1996, Loomes *et al.* 1999, Oda *et al.* 1997a, Rao *et al.* 2000, Shawber *et al.* 1996a, Shutter *et al.* 2000, Valsecchi *et al.* 1997). There does not seem to be any definite consensus in the pattern of expression between a particular ligand and receptor, indeed judging from the expression patterns all ligands may have the ability to activate all receptors. More specific experiments have been performed to study direct interactions between receptors and ligands *in vitro*. Serrate1 (Jagged1) can bind Notch1-3 in a solid phase assay (Shimizu *et al.* 1999) and Delta and Serrate have similar affinities to *Drosophila* Notch in an aggregation assay in *Drosophila* cells (Klueg and Muskavitch 1999). Furthermore, experiments in both co-culture and *in vivo*, show that both Dll1 and Serrate1 can induce HES-1 expression via Notch1 and Notch3 (Bash *et al.* 1999, Hoyne *et al.* 2000, Jarriault *et al.* 1998, Kuroda *et al.* 1999); J.L. and G.C. unpublished results). Finally, Dll1 and Serrate1 result in cellular responses indistinguishable from an activated Notch1 (N1IC) (Lindsell *et al.* 1995, Morrison *et al.* 2000, Wang *et al.* 1998).

Effects of genetic disruption of the ligands

Mutations in some of the ligands are associated with specific human genetic disorders, like Alagille's and Spondylocostal dysostosis (see 'Notch and Disease'). The function of the ligands has been analysed by gene targeting and there is a substantial variation in phenotypes among the different ligands. Serrate1 is essential for remodelling embryonic vasculature and homozygous mice die prior to E11.5 from severe haemorrhage due to defective formation of the vascular system. Heterozygous mice exhibit an eye phenotype similar to that in Alagille's, but do not exhibit other features of this disease (Xue *et al.* 1999). Serrate1 displays a genetic link to Notch2 in that double heterozygote Serrate1$^{+/-}$ Notch2$^{+/-}$ mutants show more severe phenotypes than the single mutants (McCright *et al.* 2001). Serrate2 (Jagged 2) mutant mice die at birth, with severe craniofacial and limb malformations. The craniofacial malformations manifest as cleft palate and fusion of the tongue with the palatal shelves, which prevents the pups from breathing. The mutant mice also exhibit syndactyly of the limbs (Jiang *et al.* 1998). Mutations in the Delta class of ligands result in gross developmental defects. Dll1$^{-/-}$ mice show severe segmentation defects and fail to maintain the integrity of the somites (Hrabe de Angelis *et al.* 1997). This phenotype is reminiscent of that of Notch1 mutants.

In conclusion, to date, no unique ligand-receptor interactions have been identified and both classes of ligands can activate Notch1. It has recently been shown that activation of Notch by Delta and Serrate can be modulated by differential glycosylation of the receptors (Bruckner *et al.* 2000). This is mediated by *Fringe*, a glycosyltransferase which forms a complex during maturation in Golgi (Ju *et al.* 2000). *Fringe* has been shown to influence ligand specificity of Notch2, but not Notch1, suggesting a possible mechanism for lending specificity to the system (Hicks *et al.* 2000).

Signalling mechanism- how does it start

The Notch signalling mechanism is characterised by a series of proteolytic events of which the first occurs during protein maturation in the ER and is mediated by a furin protease (Blaumueller *et al.* 1997, Logeat *et al.* 1998, Pan and Rubin 1997). The receptor is cleaved at site 1 (S1) in the extracellular domain (Figure 2B). The receptor is then presented on the cell surface as a heteromeric protein. It is not yet known whether Notch forms multimeric complexes or if the receptors exists as single entities, but it may be speculated that the receptor must be monomeric for activation and that ligands dissociate any complexes. It has been proposed that the presence of the extracellular domain negatively regulates Notch signalling (Rand *et al.* 2000) and that ligands are required for relieving the repression. It has even been suggested that the extracellular domain of Notch is "ripped" from the receiving cell and trans-endocytosed into the signalling cell (Klueg and Muskavitch 1999, Parks *et al.* 2000). Ligand binding triggers two rapid consecutive proteolytic events (S2 and S3, see Figure 2B) that result in the release of an intracellular, functionally active form of Notch. S2 cleavage is mediated by TACE, a metalloprotease of the ADAM family and results in a transient intermediate peptide (NEXT) that is liberated from oppression by the ectodomain (Brou *et al.* 2000, Mumm *et al.* 2000). S2 cleavage is required for, and rapidly followed by, cleavage at a third site (S3) that is located in the transmembrane region. Just how S3 cleavage is mediated is much debated. It is nevertheless clear that presenilins (PS) play a crucial role in this event. PSs are multispanning transmembrane proteins that have been shown to be required for γ-secretase processing of the amyloid precursor protein (APP) (for review see De Strooper and Annaert 2000). Notch processing shares several features with APP and it is speculated that γ-secretase processing and S3 cleavage is mediated by a common enzyme. PSs can be purified as multiprotein complexes with γ-secretase activity, but it is not yet clear whether S3 proteolysis is mediated by PSs or by a separate, associated γ-secretase (reviewed by Kopan and Goate 2000). Loss of PS results in reduced Notch processing at S3 (De Strooper *et al.* 1999). S3 cleavage results in a functionally active receptor referred to as NIC (Notch IntraCellular domain) which is released into the cytoplasm and then translocates, driven by the NLSs. This mechanism is referred to as RIP (Regulated Intramembrane Proteolysis) and it is shared by some other proteins, like APP and SREBPs (sterol regulatory element-binding proteins) (reviewed in Brown *et al.* 2000).

Signalling mechanism – where does it end

In the absence of N1IC, Notch-responsive genes (e.g. HES-1 and HES-5) are repressed by a repression-complex, outlined in Figure 3 (see (Mumm and Kopan 2000) for review). The key component of this complex is CSL, a DNA-binding protein recognising a single CGTGGGAA sequence (Tun *et al.* 1994). Sometimes referred to as RBP-Jk, CSL was first isolated from mouse pre-B cells and was initially believed to be involved in VDJ-recombination. However, this was not the case and later it was correctly identified as the vertebrate homologue of *Drosophila Supressor of Hairless* (*Su(H)*) (Hamaguchi *et al.* 1989). CSL binds to DNA as a monomer, forming a stable co-

repressor complex that consists of a number of co-repressors, i.e. SMRT, CIR and KyoT2 (Hsieh *et al.* 1999, Kao *et al.* 1998, Taniguchi *et al.* 1998) which can recruit the Sin3 complex of negative chromatin-remodelling regulators (for review see (Ahringer 2000)) (Figure 3, first panel). CSL is also associated with SKIP (for Ski Interacting Protein) (Zhou *et al.* 2000a, Zhou *et al.* 2000b). Ski is known as a member of the co-repressor complex in TGF-β and nuclear receptor-mediated signalling (Akiyoshi *et al.* 1999, Nomura *et al.* 1999, Tagami *et al.* 1998). SKIP has been shown to relieve this repression and to facilitate nuclear receptor-mediated transcription (Baudino *et al.* 1998). SKIP also interacts with SMRT and CIR. Interestingly, SKIP can form trimeric complexes with CSL and either Notch1 or SMRT, but never with Notch1 and SMRT in the same complex. Furthermore, point mutations in N1IC that abolish its activity (Jarriault *et al.* 1995, Kopan *et al.* 1994) have recently been shown to disrupt SKIP interactions (Zhou *et al.* 2000b). Taken together, this suggests that SKIP may play dual roles in Notch signalling, both in transcriptional repression and in activation.

It has been shown that the RAM domain of Notch1 binds to CSL and that loss of RAM significantly reduces Notch activity on a multimerised CSL reporter (Kurooka *et al.* 1998). It is believed that N1IC forms a transient, unstable complex with the co-repressors which eventually dissociate (Kao *et al.* 1998). This relief of repression is further enhanced by recruitment of histone acetyl transferases (HATs). Kurooka *et al* show that Notch1 can interact with PCAF and GCN5, two closely related HATs (Kurooka *et al.* 1998). Together with SKIP and CSL, Notch and the HATs form a stable pre-initiation complex which then can attract the basal transcription machinery that results in a stable coactivator complex (Figure 3, last panel). Further components of the activation complex are likely to be identified. MAML1, a vertebrate homologue of *Drosophila mastermind*, was recently shown to interact physically with Notch1 and to function as a positive co-activator of transcription (Wu *et al.* 2000). In *C.elegans*, another factor called LAG-3 (Lin and Glp-3) has been linked to Notch signalling. Like SKIP, it binds the fourth ankyrin repeat of Notch and it is crucial for Notch activity in *C.elegans* (Petcherski and Kimble 2000). LAG-3 is a small, glutamine-rich protein and is thus a good candidate for functioning as a transcriptional activation domain. No vertebrate LAG-3 has been identified as yet. However, the OPA-domain in *Drosophila* and vertebrate Notch receptors is glutamine-rich. Since the nematode Notch homologues Lin-12 and GLP-1 lack an OPA domain, it is possible that LAG-3 has been replaced in vertebrates and in *Drosophila* by the OPA-domain. In conclusion, Notch1 activates transcription at two levels. First, by displacing SMRT and hence relieves transcriptional repression by destabilising the co-repressor complex and second, Notch1 is an activator through the recruitment of HATs, which can positively affect chromatin remodelling.

In many situations the cell has to quickly reset its signalling status to stay responsive to new input, particularly in rapidly developing organisms like *Drosophila*. Thus, there must be an efficient system for clearing the nucleus of NICs. This is further supported by the fact that it is difficult to detect any

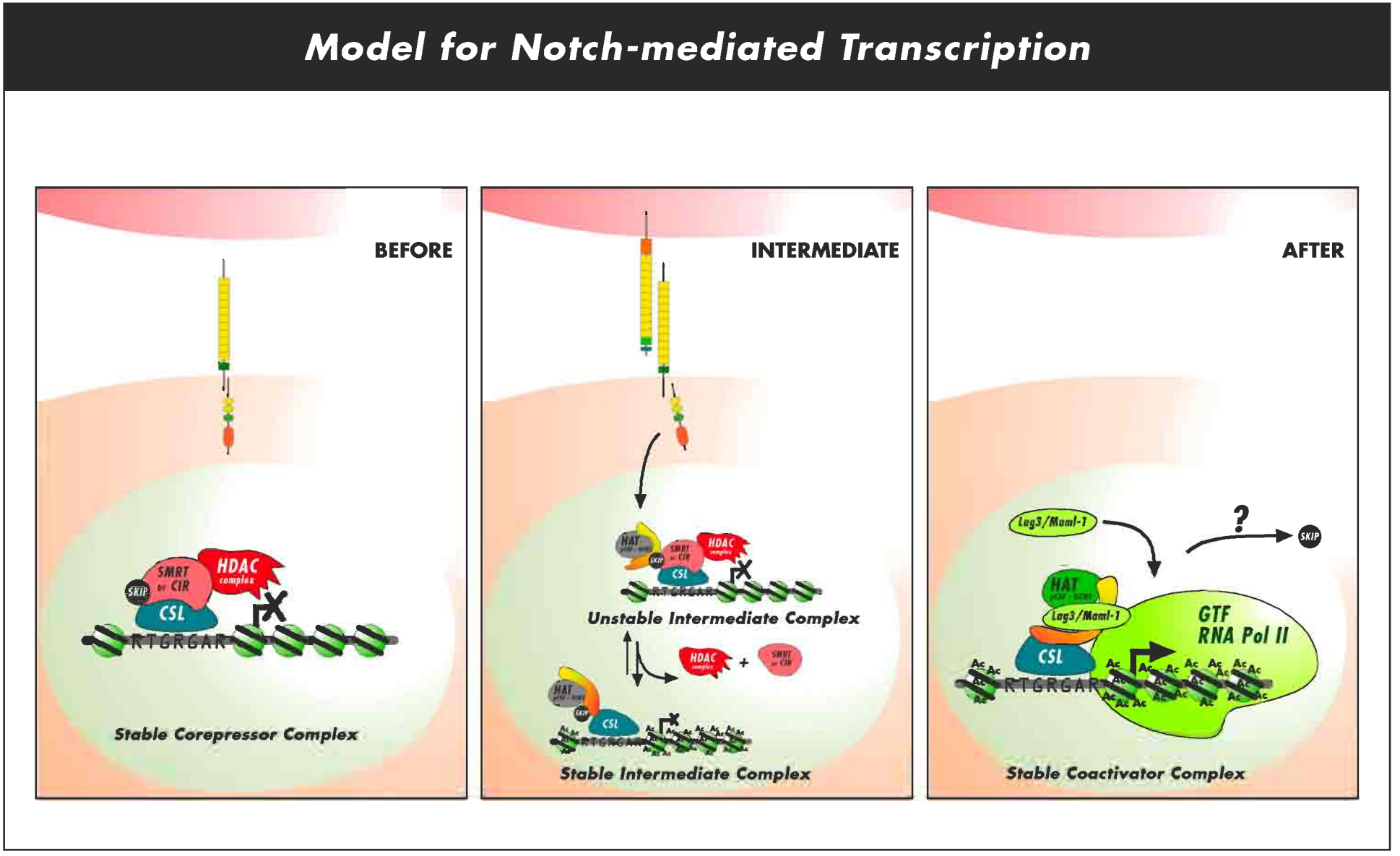


Figure 3. Model for Notch1-mediated transcription. Notch1 is shown as a yellow-orange shape.

nuclear NICs and that sub-detection levels are enough to activate transcription. Notch contains a PEST sequence that has been proposed to be involved in protein stability (Öberg *et al*, submitted). Genetic analysis in *C. elegans* has shown that SEL-10, a protein of the ubiquitin-ligase type, can negatively regulate LIN-12 (Hubbard *et al.* 1997). It has recently been demonstrated that Notch is targeted for degradation by the proteasome and that SEL-10 is required for this (Öberg *et al*, submitted). Interestingly, SEL-10 might have a more direct role in repressing Notch since Notch signalling is reduced without affecting the steady-state levels of Notch in co-expression experiments (Öberg *et al*, submitted). Other proteins in the ubiquitination pathway, like *Suppressor of Deltex* and its vertebrate homologue, *Itch*, can also negatively affect Notch signalling (Cornell *et al.* 1999).

Notch target genes

Surprisingly few target genes for Notch have been identified, considering the number of developmental processes Notch regulates. The most well-known targets are the *hairy* and *Enhancer of split* (*E(spl)*) genes (HES in mammals) (Bailey and Posakony 1995, Eastman *et al.* 1997, Furukawa *et al.* 1995, Lecourtois and Schweisguth 1995). These belong to the basic helix-loop-helix (bHLH) family of transcription factors and function as transcriptional repressors (Oellers *et al.* 1994, Ohsako *et al.* 1994, Sasai *et al.* 1992, Van Doren *et al.* 1994). In mammals, Notch has been shown to regulate expression of HES-1 and HES-5 (de la Pompa *et al.* 1997, Jarriault *et al.* 1995, Jarriault *et al.* 1998, Kuroda *et al.* 1999). The role of the HES genes has been extensively studied in neurogenesis, in which they function to inhibit differentiation (see (Kageyama and Nakanishi 1997) for review). They negatively regulate the expression and function of another group of bHLH genes, referred to as pro-neural genes, which act as positive mediators of neurogenesis. The vertebrate homologues of *Drosophila* pro-neural genes (*atonal* and the *achaete-scute* complex (*A-Sc*)) include *Mash*, *Math*, *neurogenins* and *NeuroD* (reviewed in Lee 1997). *NeuroD* is downstream of *Mash1* and *neurogenin*, and promotes terminal differentiation of neurons. It has been suggested that the HES genes regulate expression of *NeuroD* by controlling *Mash*, *Math* and *neurogenin* (Cau *et al.* 2000, Cau *et al.* 1997). Thus, the HES genes and the pro-neural genes work in a counteracting fashion to control proper rate and timing in neural differentiation.

Another group of target genes has recently been identified, the HEY genes. They are structurally related to the HES genes, but represent a distinct subfamily of bHLH proteins (Maier and Gessler 2000, Steidl *et al.* 2000). The expression of one of the HEY genes, HEYL, correlates well with regions in which Notch signalling takes place and HEYL expression is significantly decreased in embryos defective for Notch1 or Dll1 (Leimeister *et al.* 2000).

1.4 Developmental biology

Embryonic development can be described as a consecutive sequence of cell specifications. A totipotent, fertilised egg cell generates a large number of daughter cells through which stepwise specifications adopt unique properties, resulting in a growing embryo and ultimately in a mature organism. The process of cell differentiation is all about history. Just as our personality is a result of our life experiences and heredity, a particular cell type is the consequence of the external stimuli (experiences) the cell has been subjected to since the first division, combined with its genome. Cell differentiation is integrated in an advanced choreography involving cell proliferation, migration, structural remodelling and death to create a growing embryo. Notch signalling has an impact on many of these events, and I will describe some key steps in embryogenesis.

Gastrulation

The first steps in embryogenesis after fertilisation vary considerably between different vertebrate species, only converging for a brief period when they pass through a common template and then diverging again in completely different forms (Figure 4A*).* For example, the fertilised egg itself is merely 0,1 mm in mouse, 1.2 mm in *Xenopus* but 30 cm in an ostrich. In *Xenopus,* the fertilised cell divides more than 1000 times in 24 h while in humans it takes 24 h to complete the first cell division. Irrespective of which strategy nature has chosen to complete this first blastula stage, all species go through gastrulation in a very similar fashion.

During gastrulation, the cells in the early embryo learn what is up and down (dorsal/ventral), and front and back (anterior/posterior). This process of axis formation is already initiated during the blastula stage, but during gastrulation this initial information is translated into a three-dimensional matrix dividing the cells into three layers (ectoderm, mesoderm and endoderm) (Figure 4B). From now on, all further development will be based on the interplay between these three layers and the molecular signals that they produce.

An important first step for the embryo during gastrulation is to set up a signalling centre (organiser). The organiser instructs or *patterns* the mesoderm, which then in turn influences the ectoderm and endoderm. In vertebrates, a region of the mesoderm is organised into a notochord that will function as a conductor of neurulation and organogenesis (see below) by providing key molecular signals.

Neurulation and somitogenesis

The central nervous system (CNS) of all vertebrates develops from a one cell-layer-thick tube, running all the way along the embryo from the ‘head’ (anterior) to the ‘tail’ (posterior).

The process of neurulation refers to the formation of the neural tube. During neurulation, cells at the ‘top’ of the embryo (dorsal side) will start sinking down into the gastrula, forming a groove (Figure 4C). Finally, the edges of the groove will meet and fuse, forming a closed tube under the surface of an

intact sheet of ectoderm. This process is driven by factors produced by the notochord. Simultaneously, the mesoderm surrounding the groove will remodel and condense into small structures called somites (Figure D). The neural tube will give rise to all of the CNS, while the somites will develop into ribs, skeletal muscles and dermis. The anterior neural tube will develop into the brain while the somites and the posterior neural tube will form the spinal cord, the limbs and the trunk.

Organogenesis

By the onset of organogenesis, the embryo is divided into segments, of which each has a clear polarity and different identity. Thus, all cells of the embryo are divided into microenvironments where they are exposed to different molecular signals. Most organs form at the interface between mesenchyme and epithelium where both tissues contribute to the final organ. Mesenchymal and epithelial cells usually originate from two different layers e.g. mesoderm and either ectoderm or endoderm. Interestingly, the same molecular pathways are involved in the development of different organs and many of the signals that originally specified axis formation, patterning and segment identity are now involved in organogenesis (e.g. *Shh*, FGFs and members of the TGF-β family).

Pancreas development serves as a good example to illustrate organogenesis (reviewed in (Kim *et al.* 2000)). The pancreas is composed of two components: the endocrine and exocrine pancreas. The exocrine component consists of acinar and duct cells, which produce digestive enzymes that promote nutrition digestion and absorption. The exocrine pancreas is interspersed with the islets of Langerhans that consist of endocrine cell types. These include insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ–cells and pancreatic polypeptide-producing pp-cells. The pancreas develops from endoderm-derived foregut epithelium, and is formed from a dorsal and ventral anlage, which eventually fuses to form one structure. The process is initiated at E9.5 in mice by the formation of a dorsal pancreatic bud. Proliferation and branching of the dorsal pancreatic bud is stimulated by mesenchymal signals and activation of specific pro-endocrine genes, which ensures the proper development of the final organ (see below).

Another example of organogenesis is the development of the brain. During neurulation (see above), the neural tube is formed. Lining the neural tube is the neuroepithelium, which consists of epithelial cells that generate virtually all of the neurons and glia cells of the CNS. The cells lining the lumen of the tube (ventricular zone) proliferate to generate a laminated structure which is initially divided into different zones or layers: the ventricular, the mantle and the marginal zones. Immature nerve cells arise from the division of neuroependymal cells in the ventricular zone and migrate to the mantle layer. The marginal zone contains the axonal processes of developing neurons. The ventricular zone contains proliferating progenitor cells that progressively become post-mitotic and start differentiating. Among the first cells to differentiate are the radial glia cells (Choi and Lapham 1978, Levitt *et al.* 1983), which span all layers and serve as a scaffold along which immature, post-mitotic neurons can migrate. The other major cell type, glial cells (astrocytes and oligodendrocytes) are generated

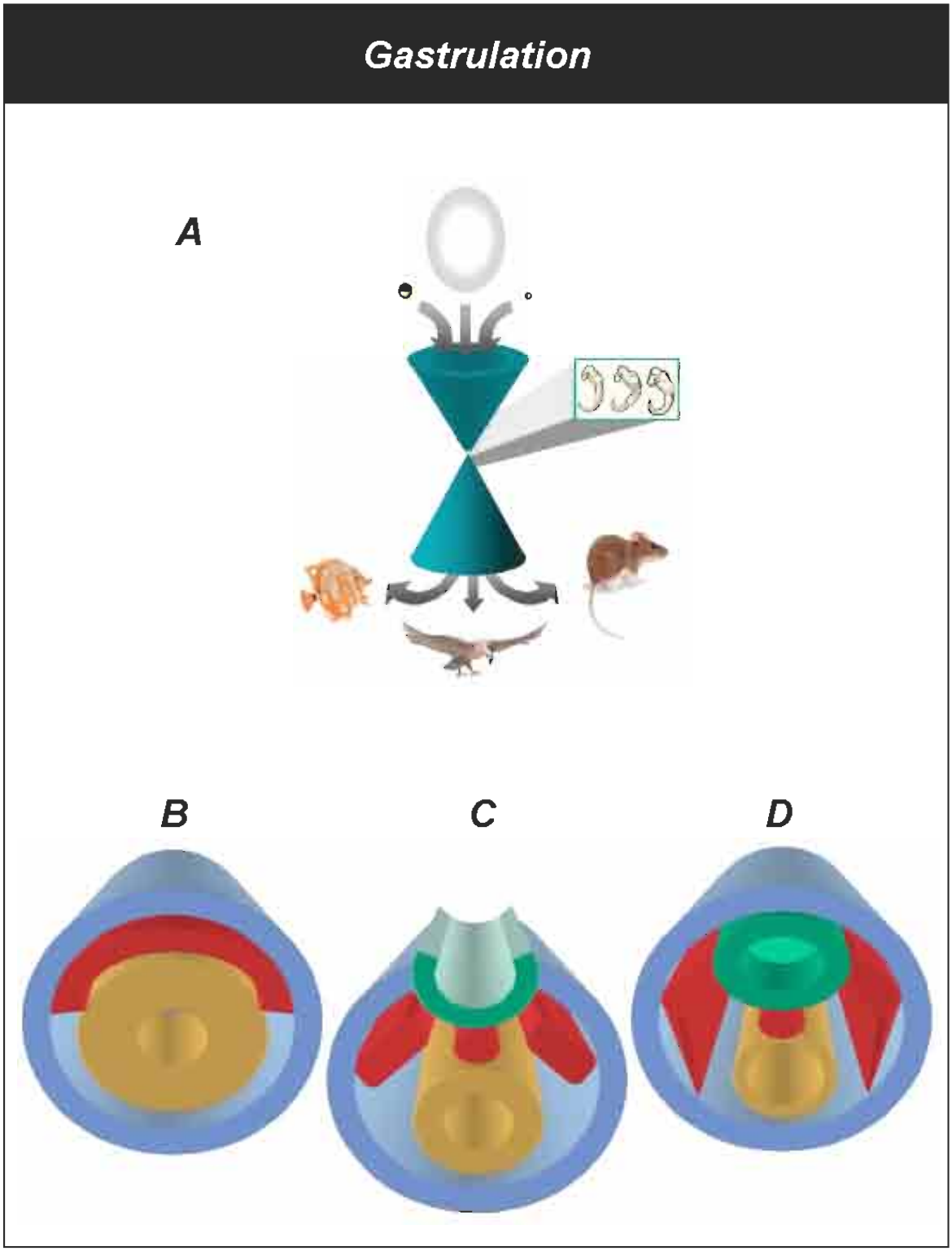


Figure 4. Gastrulation is an embryonic process common to all animals. A) Despite differences in fertilisation, gastrulation is very similar in all vertebrates. B) Gastrula stage embryo. C) Early neurula stage embryo. D) Embryo with closed neural tube. Ectoderm is blue, mesoderm is red and endoderm is yellow.

following neurogenesis. Macroscopically, brain development can be observed as swelling and flexure of the anterior neural tube, forming distinct vesicles that will eventually form all major regions, including the ventricular system of the brain.

1.5 The molecular mechanisms of development

The mechanisms mediating these fundamental embryonic processes at the molecular and cellular level can be summarised as induction, cell differentiation, proliferation, apoptosis and migration.

Induction

During gastrulation and neurulation, intense molecular signalling occurs. Molecules such as *Sonic hedgehog* (*Shh*), members of the TGF-β family and RTKs pattern the embryo, resulting in fields of cells forming equivalence groups. All cells within an equivalence group are equally competent to differentiate in response to a particular inductive signal. Usually cells at this stage are multipotent progenitors and can form all future cell types within that particular tissue, and inductive signals instruct the cells to adopt a specific fate. Inductive signals are usually soluble extracellular molecules, referred to as morphogens, and they often induce expression of specific transcription factors that then initiate migration, growth and cell differentiation programs. The morphogens form concentration gradients to which promoters of the specific transcription factors can respond. In this way, a single morphogen can induce several different cell fates. For example, the formation of motor neurons and different classes of interneurons are specified by selective activation and repression of homeodomain-containing transcription factors (*Dbx, Pax, Nkx*) by *Shh* (reviewed by Briscoe and Ericson 1999).

Induction does not always require an inductive signal; in some situations, inhibition of a repressive signal can trigger differentiation. For example, during pancreas development, exocrine and endocrine cell fates are controlled by the interplay between repression, mediated by *follistatin* or *Shh*, and induction mediated by activin signalling (for review, see Kim *et al.* 2000). *Shh* is expressed in nearly all epithelial cells lining the alimentary canal. However, it is excluded from the region corresponding to the pre-pancreatic epithelium. Misexpression of *Shh* in pancreatic epithelium results in the loss of pancreatic markers, and transformation into gut mesoderm (Apelqvist *et al.* 1997, Hebrok *et al.* 1998). Thus, *Shh* functions to repress pancreas induction. This is counteracted by activin and TGF-β signalling, which can inhibit *Shh* signalling (Hebrok *et al.* 1998). TGF-β1 promotes endocrine differentiation through the *ActR IIA* and *IIB*, which activates expression of the pro-endocrine markers *Islet-1* and *neurogenin3* (*ngn3*) (Kim *et al.* 2000). *Follistatin* is a known antagonist of TGF-β signalling and has been shown to inhibit endocrine differentiation and promote exocrine cells *in vitro* (Miralles *et al.* 1998). *Ipf1/pdx1* is another pro-endocrine gene. In the presence of *Shh*, *Ipf1/pdx1* is repressed by Patched (the *Shh* receptor), creating a permissive condition for endocrine cell

differentiation (Hebrok *et al.* 2000). Another way of promoting different cell fates by repression is by lateral inhibition, which will be described in detail later.

Cell differentiation

Cell differentiation occurs throughout development and into adulthood. For example, the earliest differentiation event during mouse embryogenesis is the formation of the extra-embryonic trophoblasts, while the cells of the immune system keep differentiating continuously throughout life. The first cells to form a vertebrate embryo are pluripotent embryonic stem cells that can develop into any tissue. They progressively lose their pluripotency and become multipotent progenitors. The multipotent progenitor cells then gradually become increasingly restricted and finally end up as highly specialised cells. Cell differentiation can be studied by analysing genetic markers that relate to the expression of specialised genes. For example, a cortical progenitor cell can adopt both neuronal and glial fates, but does not initially express either β-tubulin III or GFAP (neuronal and glial markers, respectively). However, when stimulated with FGF2, these cells start expressing GFAP, indicating that they have adopted the glial fate (Qian *et al.* 1997).

In the adult, most cells have acquired their terminal, irreversible fates. However, in many tissues a small number of very slowly dividing, multipotent cells is maintained. A classical example is haematopoetic stem cells, which constantly replenish all types of blood cells (Weiss 1997). Stem cells have also been found in other tissues including CNS and skin (Jones *et al.* 1995, Morshead *et al.* 1994, Vescovi *et al.* 1993). Evidence has emerged recently that stem cells are not only multipotent, but pluripotent, and capable of giving rise to tissues derived from all three germ layers when transplanted into early embryos (Clarke *et al.* 2000). Likewise, transplantation of haematopoetic stem cells into adult, immunodeficient mice have been shown to populate the brain and give rise to cells expressing neuronal markers (Mezey *et al.* 2000).

Proliferation and Apoptosis

The underlying basis for morphogenesis is the proliferation of progenitor cells. Most of the signalling pathways described above can, under certain conditions, stimulate the cell cycle machinery. In particular, the RTKs are potent mitogens, i.e. activators of proliferation, and they are required for amplification and maintenance of progenitor cells *in vivo* and for the growth of cells in culture. Proliferation and differentiation are tightly regulated to maintain proper growth of the embryo, with terminal cell differentiation usually requiring withdrawal from the cell cycle. In many developmental processes, like neurogenesis for example, the correct number of cells is ensured by selective survival of neurons from a large pool of ‘born’ cells. In the case of neurogenesis, cells compete for limiting amounts of survival factors and/or functional synaptic connections with other neurons or muscles. The surplus of cells that do not receive a survival signal inevitably activate the programmed cell death pathway and die through apoptosis (for review see Denecker *et al.* 2001).

1.6 Notch signalling in development

Notch signalling is deeply involved in many of the processes described above, which is reflected by the broad expression pattern of the mammalian Notch receptors. Notch1-3 are expressed early in presomitic mesoderm and then in maturing somites, where they control somite boundary formation (Jiang *et al.* 2000, Williams *et al.* 1995). Later, all three receptors are expressed throughout the neural tube and in retina, pancreas, lung, tooth, and skin (Apelqvist *et al.* 1999, Kopan and Weintraub 1993, Lindsell *et al.* 1996, Mitsiadis *et al.* 1995, Post *et al.* 2000, Williams *et al.* 1995).
An important function of Notch signalling is to mediate lateral inhibition.

Lateral inhibition at the cellular level

Morphogenic gradients affect all cells within a specific concentration level equally. These cells can often substitute for each other, and they define an equivalence group. It is not always desirable that all cells within an equivalence group will acquire the same fate, and lateral inhibition represents a mechanism to prevent this (for review see Beatus and Lendahl 1998, Greenwald and Rubin 1992, Lewis 1996). Lateral inhibition can be illustrated by the classical example of Notch function in the fly, the neurogenic phenotype (Figure 5A). Notch function is required for proper delamination of neuroblasts along the ventral midline, and in the choice between becoming a neuroblast, which gives rise to the central nervous system, or a hypodermal cell.
Loss of Notch function and thus lateral inhibition 'permits' too many cells from becoming neuroblasts at the expense of hypodermal cells. This system is almost perfectly copied in the generation of primary nervous system in amphibians. *Xenopus laevis* forms a primary nervous system that is required during the early motile life of the embryo. This primary nervous system is generated at the neural plate stage and consists of three longitudinal rows of neurons on each side of the midline, spawning motoneurons, interneurons and sensory neurons (Rohon-Beard neurons). The regularly-spaced primary neurons differentiate from a sheet of cells in the neural plate, while the non-neural cells become epidermal cells. Activation of Notch signalling, achieved by expression of Delta-1 or the intracellular domain of Notch1, leads to a reduced number of primary neurons (Chitnis *et al.* 1995, Coffman *et al.* 1993, Wettstein *et al.* 1997). Conversely, inhibition of Notch signalling, mediated by expression of dominant-negative versions of Delta or CSL, results in the production of supranumerous primary neurons in the neural plate (Chitnis *et al.* 1995, Wettstein *et al.* 1997).

Lateral inhibition at the molecular level

The molecular basis for lateral inhibition has been proposed in a model referred to as 'lateral inhibition with feedback' (Lewis 1996). According to this model, cells with initially small differences in receptor/ligand expression can switch to become either receiving (Notch expressing) or signalling (Delta/Serrate expressing) (Figure 5B). This is mediated via a feedback loop that switches off ligand expression on the receiving cell, preventing it from signalling to its neighbours. Ligands and receptors mutually repress each other's expression on neighbouring cells via *E(spl)/*HES and *Achaete-Scute* (*A-Sc/Mash*). *A-Sc/Mash* is a positive regulator of Delta expression. Cells in these clusters that express slightly higher levels of Delta will induce stronger *E(spl)*/HES expression in neighbouring cells via the Notch pathway. The latter cells, with increased levels of *E(spl)*/HES, repress expression of *A-Sc/Mash* and consequently that of Delta. Eventually these cells will lose their signalling capacity and become receiving cells. In contrast, the signalling cells, which initially expressed only slightly higher levels of Delta than the surrounding cells, will receive fewer stimuli from their Notch receptors because of lower levels of Delta ligands on the surrounding cells. This leads to lower levels of *E(spl)/*HES, which increases *A-Sc/Mash* activity and Delta expression.

Notch as an inhibitor of differentiation

Another aspect of lateral inhibition is the discrimination between differentiation and proliferation rather than between two differentiated states. In this way, a subset of equivalent cells is allowed to differentiate at a given time-point without totally depleting the pool of progenitor cells. This manifests itself as a block of differentiation mediated by Notch signalling during neurogenesis and myogenesis. A number of muscle-specific bHLH transcription factors control the progression of muscle cell differentiation, which involve exit from the cell cycle, migration of myoblasts and fusion of cells into myotubes (see Perry and Rudnick 2000 for review). Primary Myogenic Regulatory Factors (MFRs), such as *MyoD* and *Myf-5*, specify uncommitted mesenchymal cells to adopt the myogenic fate and to form myoblasts. *MyoD* alone can induce myogenesis in 3T3 fibroblasts. Later, *myogenin* and *MRF4* gene-products, which represent secondary MFRs, mediate terminal differentiation and maturation into myotubes. Several of the Notch receptors and ligands are expressed during myogenesis (Hirsinger *et al.* 2001, Palmeirim *et al.* 1998). Early experiments with truncated Notch1 showed that N1IC could inhibit muscle cell differentiation in cultured cells *in vitro* (Kopan *et al.* 1994). The blocked cells continued to proliferate and failed to form myotubes. Activation of endogenous Notch receptors by Dll1 and Serrate1 (Jagged1) also inhibits myogenesis (Hirsinger *et al.* 2001, Kuroda *et al.* 1999, Nofziger *et al.* 1999). These experiments were corroborated *in vivo* in *Xenopus* and chicken embryos. The exact mechanism of how Notch inhibits myogenesis is not yet clear, but Notch seems to mediate this effect at two levels. First, activation of Notch signalling in C2C12 has been shown to reduce the expression of MyoD itself (Hirsinger *et al.* 2001, Kuroda *et al.* 1999). Second, HES–1 can inhibit the function of MyoD by direct interaction and it has been suggested that Notch inhibits myogenesis by

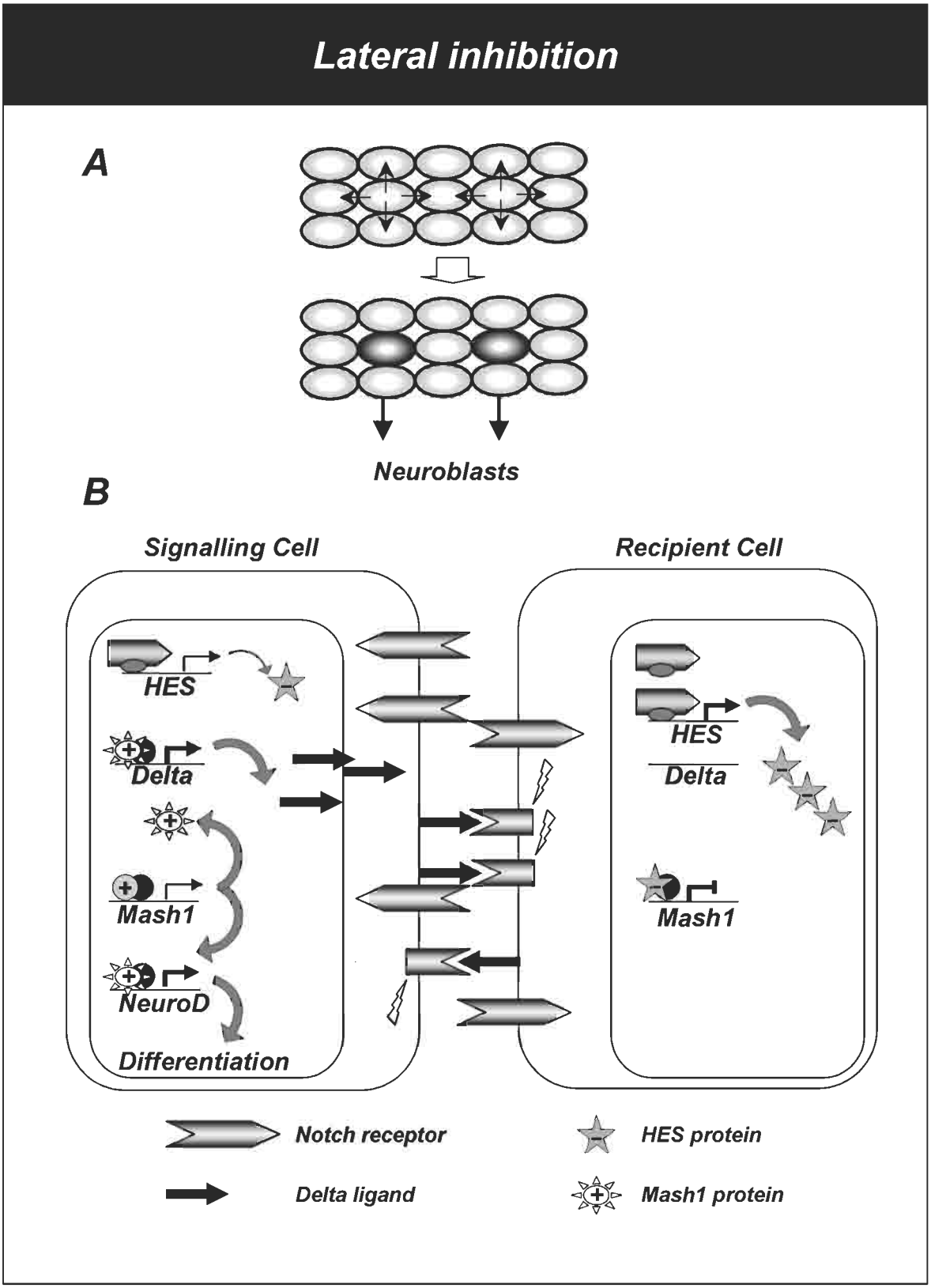


Figure 5. Lateral inhibition on the cellular and molecular levels. A) Neuroblast formation in *Drosophila*. B) Lateral inhibition with feedback.

inducing HES-1 expression via CSL (Jarriault *et al.* 1995, Kopan *et al.* 1994, Sasai *et al.* 1992). Interestingly, two reports show that Notch deletion mutants lacking the CSL-binding domain can still block myogenesis in a CSL-independent pathway (Nofziger *et al.* 1999, Shawber *et al.* 1996b). The authors also suggest that the two pathways inhibit myogenesis at two distinct phases of muscle cell differentiation. The idea of a CSL-independent pathway is possibly supported by Notch's ability to inhibit another bHLH transcription factor, E47, which is important for B-cell development in the immune system (Ordentlich *et al.* 1998). It is suggested that Notch inhibits E47 via Ras signalling and it is possible that a similar mechanism could account for a CSL-independent pathway in muscle cell differentiation. Regulation of MyoD expression reaches higher levels of complexity since it has been shown that MyoD itself stimulates Delta expression and triggers Notch signalling in *Xenopus* gastrula (Wittenberger *et al.* 1999). This also provides evidence for a functional feedback loop in myogenesis.
The function of Notch in myogenesis is strongly supported by the expression of Notch pathway components in somites in addition to the severe somitic phenotypes observed in Notch and Delta knock-out mice.

There are many similarities between myogenesis and neurogenesis at the molecular level and it has been demonstrated that Notch plays important roles during several stages of vertebrate neurogenesis. N1IC was initially shown to inhibit the neuronal differentiation of induced P19 cells in culture (Nye *et al.* 1994). This initial work has since been corroborated *in vivo* by experiments in *Xenopus* (Chitnis *et al.* 1995, Chitnis and Kintner 1996, Dorsky *et al.* 1995), Chick (Austin *et al.* 1995, Henrique *et al.* 1997) and mammals (de la Pompa *et al.* 1997, Wang *et al.* 1998). In essence, this body of work presents a model by which Notch controls the rate and timing of neuronal differentiation by maintaining a pool of undifferentiated progenitor cells (for reviews see Beatus and Lendahl 1998) and (Lewis 1998). This is best illustrated by Notch function in the retina, where the birthdates of a limited number of neuronal cell types are well established (reviewed in Beatus and Lendahl 1998) and (Rapaport and Dorsky 1998). Notch signalling can be antagonised by overexpression of a dominant-negative derivative of the ligand Delta ($Delta^{dn}$) or by applying antisense oligonucleotides that reduce expression of Delta or Notch1. This leads to a reduced thickness of the retina due to precocious differentiation of the progenitor pool (Ahmad *et al.* 1997, Austin *et al.* 1995, Henrique *et al.* 1997). A similar effect can be observed in mutant mice that lack the HES-1 gene (Tomita *et al.* 1996). Conversely, misexpression of Delta or constitutively-active Notch has the opposite effect, resulting in an increased number of cells with neuroepithelial morphology (Dorsky *et al.* 1997, Ishibashi *et al.* 1994). In summary, Notch inhibits differentiation by blocking the expression and function of pro-neural genes via HES activation. Simultaneously, lateral inhibition ensures that Notch is only expressed in the progenitor cells, whereas Delta is observed on the delaminating prospective neurons (Henrique *et al.* 1995).

Notch as an inducer of differentiation

This classical view of Notch as an inhibitor of differentiation has recently been enriched in that it also can function as an inducer of glial differentiation in both the PNS and the CNS. Interestingly, it had been noted early on that Notch permits glial differentiation of P19 cells in culture and of Müller Glia cells in developing retina, even though neuronal fates were inhibited (Dorsky *et al.* 1995, Nye *et al.* 1994). However, it appears that Notch has a more direct influence on glial fates. Furukawa *et al* found that misexpression of N1IC or HES-1 forced progenitor cells in the retina to become Müller Glia cells (MGC) (Furukawa *et al.* 2000). Another group has shown that misexpression of HES-5 had the same effect. Conversely, the authors have also shown that loss of HES-5 expression results in decreased numbers of MGCs (Hojo *et al.* 2000). Besides the retina, gliogenic roles for Notch have also been demonstrated in the developing neural tube and in CNS and PNS stem cells. Introduction of N1IC into cells in the developing mouse brain at embryonic day 9.5 shows that N1IC-expressing cells later developed into radial glial cells (Gaiano *et al.* 2000). Radial glial cells are a cell population with some glial characteristics, but which are also most likely a progenitor population for other neural cell types, including neurons (Malatesta *et al.* 2000).

Work from two groups has shown that Notch can induce gliogenesis in stem cells. Morrison *et al* have shown that transient activation of Notch in neural crest cells (PNS), either by N1IC or by recombinant, soluble ligands, causes an irreversible switch to the glial fate (Morrison *et al.* 2000). Moreover, this work demonstrates that a transient 'flash' of N1IC can even overcome a strong neurogenic signals mediated by BMPs. Finally, CNS stem cells derived from adult hippocampus develop into astrocytes in response to transient pulses of either N1IC or N3IC (Tanigaki *et al.* 2001). In addition, the authors demonstrate that Notch induces astrocyte differentiation independently of ciliary neurotrophic factor (CNTF), which is also a potent astrocytic differentiation factor.

Thus, in contrast to the classical situation, it seems like in some cases, Notch can function as an inducer of differentiation. Interestingly, the original work by Nye *et al* showed a slightly higher frequency of glial differentiation in RA-induced P19 cells expressing N1IC (Nye *et al.* 1994). This hints that Notch can inhibit differentiation and promote it in the same cell type. It will be interesting to learn which factors influence Notch's specificity as an inhibitor and, conversely, as an inducer of differentiation.

The role of Notch in neuronal maturation

Recently, an atypical case of lateral inhibition was demonstrated in which Notch influenced neurite (dendrites or axons) outgrowth of differentiated neurons. Cultured neurons, grown at high density, eventually display shorter and more branched neurites than neurons cultured at low density (Sestan *et al.* 1999). Growth at high density represents numerous cell-cell contacts and thus implicates Notch signalling. Indeed, the levels of intracellular Notch increased during neuronal differentiation (Redmond *et al.* 2000, Sestan *et al.* 1999). Furthermore, it was shown that the same effect could be

reached by activated Notch signalling, either by expression of N1IC and N2IC or by activating endogenous receptors with soluble ligands. Conversely, antagonising Notch function with *Numb*, a repressor of Notch function (Frise *et al.* 1996), had the opposite effect (Sestan *et al.* 1999). These results were repeated in a neuronal cell line. Franklin *et al* also provide evidence that expression of the Notch1 intracellular domain negatively affects neurite length (Franklin *et al.* 1999). They also made the interesting observation that when Notch receptors interact with Delta1 ligands on neighbouring cells neurites get shorter, but when Delta1 is expressed in the same cell as Notch, neurites become longer. This may be a result of Delta blocking Notch receptor function when expressed in the same cell, and activating it only when expressed *in trans*, i.e. on a neighbouring cell. The exciting conclusion from these three reports is that Notch plays a critical role in the control of neurite growth. As the reports deal exclusively with neuronal cells cultured *in vitro*, it is important to seek corroboration *in vivo*, where such supporting evidence is now emerging. In *Drosophila*, overexpression of the gene, *atonal*, increases neurite arborisation, and this function is counteracted by expression of the intracellular domain of Notch. These results show that Notch not only influences glial and neuronal precursor differentiation but also maturation of terminally-differentiated neurons. This opens a totally new dimension of Notch function in synaptogenesis and remodelling of the mature dendritic tree (Sestan *et al.* 1999).

In conclusion, Notch is involved at all levels of cell differentiation and maturation and it appears that Notch signalling can be employed whenever the cell has a decision to make. This can be the choice between two different cell fates, or between differentiation and maintenance as a progenitor, or indeed death. It can also involve choosing which cells to contact and which directions to extend neuronal processes.

1.7 Notch and disease

Considering the importance of Notch signalling during development, it is not surprising that several disorders are linked to mutations in components of the Notch signalling pathway.

CADASIL

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephaleopathy (CADASIL) is a genetic vascular dementing disorder, with clinical onset between 30 and 50 years. Recurring strokes due to brain infarcts in the white and deep grey matter lead initially to dementia and finally to death (reviewed in Kalimo *et al.* 1999). CADASIL has been linked to missense or small in-frame deletions of the human Notch3 gene (Joutel *et al.* 1996). All of these mutations are located in the extracellular domain and result in unpaired cysteine residues. The mutations are strongly clustered

to exon 4, which encodes EGF-like repeats 3-5, but can be found in the entire extracellular domain (Joutel *et al.* 1997). In adult humans, Notch3 is expressed only in vascular smooth muscle cells (VSMCs) (Joutel *et al.* 2000), the degeneration of which is the key pathogenic feature of CADASIL. The disorder is characterised by a number of pathological hallmarks. First, histopathological analysis of medium-sized arterioles in many tissues reveals accumulation of granular osmiophilic material (GOM) between the degenerating VSMCs (Goebel *et al.* 1997). Second, cerebrovascular disturbances lead to a cognitive decline and finally dementia (reviewed in Kalimo *et al.* 1999). Cognitive decline becomes clinically manifest between 40 and 70 years of age. Third, even in asymptomatic carriers, MRI (Magnetic Resonance Imaging) reveals small periventricular hyperintensities that are indicative of CADASIL (Chabriat *et al.* 1995).

The precise mechanism through which Notch3 mutations lead to VSMC degeneration, stroke and dementia remains to be established.

Notch receptors in tumourgenesis

All Notch receptors have been implemented in tumourgenesis. The human Notch1 (TAN-1) was originally identified by analysis of recurrent chromosomal translocations in T-cell lymphoblastic leukaemias (Ellisen *et al.* 1991). Notch2 has recently been identified in thymic lymphomas in Feline leukaemia virus-infected cats (Rohn *et al.* 1996). Notch3 has been shown to cause aggressive T-cell lymphomas in transgenic mice (Bellavia *et al.* 2000) and it has been identified in renal cell carcinomas (Rae *et al.* 2000). Finally Notch4, which was previously called *int3*, forms mammary tumours in mice when misregulated following integration of the mouse mammary tumour virus (Sarkar *et al.* 1994).

Ligand-associated disorders

Mutations in human Serrate1 (Jagged1) underlie Alagille syndrome, a dominant disorder associated with abnormalities of the liver, heart, skeleton, eyes and face (Li *et al.* 1997, Oda *et al.* 1997b). The disorder is caused by haploinsufficiency of Serrate1, since the mutations usually result in frame-shifts or deletions of the gene (Crosnier *et al.* 1999, Krantz *et al.* 1998).

The Dll3 gene has recently been linked to human Spondylocostal dysostosis (SD) (Bulman *et al.* 2000). SD is a group of vertebral malsegmentation syndromes that are characterised by multiple hemivertebrae, rib fusions and deletions (Duru *et al.* 1999). The mutations result in extracellular deletions or missense mutations of highly conserved residues, further implicating haploinsufficiency as a cause of the syndrome.

2. PRESENT INVESTIGATION

Aim of this study

The aim of this study has been to analyse the extent of functional diversity in the Notch receptor family. More specifically, we have investigated the molecular differences between the Notch1 and Notch3 receptors in terms of transcriptional activity and interactions with transcriptional activators and repressors. Given the importance of Notch signalling for many cellular differentiation programmes, it is of considerable interest to learn about the specific functions of the individual Notch receptors. We have consolidated knowledge from *in vitro* studies by investigating the effects of Notch3 signalling during pancreas development and in CADASIL pathogenesis.

2.1 Functional differences between Notch1IC and Notch3IC (Paper I)

Notch3 is a poor activator of HES expression

N1IC represents a dominant gain-of-function receptor and it functions as a potent activator of HES transcription (Hsieh *et al.* 1996, Jarriault *et al.* 1995, Jennings *et al.* 1994, Wettstein *et al.* 1997). We analysed N1IC activity on a HES-luciferase reporter plasmid in a number of transformed cell lines (JEG, HeLa, P19, COS-7, 293T). As expected, N1IC induces expression from the reporter at significant levels compared to mock transfected cells. Interestingly, there was a considerable difference in activity between the cell lines which could not be satisfactorily explained by different transfection efficiencies. We then wanted to establish whether N3IC would behave in a similar way. Therefore, we analysed Notch3 IC's effect on HES reporter activation in the same cell lines. Unlike N1IC, N3IC proved to be a very weak activator of HES-1 and HES-5 reporters in the tested cells. We found that in all cell lines tested, the activity was never higher than four-fold (compared to 15-75 fold for N1IC) (Paper I). This low activity could not be explained by improper expression of the N3IC-expression plasmid or by mislocalisation of the protein, since immunocytochemistry indicated that N3IC was properly localised to the nucleus. Because Notch activity is dependent on CSL, we demonstrated that N1IC and N3IC bind CSL with similar affinities. We then tested whether reporter activation could be enhanced by co-transfecting exogenous CSL. N3IC-mediated activation was not improved even at five-fold levels of CSL. Surprisingly, N1IC activity was markedly reduced by exogenous CSL, suggesting that free CSL sequester other necessary factors and that the stoichiometry of the Notch activation complex is important.

Notch3 can suppress N1IC activity

The Notch genes have distinct but overlapping patterns of expression. Notch1 and Notch3 are co-expressed in several tissues during development (CNS, pancreas, teeth and haematopoesis) and we were curious to see how signalling is affected in situations in which receptors have been activated. Several examples from other signalling pathways show that two members of a gene family can either augment each other's effect or suppress it (rSMADs and iSMADs see above). Thus, we co-expressed the intracellular domains of Notch1 and Notch3 and analysed HES-reporter activation. We found that in the presence of equal amounts of N3IC, reporter activation is substantially reduced compared to N1IC alone. In JEG and HeLa cells, suppression was more than 90% of the N1IC. Interestingly, suppression of N1IC activity was almost fully penetrant (84 %) even at 50 % lower amounts of N3IC than N1IC. In several cases, the suppressed activity was similar to the activity of N3IC alone, suggesting that N3IC exerts a dominant effect over N1IC (Paper I). A plausible explanation for the suppression effect would be competition for common factors necessary for reporter activation. The obvious candidate factor is CSL, which mediates promoter binding to Notch. To address this issue, we designed a Notch1 molecule that would be independent of CSL for DNA binding, by fusing N1IC to the DNA-binding domain of the yeast transcription factor GAL4 (G4-N1IC) (Figure 6). This construct proved to be a potent activator of a UAS-reporter. Co-transfection of G4-N1IC and increasing levels of N3IC showed that N3IC efficiently suppresses reporter activation. This system, using G4-N1IC, allowed us to compare the competitive features of N3IC with N1IC. Co-transfection of G4-N1IC and free N1IC resulted in a linear suppression that was poorer than that observed for N3IC. Thus, there is yet another difference between the two NICs: not only is N3IC a weaker activator of transcription, but it is also a stronger antagonist of G4-N1IC than N1IC itself. In conclusion, we find two major differences between N1IC and N3IC. First, N3IC is a weak activator of HES promoter activation compared to N1IC and the low transcriptional activity of Notch3 IC is dominant over Notch1 IC-mediated activation. Hence, Notch3 IC behaves as a functional repressor of Notch1 signalling with respect to the HES genes. Second, N3IC is a considerably better repressor of G4-N1IC than N1IC. From these data, we also conclude that N3IC represses N1IC-mediated activation of HES promoter expression at two levels (Fig A-model 1). First, by competing for CSL and second, by competing for a common factor other than CSL. We predict that N3IC would bind this factor X with a higher affinity than N1IC does, but that the interaction does not pay off in terms of transcriptional activation.

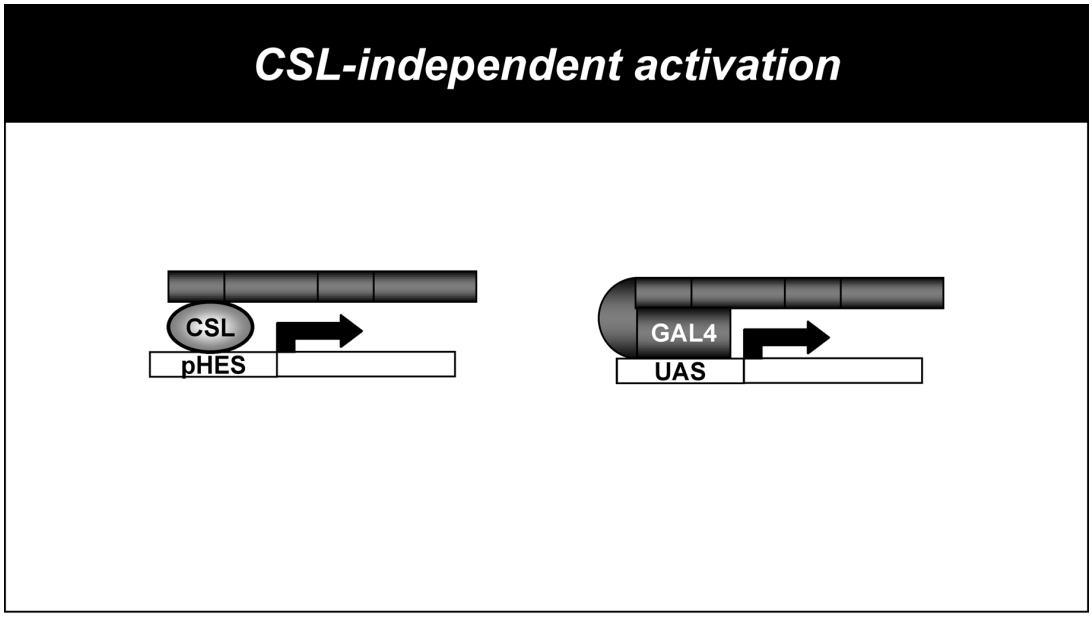


Figure 6. N1IC activates transcription independently of CSL when fused to yeast the GAL4 DNA-binding domain.

2.2 Molecular dissection of the Notch ICs (Paper II)

Identification of RE/AC

N1IC and N3IC are structurally very similar and share 50 % amino acid identity. Given the substantial differences in HES promoter activation, we wished to understand the molecular mechanism for this difference. Two obvious questions arise: first, why is N3IC a weaker activator of the HES promoter and second, why is it a better repressor?

To address these issues, we designed a number of C-terminal deletion mutants of N1IC and N3IC. We found that a 125-amino acid (aa) region, immediately C-terminal to the ankyrin repeats, was crucial for N3IC's ability to suppress G4-N1IC activity. This region (referred to as RE/AC, see below) is necessary, but not sufficient for suppression and the minimal construct that would mediate the N3IC effect consists of RE/AC and the last four ankyrin repeats. The corresponding region in N1IC proved to be crucial for activation of the HES promoter. Specific deletion of this 100-aa-residue-long region alone totally abolished reporter activation. Hence, we refer to this region as RE/AC, for repression/activation. We found that deletions downstream of the RE/AC only affected transcriptional activity moderately. This is in contrast to what has been shown earlier. Kurooka *et al* showed that the C-terminal region of N1IC could function as a Transcriptional Activation Domain (TAD) when fused to GAL4. The authors also showed that loss of the TAD markedly reduced the activity of a multimerised CSL response element (Kurooka *et al.* 1998).

From these experiments we have learned that RE/AC, a common region in N1IC and N3IC, is crucial for activity both in terms of activation and repression. Furthermore, N3IC's poor activation capacity cannot simply be explained by a missing TAD. Given the structural similarities between N1IC and N3IC, the answer to the questions above could be expected to lie in subtle differences within the different domains.

Identifying the regions that confer activation and repression

To further address the influence of the individual regions on activation and repression, we conducted a number of region-swapping experiments. The C-terminus, RE/AC and the ankyrin repeats were exchanged in different combinations between N1IC and N3IC. We introduced a special nomenclature for these experiments, which is outlined in figure 7. All hybrid molecules were tested for their ability to activate a HES reporter or to repress G4-N1IC. Some of the hybrid molecules have been designed without the C-terminal region, since it has little effect on the HES promoter. We demonstrated that the origin of the ankyrin repeats has most influence on both activation and repression. For example, molecule 3130, in which the ankyrin repeats from N3IC have been exchanged for N1IC, is almost as good an activator (83%) as N1IC. Conversely, 1310 is a considerably weaker activator than N1IC. These results are even more pronounced in terms of G4-N1IC repression. Even though the RE/AC

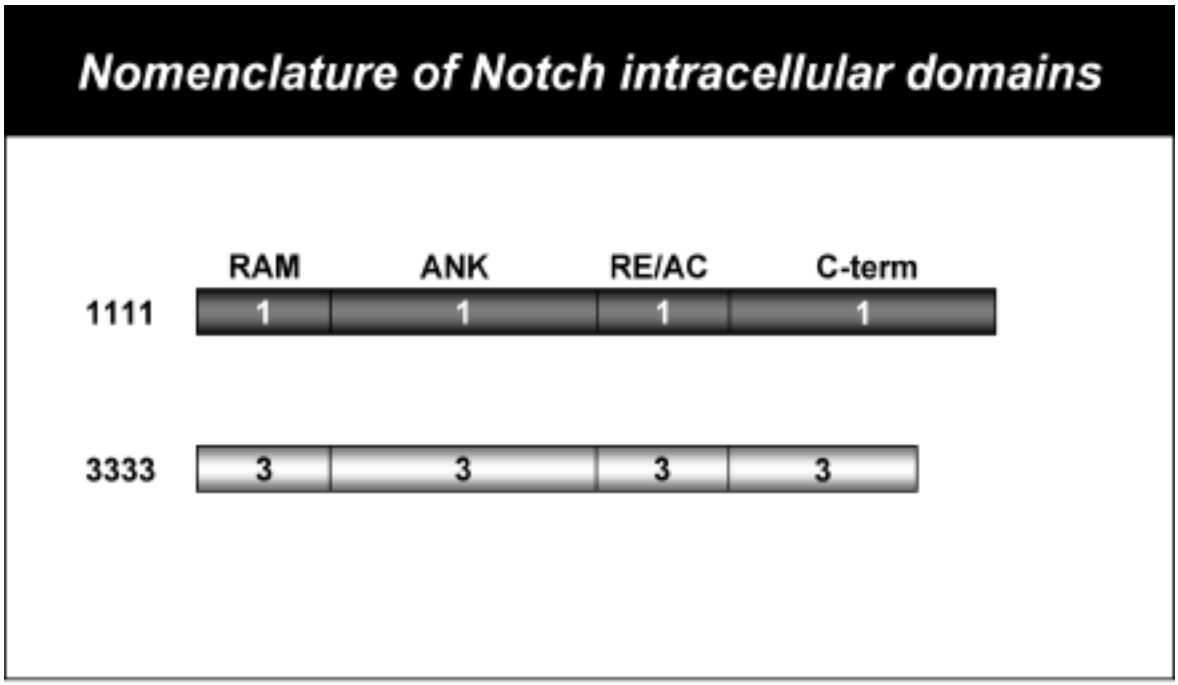


Figure 7. We divide the Notch IC into four regions: the RAM region, the ankyrin repeats, the RE/AC region and the C-terminal region. Each region in Notch 1 is denoted by 1 (for Notch 1) and by 3 for Notch 3. Deletion of a region is represented by 0 (see below). Thus, the entire ICs of Notch 1 and Notch 3 are referred to as 1111 and 3333, respectively.

domain is crucial for Notch function, it seems to be largely exchangeable between the two receptors. No significant change in activity could be detected when RE/AC was swapped. Replacement of N3IC's C-terminus with that of N1IC had only a minor effect on HES-promoter activation. This is noteworthy and indicates that the putative TAD has no major influence on HES promoter activation *in vitro*. Exchange of both RE/AC and the C-terminus resulted in a minor enhancement, compared to when only one domain was exchanged, suggesting that the effects are additive. In conclusion, we have learned that the origin of the ankyrin repeats have most influence on activation and repression while the RE/AC and C-terminal regions are interchangeable.

Searching for factor X

It is possible that the question of the poor HES activation and the inhibition of N1IC-mediated transcription have a common answer. It has been suggested that Notch1 mediates transcriptional activation at several levels. First, repression mediated by the SMRT co-repressor must be overcome (Kao *et al.* 1998) and second, positive co-activators (HATs) have to be recruited (Kurooka and Honjo 2000). Finally, a third factor, SKIP has been shown to interact both with the repressor complex and the activator complex and to be crucial for N1IC activity (Zhou *et al.* 2000b). N3IC's poor transcriptional activity could be explained if N3IC failed to destabilise the SMRT co-repressor complex or if N3IC failed to bind either PCAF (HAT) or SKIP. Using a 'three-hybrid' assay, we demonstrated that N3IC can indeed displace SMRT from CSL. Thus, it is unlikely that N3IC's weak activation is due to failed de-repression. The poor activity of N3IC cannot be explained by loss of SKIP binding, since N3IC shows a solid interaction with SKIP in both GST-pulldown and mammalian two-hybrid assays. We also tested whether N3IC suppressed N1IC activity by competing for SKIP. This is not likely, since increased levels of SKIP did not rescue repression of N1IC on a HES reporter.

PCAF has been shown to interact with the ankyrin repeat region and the C-terminus of N1IC (Kurooka and Honjo 2000). Given that N3IC lacks the OPA domain in the C-terminus and that the origin of the ankyrin repeat region has large influence on transcriptional activation, we were interested to learn whether N3IC would interact with PCAF. Both GST-pulldown and mammalian two-hybrid experiments indicate that N3IC binds to PCAF. However, the interaction seems slightly weaker than for N1IC. As for SKIP, we tested whether increased levels of PCAF could rescue repression of N1IC activity. N3IC repression could not be relieved here either. The reason for N3IC's weaker activity and repressive features remains elusive, but we strongly suspect that the two issues are closely related and that answering one will also reveal the answer to the other.

2.3 Notch3 IC also represses Notch1 IC function *in vivo* (Paper I + III)

Based on our experiments in transfected cells, we wanted to analyse the effects of N3IC *in vivo*. We were careful to choose tissues in which co-expression of Notch1 and Notch3 naturally occurred, such as the CNS. We also tested N3IC in pancreas development, which represents a reasonably 'simple' system with respect to cellular complexity, and where a change of fate may be possible to interpret in terms of Notch activation/repression.

Overexpression of N3IC in the neural tube reduces HES-5 expression

It has been shown previously that overexpression of N3IC in the neural tube in transgenic mice has severe effects on CNS development (Lardelli *et al.* 1996). Notch1 is heavily expressed in the neural tube at this stage (Lindsell *et al.* 1996, Williams *et al.* 1995) and the observed effects could possibly be due to affected Notch1 signalling. New N3IC-overexpressing transgenic mice were produced and we analysed endogenous HES-5 expression in E10.5 embryos (Paper I).

We found that HES-5 expression was abolished in a region caudal to the rhombic lip. Strikingly, expression was not abolished in the entire neural tube, suggesting that either HES-5 is controlled by other factors than Notch1 in those regions or alternatively, that N3IC repression is blocked for some reason.

This experiment demonstrates that N3IC can also repress N1IC activity *in vivo*, which may be of great importance in tissues where the two receptors are co-expressed (see above).

N3IC affects cell fate in the developing pancreas by repressing Notch1 signalling

The effects of N3IC were also studied in a completely different system. The developing pancreas is a great model system to study patterning and cell differentiation. It consists of a limited number of cell types which are well characterised in terms of when they differentiate (Wessells 1967) and see above). Endocrine cells develop from a field of equivalent progenitor cells and end up as scattered islets intermingled with exocrine tissue. Endocrine cells are singled out several days before exocrine cells in an ordered process (reviewed in Kim and Hebrok 2001). This process is reminiscent of lateral inhibition and suggests that Notch signalling may be involved in the differentiation of endocrine cells. The expression of various members of the Notch signalling pathway was studied by whole mount *in situ* hybridisation at two developmental stages (E9.5 and E13.5). We found that N1, N2 and HES-1 are uniformly expressed in the epithelium at the level of the future pancreatic bud at E9.5. Interestingly, Dll1 was only detected in the dorsal epithelium despite the uniform HES-1 expression. Neither Serrate1 nor Notch3 were expressed at this stage. To analyse the role of Notch signalling in pancreatic development, we studied mice deficient in RBP-Jk and Dll1. These animals have similar phenotypes to Notch1$^{-/-}$ animals and they are believed to be deficient in Notch1 signalling (de la Pompa *et al.*

1997, Hrabe de Angelis *et al.* 1997, Oka *et al.* 1995). In keeping with this, we found that HES-1 was downregulated in Dll1$^{-/-}$ pancreas. We also found that pancreatic development was obstructed in RBP-Jk$^{-/-}$ and Dll1$^{-/-}$ embryos (Paper III). The pancreatic bud was small and poorly branched, and excessive endocrine cell differentiation could be observed. The small size of the pancreatic bud and the increase in numbers of cells positive for an endocrine marker suggests premature differentiation of pancreatic progenitor cells. To test whether Notch3 could inhibit Notch1 activity *in vivo*, we misexpressed the N3IC from the *ipf1/pdx1* promoter. If inhibition would be the case, we would expect similar phenotypes to the RBP-Jk$^{-/-}$ and Dll1$^{-/-}$ mutants. We found that the phenotype of *ipf1/pdx1*-N3IC pancreas was indeed indistinguishable from that of the RBP-Jk$^{-/-}$ and Dll1$^{-/-}$ mutants. This clearly supports the notion of N3IC as a repressor of N1IC activity *in vivo*.

Interestingly, we observed an increase in the number of *neurogenin 3* (*Ngn3*) positive cells in all the mutants. *Ngn3* is a homologue of the pro-neural gene *Ngn1*, and its upregulation in the progenitor cells suggests that it might be an early inducer of endocrine fate. To investigate this, we misexpressed *Ngn3* in early progenitors using the *ipf1/pdx1* promoter. Indeed, the pancreas of transgenic mice was morphologically similar to the *ipf1/pdx1*-N3IC, RBP-Jk$^{-/-}$ and Dll1$^{-/-}$ animals, containing significantly increased numbers of endocrine cells. This suggests that *Ngn3* functions to promote endocrine cell differentiation and that it might be analogous to the function of *Ngn1* in neuronal specification in the CNS.

2.4 Notch and CADASIL (Paper IV)

Notch3 plays an important role in the pathogenesis of CADASIL, a familial form of stroke.

CADASIL is caused by specific missense mutations in the extracellular domain of Notch3. There are a number of obvious limitations to studying the pathogenesis of CADASIL in patients. For example, it is very difficult to assess the relationship between the structural changes of the arterioles with the onset of neurodegenerative symptoms, i.e. do GOM accumulate over time or are they already present early in life? Another issue is to understand if and why the VSMCs degenerate or fail to regenerate. Finally, experimental treatment of CADASIL is not suitable on patients for obvious reasons. To address these questions, we have generated a murine 'knock-in' model for the most prevalent CADASIL mutation (R140C). We modified the genomic locus of Notch3 by changing an arginine residue, corresponding to the human arginine 140, to a cysteine. The modified genomic fragment was introduced into ES cells by homologous recombination and correctly targeted cells were subsequently used to generate CADASIL$^{+/R140C}$ and CADASIL$^{R140C/\,R140C}$ mice. We have analysed these mice with respect to the effects observed in patients. CADASIL$^{+/R140C}$ and CADASIL $^{R140C/\,R140C}$ mice were viable and fertile and showed no overt gross phenotypes. Patients show characteristic pathological changes in the VSMCs. We therefore analysed the arteries of 10- and 14-month old transgenic mice. Light

microscopic analysis of the vessel wall in our mouse model showed several abnormalities. The vessel wall was considerably thicker in CADASIL$^{+/R140C}$ than in wildtype littermates and the elastic laminae showed frequent fragmentation and illegitimate branching. The VSMCs were also irregularly orientated between the layers of elastic laminae. The increased thickness of the vessel wall was not due to increased number of layers of VSMCs, but rather to increased deposits of extracellular matrix (ECM) surrounding the VSMCs. Preliminary results indicate that there does not seem to be any gross change in the number of VSMCs within individual layers (unpublished data). At the electron microscopic level, VSMCs in the CADASIL mice were more irregular in shape, with cell debris observed both intracellularly and in the extracellular space. A characteristic feature of CADASIL in patients is the presence of GOM. Interestingly, no unequivocal GOM could be detected in the mouse model, even though granular material reminicent, but distinct from GOM was present in CADASIL mice.

Another difference between human and mouse CADASIL was that no signs of brain infarcts were observed in either MRI scans or in paraffin sections of CADASIL and wildtype mice.

CADASIL patients show a cognitive decline, finally leading to dementia. We found that CADASIL mice exhibited an age-dependent deficit in exploratory behaviour (open field test) suggesting that mice also develop cognitive dysfunctions.

We conclude that CADASIL mice share several important features with human patients in that they develop an arteriopathy and alterations in VSMCs, which may be reflected in an age-dependent deficit in behaviour. However, we also notice a few differences in pathogenesis, since the mice do not exhibit true GOM and they do not develop brain infarcts.

3. DISCUSSION

The existence of multiple homologues within a gene family is a general issue in biology. Understanding their individual functions and interplay is important from clinical, developmental and evolutionary perspectives. Most of the work on Notch receptor function and signalling has been focused on the *Drosophila* Notch and Notch1 receptors, which seem to have similar functions in terms of lateral inhibition and cell differentiation. The data presented in this thesis address the issue of the functional differences and similarities between the Notch1 and Notch3 receptors. Notch3 is also unique in the sense that mutations in this gene cause a vascular dementia syndrome. We discuss the effects of a genetically introduced CADASIL-allele in a mouse model.

3.1 Functional differences in Notch1 IC and Notch3 IC signalling

Notch3 IC is a functional repressor of Notch1 IC-mediated activation

We have analysed the molecular mechanisms whereby Notch receptors activate transcription of downstream target genes like HES-1 and HES-5 and we find that there is a distinct difference in activity between N1IC and N3IC. Several groups have shown that N1IC is a reasonably good activator of HES-1 transcription in transfected cells (see above). In contrast, we show that N3IC has a much weaker capacity for transcriptional activation. Furthermore, we find that the low transcriptional activity of Notch3 IC is dominant over Notch1 IC-mediated activation. Hence, Notch3 IC behaves as a functional repressor of Notch1 signalling with respect to the HES genes. We also show that low levels of Notch3 IC are enough to efficiently repress Notch1 IC activity. This is important since Notch1 and Notch3 are co-expressed in several tissues during development. One function for Notch3 could thus be to modulate Notch1 signalling. Today, we still do not know which ligands activate Notch1 and Notch3 *in vivo* in specific tissues and thus it is difficult to understand the extent of such modulatory effects. It should also be kept in mind that these experiments have been conducted using the ICD of N3. Thus, we do not know how this relates to the receptor levels and the extent of activation by the natural ligands. Even though relatively low levels of N3IC repress N1IC, it is possible that activation of the Notch3 receptor is a very rare event and thus does not play any significant role under natural circumstances. Curiously, there is no solid evidence to date that Notch3 is processed in the same way as Notch1 in response to ligand activation, even though strong indications for this exist (Joutel *et al.* 2000).

We have tried to understand the molecular reasons for the difference in activity between the two receptors. We have systematically deleted specific regions of N1 and N3ICs in addition to generating 'chimeric' N1/N3Ics and we make two observations.

The importance of having the right ankyrin repeats

We conclude that the origin of the ankyrin repeat region, in particular ankyrin 3-6, determines whether Notch will function as either a good activator or a repressor of Notch-mediated activation. It is well established that the ankyrin repeats are crucial for Notch function and that they are also the most highly conserved region of the receptor (Aster *et al.* 2000, Kurooka *et al.* 1998, Rebay *et al.* 1993). The fourth ankyrin repeat is of particular interest, since missense mutations in this region have been shown to negatively affect receptor function in *C. elegans* as well as inhibition of myogenesis and HES-1 promoter activation in mammalian cells (Jarriault *et al.* 1995, Kato *et al.* 1997, Kodoyianni *et al.* 1992, Kopan *et al.* 1994). It has recently been demonstrated that these missense mutations disturb the interaction between Notch and SKIP, a co-factor in transcriptional activation (Zhou *et al.* 2000a, Zhou *et al.* 2000b). However, SKIP is not likely to be the crucial factor for the differences in N1's and

N3IC's activities, since we show that both molecules bind this factor with similar affinities. In addition, increased levels of exogenous SKIP do not 'rescue' N3IC-mediated repression.

No action without RE/AC

We have shown that the RE/AC is crucial for both N1 and N3 function, but that the origin of this region is of less importance. The true function of this region still remains to be elucidated, but several lines of evidence indicate that it might also be involved in functions other than transcriptional activation. Wettstein *et al* show that C-terminal deletions affecting the RE/AC region reduce ESR-1 (*Xenopus* homologue of HES-1) induction in neutralised animal caps (Wettstein *et al.* 1997). Another group has studied N1IC's capacity to cause neoplastic transformation in cell lines and they demonstrate that this feature is totally dependent on the presence of 9 aa residues within RE/AC. Internal deletion of this region completely abolishes the foci-forming capacity (Jeffries and Capobianco 2000). Finally, an interesting report addresses Notch1 and Notch2 function in myeloid differentiation. The myeloid progenitor cell line 32D can differentiate to granulocytes in response to two different cytokines, G-CSF and GM-CSF. Bigas *et al* have shown that Notch1 inhibits differentiation induced by G-CSF alone, while Notch2 does the opposite (Bigas *et al.* 1998). They have mapped the region responsible for this cytokine specificity (referred to as NCR) and it correlates very well with RE/AC and the regions identified by Wettstein *et al* and Jefferies *et al*

All data indicate that the RE/AC is a rather small region, perhaps as small as nine aa residues. It is, however, unlikely that it represents a true domain in the sense of a specific fold. Some experiments suggest that this region is subject to post-translational modifications. Bigas *et al* show that part of the RE/AC region in N1 and N2 is responsible for differences in migration in SDS-PAGE and they suggest that this may reflect different post-translational modifications.

In conclusion, the minimal region required for repression of N1IC-mediated HES activation consists of ankyrin repeats 3-6 and 40 aa residues (or possibly, as few as 9 aa residues) downstream of those, corresponding to the RE/AC region.

It has been shown that N1IC contains a transcriptional activation domain (TAD) and that it is required for proper transcriptional activation under certain circumstances (Kurooka *et al.* 1998). We find that the TAD can indeed function as an activation domain, but that it only contributes to a minor extent to activate the HES promoters. This contradiction could be explained by the use of different promoters. Kurooka *et al* used a hexamerised EBNA2 element from the TP-1 promoter, whereas we have used a 350bp-region from the endogenous HES-1 promoter.

It should be remembered that proteins are not linear arrays of domains and thus care is needed when doing deletion experiments. It is easy to misinterpret specific loss of activity with a collapsed protein

due to deletion of a structurally crucial domain. However, we have tried to guard against this by testing our mutants for retained protein-interaction capacities and proper intracellular localisation.

Notch out for factor X

The identification of the ankyrin repeat region and RE/AC as critical domains for activation and repression does not explain why N3IC is a poorer activator than N1IC. We have systematically tested various steps in the process of Notch-mediated transcriptional activation. We conclude that the poor transcriptional activity of N3IC is due neither to impaired access of CSL nor to failure to alleviate repression by SMRT. However, since we have not yet tested whether N3IC is competent to displace any of the other two known co-repressors, CIR and KyoT2, it is possible that one of them is the crucial factor. In addition, various protein-protein interaction approaches can be used to systematically search for novel interacting factors. Once repression is relieved, positive co-factors have to be recruited for proper transcriptional activation. Kurooka *et al* show that the histone acetyl transferase, PCAF, functionally interacts with Notch1 IC and that inhibition of PCAF decreases N1IC-mediated transcriptional activation (Kurooka and Honjo 2000). We show that N3IC can also bind PCAF, possibly with slightly lower affinity. This suggests that the weak activation is not entirely due to failed recruitment of HATs. The role of SKIP has already been discussed above.

Assuming that both Notch1 IC and Notch3 IC have access to RBP-Jk *in vivo*, we propose three different models to explain the differences in activation (Figure 8). These models take into account that the origin of the ankyrin repeat regions is important and that a Notch IC requires the presence of a RE/AC region, which binds a factor that is present in limiting amounts. In the first model (Figure 8A), the ankyrin repeat region would be important for the conformation of the Notch IC/factor complex, and the factor binding to the RE/AC region would only be presented optimally to the transcription machinery when the ankyrin repeat region is of Notch1 IC origin. In the second model (Figure 8B), the ankyrin repeat region of Notch1 IC serves as a docking site for a second co-activator, which cannot bind or binds less well to the ankyrin repeat region of Notch3 IC. Only the cooperative binding of the co-activator on the Notch1 IC ankyrin repeats and the factor binding to the RE/AC region would lead to potent activation. PCAF binds less well to Notch3 IC than to Notch1 IC, and could thus be a candidate factor in Figure 8B. PCAF has indeed been shown to bind both the ankyrin repeats and the C-terminal region in Notch1 IC. The less efficient PCAF binding of Notch3 IC could result in a more compacted chromatin structure at the promoter, compared to when Notch1 IC-PCAF is present. In the third model (Figure 8C), the presence of an additional factor is also postulated, but in this model the co-factor would be a co-repressor specifically recruited to the Notch3 IC ankyrin repeat region. This could quench the activity of the factor binding to the RE/AC region, thus rendering Notch3 IC incapable of activating transcription.

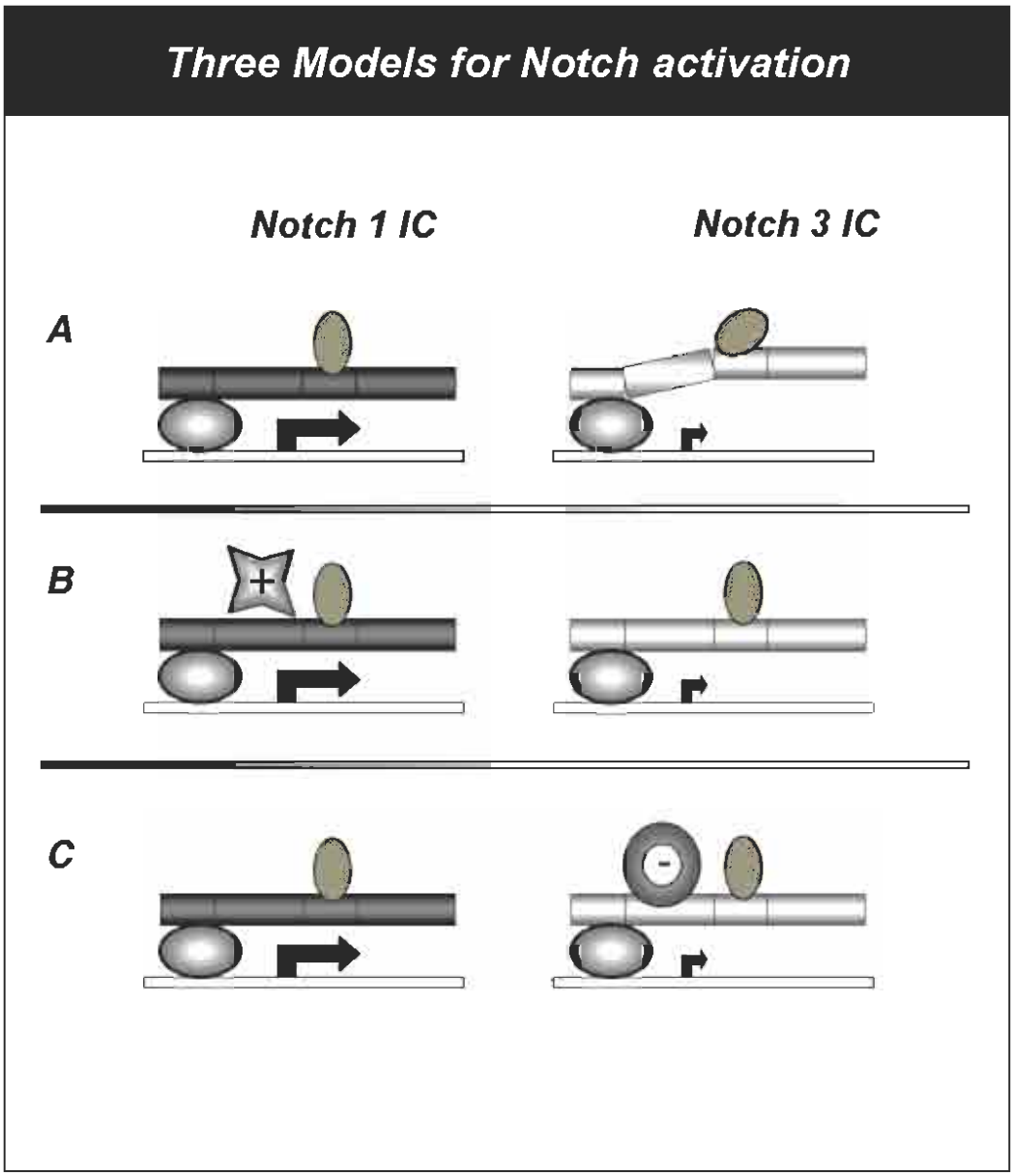


Figure 8. Three models to explain the difference in activation from the HES promoter between N1IC and N3IC. Striped oval= common co-factor for Notch 1 and Notch 3. "+" denotes a Notch 1-specific Co-activator. "-" represents Notch 3-specific repressor.

A complicating factor in the analysis of Notch signalling is the relatively low level of activation from Notch receptors even under optimal conditions, which makes the system more vulnerable to variability in the testing conditions compared to receptor systems with very high degrees of activation. We have observed variability in the results not only between different cell lines, but sometimes also within different batches of the same cell line. This should be taken into account, in particular when transient transfections are used. The variability suggests that the stoichiometry of the signalling components is very important and that the levels of these components may be affected by the 'health status' of the cell or by small variations in cell density and the amount of serum. This might reflect the sensitivity of Notch signalling and also be a consequence of the relative low levels of activation naturally occurring in this signalling system. For example, Schroeter *et al* have shown that levels barely above detection of N1IC are enough to mediate efficient activation of the HES promoter (Huppert *et al.* 2000, Schroeter *et al.* 1998). The amounts of cDNA commonly used in transient transfections are several magnitudes higher than that shown to be enough for activation and probably represents a saturated state.

Low levels of nuclear N1IC also brings about the issue of proper clearing of the ICD. As discussed in the introduction, in many situations the cell must be able to quickly reset the Notch signalling status to be ready to respond to coming signalling events. Several recent studies indicate that Notch turnover is regulated by the ubiquitin pathway (Cornell *et al.* 1999, Hubbard *et al.* 1997, Qiu *et al.* 2000). Interestingly, in some situations Sel-10 can downregulate N1IC activity without affecting N1IC protein levels, suggesting that Sel-10 can also target other components of Notch signalling for degradation (Öberg *et al*, unpublished results).

Notch3 is also a repressor in vivo

We demonstrate that N3IC can suppress HES expression in transgenic mice both in the pancreas and in the developing CNS and presumably this is mediated by interference with Notch1 signalling. Misexpression of N3IC in the neural tube results in severe haemorrhage and defective closure of the anterior neural pore (Lardelli *et al.* 1996). In addition, these transgenic embryos display a characteristic zig-zag shaped posterior neural tube, a pattern which can also be observed in embryos with a processing-deficient Notch1 allele or in HES-1 deficient embryos (Huppert *et al.* 2000, Ishibashi *et al.* 1995). Furthermore, N3IC-misexpressing mutants share another feature with HES-$1^{-/-}$ embryos. Both types of mutations result in a holoencephalic, non-closure of the anterior neural pore (Ishibashi *et al.* 1995, Lardelli *et al.* 1996). As described above, misexpression of N3IC in the developing pancreas results in precocious differentiation of endocrine cells and hence an underdeveloped pancreas anlage. This phenotype is compatible with loss of Notch1 function, which supports the role of Notch3 as an inhibitor of Notch1 signalling. We show that N3IC affects HES-5, but not HES-1 expression in the developing CNS, which is in keeping with previous work (de la Pompa *et al.* 1997). In contrast, we find that HES-1 is repressed during pancreas development.

Moreover, the repression of HES-5 in the CNS can only be observed in a part of the neural tube, suggesting that HES-5 is also regulated by other factors which could be other Notch receptors. This clearly illustrates the complexity of HES regulation.

Is Notch3 always this negative?

We have shown that N3IC is a poor activator of HES expression in transfected cells and that it can repress HES expression *in vivo* by blocking N1IC activity. However, this may not always be the case. Two recent reports show that N3IC can induce HES-1 expression in two different cell systems, namely adult hippocampus-derived multipotent progenitor cells (AHP) and T-cells (Bellavia *et al.* 2000, Tanigaki *et al.* 2001). As discussed in the introduction, Tanigaki *et al* show that Notch seems to function instructively to promote astrocyte differentiation of AHP cells. They have analysed the roles of N1IC and N3IC in parallel in this process and they conclude that both proteins behave in a similar fashion. In one experiment, they analyse HES-1 expression in response to Notch1 and 3 ICs and find that both proteins induce expression of endogenous HES-1 to the same degree. This is interesting and it further supports the idea that Notch functions differently in different tissues. The difference between pancreas and thymus/CNS could possibly be explained by different repertoires of co-activators and/or repressors in thymus/CNS progenitor cells compared to pancreatic progenitors. It should be noted that the authors have used RT-PCR to analyse HES-1 induction. However, it is difficult to compare the potency of activation measured by RT-PCR to that of luciferase reporter activation. Thus, in this case it is difficult to tell if N3IC is an equally strong activator or whether N1IC happens to be an equally weak activator.

In contrast to the situation in neuronal progenitor cells, N1IC and N3IC seem to have distinct effects on tumour induction and progression in the immune system. Misexpression of N3IC in the thymus/early T-cells in transgenic animals disrupts proper thymocyte differentiation (Bellavia *et al.* 2000). These animals have increased numbers of immature T-cells, which fail to downregulate CD25 and maintain highly active NF-κB signalling. Most animals develop malignant T-lymphomas within 12 weeks of birth. The lymphoma cells display constitutive activation of NF-kB signalling via IKKα-dependent degradation of IκBα and tumourgenesis can be inhibited by overexpressing IκBα. Thus, it is possible that Notch3 regulates NF-κB signalling by activating IKKα. How this is mediated, however, remains unknown. It also remains to be clarified if these effects observed in thymocytes are due to repression of Notch1 function or if they are Notch3-specific. Notably, HES-1, but not HES-5, is strongly upregulated in the transgenic mice, suggesting that N3IC can activate this promoter in a different cellular context. Misexpression of N1IC from the same promoter also leads to increased numbers of immature T-cells. However, these seem to be blocked at an earlier stage of T-cell differentiation (see Rothenberg 2001 for review).

3.2 The mouse CADASIL model – a strike on stroke or stroke on strike

Different CADASILs in mice and men

The CADASIL knock-in mice develop some aspects of the disease, but we still do not understand why CADASIL knock-in mice do not develop true GOM and brain infarcts in spite of the vascular defects (Paper IV). However, it is possible that fundamental differences in anatomy and life span between mice and men can explain these facts. For example, the sheer difference in the size of human and mouse brain mean that there are no comparable arteries with respect to diameter and VSMC-content in the mouse. Another important difference is that in CADASIL patients, degenerative changes occur mainly in white matter. The mouse brain contains only very little white matter, which is likely to affect the occurrence of brain infarcts in mice. It appears that degeneration of the VSMCs is a slow process and it may be argued that the life span of a mouse is too short to accumulate a loss of VSMCs necessary for infarct development. The earliest signs of GOM have been observed at the age of 18 (H. Kalimo, personal communication). Even though GOM might develop much earlier than that, it is possible that the two-year life span of mice is too short to accumulate GOM.

What happens to the VSMCs?

As described in the introduction, there is a loss of VSMCs in CADASIL that eventually leads to vessel wall abnormalities and brain infarcts. It is of great importance to learn why and how VSMCs disappear. The number of VSMCs is controlled by two factors: the rate of VSMC birth and death. The normal turnover of VSMCs is very slow. In CADASIL, little is known about how VSMCs die and whether death is mediated via necrosis or apoptosis. There are no clear signs of inflammation associated with arterial lesions in CADASIL patients, arguing against necrosis. The VSMCs most likely die through apoptosis at very low rates. This is supported by our preliminary results, which do not indicate any increased levels of apoptosis in arterioles of CADASIL patients compared to control individuals as detected by TUNEL assay (P.B. unpublished results). The late onset of the disease also favours this hypothesis. The slow loss of cells suggests that CADASIL might be a deficiency in regeneration of VSMCs rather than in abnormal cell death. Notably, experiments with induced injuries in rat arteries show a dramatic temporary increase in ECM remodelling and deposition followed by increased immigration of VSMCs (Godin *et al.* 2000). We observe an increased thickness in the vessel wall, both in patients and knock-in mice, which is mainly due to abnormal ECM deposition. It is possible that CADASIL arteries fail to respond normally to injuries and compensate by overproduction of ECM. It would thus be very interesting to induce experimental injury in the CADASIL knock-in model in order to assess the capacity of regeneration in these mice. Detailed analysis at the molecular level with respect to ECM remodelling, VSMC proliferation and migration,

and Notch signalling effects on these processes is required to further understand the pathogenesis of CADASIL.

Molecular mechanisms of Notch3 in CADASIL

As the name implies, CADASIL is a dominantly inherited disorder that is fully penetrant in the heterozygous state. This raises the question of how missense mutations in Notch3 mediate pathogenesis. Three possible scenarios are currently being debated, receptor gain-of-function (*gof*), loss-of-function (*lof*) and receptor accumulation. The mutations result in one unpaired cysteine residue, which presumably affects proper disulphate bond formation. In theory, this could render a receptor constitutively active. There are currently no good assays for measuring endogenous Notch3 signalling and unfortunately no reliable antibodies exist either. It is thus difficult to assess this explicitly. However, this possibility is less likely since neither CADASIL patients nor our CADASIL mouse model display any features reminiscent of the effects observed when the N3IC is over expressed (Paper III; Bellavia *et al.* 2000, Lardelli *et al.* 1996). Neither does this scenario explain the late onset of the disease.

It can be argued that CADASIL is caused by *lof* due to a dominant-negative (*dn*) receptor mutation. A group of mutations in *Drosophila* Notch called lethal-Abruptex favour this hypothesis. All of the lethal-Abruptex alleles have amino acid substitutions that lead to unpaired cysteine residues and they function as antimorphs, i.e. to partially block the non-affected allele (Portin 1981). One weakness with this explanation is that to date, no CADASIL patient has been identified with an obvious null allele of Notch3. However, lethal-Abruptex mutations only partially suppress the wildtype copy and it could be argued that 100% loss of function is embryonically lethal and that is the reason why it has not been observed. The *lof* model is further supported by experiments that show that the extracellular domain of Notch alone can function as an antimorph by sequestering ligands (Rebay *et al.* 1993). A recent report demonstrates that the ectodomain of Notch3 accumulates in CADASIL patients and suggests that oligomerisation is defective, resulting in monomeric ectodomains on the cell surface (Joutel *et al.* 2000). Thus it is possible that normal Notch3 signalling could be blocked by fake receptors.

Finally, a third model for explaining CADASIL has been suggested. This model is based on the fact that many neurodegenerative diseases like Alzheimer's, Parkinson's and Huntington's are caused by abnormal protein deposition. As mentioned above, CADASIL is characterised by increased deposition of ECM and characteristic aggregates called GOM.

Joutel *et al* demonstrated recently that CADASIL patients have elevated levels of the extracellular domain of Notch3 (N3EC), suggesting that it is (Joutel *et al.* 2000). The N3IC did not accumulate in the CADASIL mice suggesting that the N3EC fails to be cleared from the cell surface, rather than an overall decrease in receptor synthesis being the case. This observation invokes another issue concerning the processing of Notch3. Notch maturation occurs in trans-Golgi and is crucial for receptor function. In CADASIL, the receptor seems to be processed correctly and is presented on the

cell surface (Joutel *et al.* 2000). However, our preliminary results indicate that there is a difference in intracellular localisation between wildtype and CADASIL receptors in transfected cells. Mutated receptors tend to form perinuclear aggregates to higher extent than wildtype proteins (P.B. and J.L. unpublished results). This is similar to what can be observed in experiments with mutated Serrate1. Specific mutations in Serrate1 that change the number of cysteine residues also result in perinuclear aggregates (Morrissette *et al.* 2001). This report shows that the glycosylation of mutated Serrate1 is affected, further supporting defective maturation and hence possible intracellular accumulation. Curiously, in Joutel *et al*'s study, Notch3 immunoreactivity did not label the GOM per se, but was instead closely arranged around the deposits. CADASIL knock-in mice (paper IV) develop arteriopathy, but do not display distinct GOM, which could be expected if they were aggregates of mutated Notch3 receptors. This suggests that GOM may be an effect of CADASIL rather than the cause of VSMC degeneration. Taken together, these observations strongly suggest that mutated Notch3 receptors might form high molecular weight complexes in CADASIL patients that accumulate over time and affect vessel wall morphology. This model would also explain the late onset of the disease.

In conclusion, further experiments are required to distinguish between these different models, but it is reasonable to believe that the pathology is caused by a combination of protein accumulation and defective signalling.

3.3 Peering into the future of Notch research

The number of biological processes that Notch is involved in continues to grow. It is surprising how a single receptor can mediate such varied responses as boundary formation, cyto-architectual remodelling, induction and inhibition of differentiation in a number of tissues. However, we are only beginning to understand the molecular details of these processes and still many pieces of the Notch puzzle are missing. For example, it will be very important to learn which ligands activate the endogenous Notch receptors. Are there any unique ligand/receptor interactions or is ligand-specificity totally mediated by other proteins like *Fringe*? Furthermore, many experiments indicate that gene-dosage and the level of Notch signalling has great influence on its biological function. It will thus be important to know whether different ligands activate Notch to different degrees.

The extracellular domain of Notch plays important roles in receptor regulation and mutations within it have a great impact on receptor function, having been shown to cause degeneration of VSMCs. Clearing of the ectodomain is a unique problem for the Notch signalling pathway and how it is disposed of is still not known. It is likely, however, that this is a rapid process since the extracellular domain alone represents a dominant-negative receptor. It has been suggested that trans-endocytosis is

required for activation of the receptor and this would also solve the issue of ectodomain-clearing. Furthermore, this links to the importance of quickly resetting the Notch system, so that the cell can be ready to respond to new Notch stimuli. This is particularly true for gliogenesis and neurite organisation.

The Notch signalling pathway has been described as a “molecular Swiss army knife”. The “core components” of the pathway can be used as different tools to interact with and influence components of other signalling pathways. It still remains to be elucidated how this is conferred, but most likely it involves as yet unidentified intracellular factors and post-translational modifications of the core components. Several groups have reported that Notch is phosphorylated and it is very likely that protein modifications will play a crucial role in the multifunctionality of Notch signalling. It is well established that the HES genes are potent modulators of cell differentiation and that HES-1 and HES-5 expression is regulated by Notch. However, sometimes HES-1 expression, but not HES-5 is affected by Notch, while in other situations the converse is true. This suggests a complex regulation of the HES genes. It is not yet clear which other factors cooperate together with Notch to confer this differential expression. It is also surprising that so few target genes for Notch have been identified. Could this reflect the power of Notch as a molecular Swiss army knife in that no other downstream targets are required? Using DNA-array and DNA-chip technologies to unravel the dynamics of gene expression in individual cells at different developmental stages will most likely answer that.

Gene targeting of the components of Notch signalling has taught us a lot about their functions. However, it has been difficult to study the role of Notch signalling in e.g. neuronal differentiation and maturation, due to early lethal phenotypes. This will hopefully be resolved using conditional knock-outs. Conversely, forced activation of Notch signalling has been an important tool to study receptor-function and differentiation. Accordingly, conditional systems for Notch-receptor activation would be convenient to overcome early-lethal effects of a broadly-activated Notch.

Another problem in studying the function of Notch3 arises because there is no simple read-out for its signalling. It is possible to overcome this by turning Notch3 into an artificial activator. This is accomplished by insertion of the yeast GAL4 and VP16 domains immediately downstream of the transmembrane domain. In this way, it is possible to monitor Notch3 processing and activation on a UAS-reporter. It would be very interesting to study transgenic mice expressing such a receptor on a UAS-lac Z reporter background. This would hopefully reveal the spatiotemporal pattern of Notch3 activation, which could then be compared to the pattern of expression. Such an assay may help to identify novel, hitherto unknown ligands.

Given the importance of Notch in the control of cell differentiation, it would be interesting to explore the possibilities of experimentally modulating cell fates by activating different Notch receptors. It would be of great clinical interest if e.g. neuronal stem cells could be directed to specific neuronal fates so that they could be transplanted to Parkinson patients. Likewise, misexpression of N3IC has been shown to increase the numbers of endocrine cells, maybe this could be used to replenish the β-

cells in diabetes patients. Moreover, gliomas and other aggressive brain tumours exhibit features of immature neuronal precursors. Since Notch can induce gliogenesis, perhaps forced activation of Notch in tumour cells would terminally differentiate them into astrocytes. In summary, there are many examples of how experimental modulation of Notch can be applied clinically.

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