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The role of *hes* genes in the Controlled Production of Neurons

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1. **Fior, R**. and Henrique, D. (2005). A novel *hes5/hes6* circuitry of negative regulation controls Notch activity during neurogenesis. *Dev Biol.* 15;281(2):318-33.

2. Bekman, E, Valente de Castro, C, **Fior, R** and Henrique, D. (2006). Neuronal production *in vitro* from embryonic stem cells. *Actas Bioq.* 7:61-66.

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PEDRA FILOSOFAL

Eles não sabem que o sonho
é uma constante da vida
tão concreta e definida
como outra coisa qualquer,
como esta pedra cinzenta
em que me sento e descanso,
como este ribeiro manso
em serenos sobressaltos,
como estes pinheiros altos
que em verde e oiro se agitam,
como estas aves que gritam
em bebedeiras de azul.

eles não sabem que o sonho
é vinho, é espuma, é fermento,
bichinho álacre e sedento,
de focinho pontiagudo,
que fossa através de tudo
num perpétuo movimento.

Eles não sabem que o sonho
é tela, é cor, é pincel,
base, fuste, capitel,
arco em ogiva, vitral,
pináculo de catedral,
contraponto, sinfonia,
máscara grega, magia,
que é retorta de alquimista,
mapa do mundo distante,
rosa-dos-ventos, Infante,
caravela quinhentista,
que é cabo da Boa Esperança,
ouro, canela, marfim,
florete de espadachim,
bastidor, passo de dança,
Colombina e Arlequim,
passarola voadora,
pára-raios, locomotiva,
barco de proa festiva,
alto-forno, geradora,
cisão do átomo, radar,
ultra-som, televisão,
desembarque em foguetão
na superfície lunar.

Eles não sabem, nem sonham,
que o sonho comanda a vida,
que sempre que um homem sonha
o mundo pula e avança
como bola colorida
entre as mãos de uma criança.

António Gedeão
in Movimento Perpétuo, 1956

PHILOSOPHER'S STONE

They do not know that dreams
are a constant of life
as concrete and definite
as any other thing,
like this grey boulder
where I sit down and rest,
or like this smooth brook
in its calm somersaults,
or these tall pines
that sway in green and gold
or these birds that shriek
in drunken bouts of blue.

they do not know how dreams
are wine, are foam, are yeast,
a happy and thirsty bug
that tunnels its pointy snout
through everything
in a perpetual motion.

they do not know how dreams
are canvas, colour, paintbrush,
base, shaft, capitel,
ogival arch, stained glass,
cathedral spire,
counterpoint, symphony,
Greek mask, magic,
the alchemist's retort.
the map of the faraway world,
compass plate, crown Prince,
sixteenth-century caravel,
the Cape of Good Hope,
gold, cinnamon, ivory,
the swordsman´s foil,
stage set, dance step
Harlequin and Columbine,
bird styled flying balloon,
lightning-rod, locomotive,
ship with festive prow,
blast-furnace, generator,
atom-splitting, radar,
ultrasound, television,
the rocket landing
on the moon face.

They do not know, nor dream,
that dreams command life
that whenever man dreams
the world jumps and moves forward,
like a coloured ball
in the hands of a child.

António Gedeão
in *Movimento Perpétuo*, 1956

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SUMMARY

Cell–cell signalling mediated by the Notch pathway is essential for the spatial and temporal coordination of cellular behaviour in a variety of processes, from embryonic development and stem cell biology to disease.

At the core of the Notch signalling pathway are the transmembrane Notch receptors and their ligands from the DSL (Delta–Serrate–Lag-2) family. Upon ligand–receptor interaction, two proteolytic cleavages occur, releasing the Notch intra-cellular domain (NICD). The NICD contains nuclear localization signals that render it to the nucleus where it associates with the DNA–binding protein CSL (mamalian CBF-1, *Drosophila* Supressor of Hairless, *C. elegans* Lag-1) to activate expression of its target genes (Kopan, 2002).

Notch signalling regulates neurogenesis in animals as different as flies and mammals. Notch signalling acts through a process known as Lateral Inhibition to balance the antagonistic activities of two different sets of bHLH proteins: the proneural proteins that play a positive role, promoting the commitment to a neural fate, and HES proteins that repress this cell fate decision.

Cells that express Delta can activate the Notch receptor in neighbouring cells and this leads to transcriptional up–regulation of genes encoding HES proteins, which suppress the activity of the proneural genes and, thereby, keep these cells undifferentiated. In this way, the cell that expresses the ligand Delta realizes its neural potential, becoming a neuron, but simultaneously ensures that the neighboring cells are prevented from doing so. By restraining differentiation, Notch signalling not only ensures the maintenance of a progenitor population but enables cells to be exposed to the various environmental cues that are continuously changing over time, thus, allowing the production of the different types of neurons during the whole embryonic development.

The aim of this work is to understand the molecular events downstream of Notch signalling used to control the production of neurons during vertebrate embryonic development. I have concentrated on the *hes* genes, which are the best characterized Notch targets and effectors. However, many questions remain regarding their general regulation and function.

In this work, I describe four novel chick *hes* genes that are expressed in the developing nervous system: three *hes5*-like genes (*hes5-1*, *hes5-2* and *hes5-3*) and one *hes6*-like (*hes6-2*). All four genes are expressed in the ventricular zone of the embryonic neuroepithelium, where neural progenitors are located and where the *Notch1* receptor is expressed. I show that Notch signalling positively regulates the *hes5* genes but reduces expression of *hes6-2*. Overexpression of HES5 proteins, like the constitutive activation of Notch signalling, leads to inhibition of neurogenesis, implying that *hes5* genes are bonafide Notch effectors. Furthermore, this work unravelled a novel circuitry of auto- and cross- regulation between *hes* genes: each *hes5* gene is able to repress *hes6-2*, and all three *hes5* genes seem to be repressed by *hes6-2*. Moreover, *hes5-1* and *hes5-2* genes are subjected to negatively auto–regulation.

This work proposes that the function of the HES5/HES6 circuitry is a conserved feature of the Notch pathway, modulating the response of neuroepithelial cells to Notch signals at different phases of their development. Neuronal progenitors seem to go through successive Notch activation events until they finally differentiate. In this work, I propose a model in which the HES5/HES6 circuitry of negative auto- and cross-regulation contributes to shut down the pathway after each event of Notch activation. This would enable the progenitors to go back to a "neither–ON–nor–OFF" steady state where they are ready to interact again through lateral inhibition or, in the absence of a Delta inhibitory signal, enter the neuronal differentiation program.

In order to test this model and determine if these pulses of Notch activity indeed occur, I developed a real–time imaging system to monitor Notch activity *in vivo*. A reporter construct composed of the *hes5-1* promoter, fused to a destabilized VENUS protein was generated. This reporter recapitulates the endogenous pattern of *hes5-1* and responds to Notch signalling, providing a valuable tool to monitor Notch activity in real time. Analysis of time-lapse imaging of neuroepithelial cells containing this reporter revealed a dynamic gene expression pattern suggestive of pulses of Notch activity. The possible functional relevance of these oscillations to control the neurogenesis process is discussed in detail in the general discussion of this work.

SUMÁRIO

O desenvolvimento de um organismo multicelular depende da capacidade de formar padrões biológicos organizados e reprodutíveis – e isto apenas é possivel se as células 'falarem' umas com as outras e influenciarem reciprocamente o seu comportamento e destino celular. Nestes processos de comunicação celular, uma das vias mais importantes é a via de sinalização Notch.

A comunicação célula–célula mediada pela via de sinalização Notch é essencial para a coordenação espacial e temporal de uma variedade de processos celulares durante o desenvolvimento embrionário, na regulação da população de células 'estaminais' assim como em situações patológicas (Lai *et al.,* 2004). Perturbações na via de sinalização Notch podem resultar em várias patologias humanas, como o síndrome de Alagile e CADASIL (Arteriopatia Cerebral Autosomal Dominante com Infartes Subcorticais e Leucoencefalopatias), afectando inúmeros orgãos e sistemas (Gridley, 2003). Defeitos na via de sinalização Notch estão também implicados em vários cancros, como leucemias de células T e cancro da mama (Gridley, 2004; Mastronardi and Moscarello, 2005; Radke, 2006).

No centro da via de sinalização Notch encontram–se as proteínas transmembranares Notch e os seus ligandos, pertencentes à família DSL (Delta–Serrate–Lag-2). Quando os ligandos numa célula interagem com os receptores de outra célula desencadeiam uma série de clivagens proteolíticas que levam à libertação do domínio intracelular do receptor Notch (NICD). Este domínio é então capaz de se dirigir ao núcleo, onde se associa com o factor the transcrição CSL (CBF-1 de mamíferos, Supressor of Hairless de *Drosophila*, Lag-1 de *C.elegans*) e MAM (Mastermind) para activar a expressão dos seus genes alvo (Kopan et al, 2001).

A neurogénese é um dos muitos processos que é regulado pela via de sinalização Notch, em animais tão diferentes como moscas e mamíferos. Neste processo, a via Notch actua através do processo de Inibição Lateral, balanceando a actividade antagonística de dois tipos de factores de transcrição: as proteínas proneurais que promovem a diferenciação neural e as proteínas HES que reprimem este processo.

As células que expressam o ligando Delta podem activar o receptor Notch nas células vizinhas, conduzindo à activação transcriptional dos genes que codificam as proteínas HES que, por sua vez, suprimem a actividade dos genes proneurais, mantendo assim

estas células num estado não diferenciado. Desta forma, a célula que expressa o ligando Delta concretiza o seu potencial neural, diferenciando-se num neurónio, mas simultaneamente assegura que as células vizinhas não fazem o mesmo, ou seja, não se diferenciem em neurónios. Esta diferenciação contida e controlada assegura a manutenção de uma população de células progenitoras durante todo o desenvolvimento. Este processo prolongado no tempo permite que as células progenitoras vão sendo expostas aos vários sinais exteriores que se encontram em contínua mudança, assegurando assim a produção dos diferentes tipos de neurónios durante todo o desenvolvimento embrionário.

O objectivo do presente trabalho foi compreender melhor os eventos moleculares que ocorrem por consequência da activação da via de sinalização Notch, utilizados para controlar a produção de neurónios durante o desenvolvimento embrionário de animais vertebrados. O meu trabalho incidiu no estudo dos genes *hes*, sobre os quais, apesar de serem os alvos e efectores melhor caracterizados da via de sinalização Notch, muitas interrogações ainda permanecem a respeito da sua função e regulação.

No murganho existem quatro genes *hes* que são expressos no tubo neural (*hes1, hes3, hes5* e *hes6*), no entanto só um – o gene *hes5*, parece ser um verdadeiro alvo da via Notch *in vivo* (de la Pompa *et al.*, 1997; Lutolf *et al.*, 2002). No entanto, a delecção do gene *hes5* não tem o mesmo fenótipo que a mutação no gene *Notch1* durante a neurogénese, sendo necessária a inactivação simultânea do gene *hes5* e de outros dois genes *hes* – *hes3* and *hes1,* para se verificar o fenótipo esperado para a perda total da via Notch (Lewis, 1998) – a completa eliminação da população de progenitores neurais (Hatayama, 2004). Estes resultados sugerem que outros genes *hes* poderão participar na via Notch durante a neurogénese e que a sua regulação pela via Notch poderá ter sido mascarada por possiveis regulações inter–génicas. De acordo com esta hipótese, foi mostrado que o gene *hes6* regula negativamente o gene *hes1* (Bae *et al.*, 2000; Koyano–Nakagawa *et al.*, 2000; Gratton *et al.*, 2003). No entanto, se o gene *hes6* interage com outros genes *hes* é ainda uma questão em aberto.

Mais, no contexto da segmentação foi demonstrado que os genes *hes* têm a capacidade de regularem a sua própria expressão (Hirata *et al.*, 2002; Bessho *et al.*, 2003). Este mecanismo de feedback negativo gera oscilações na expressão génica destes genes, com o mesmo periodo que a formação dos sómitos. Estas oscilações génicas parecem estar na base do relógio biológico que controla o processo da segmentação (revisto por

Dubrulle and Pourquie, 2004). No entanto, durante a neurogénese, desconhece–se totalmente se estes genes *hes* têm um comportamento oscilatório ou sequer esta capacidade auto–regulatória .

Assim, de modo a responder a estas questões e entender melhor a lógica inerente à produção controlada dos neurónios, eu comecei por caracterizar os genes *hes* de galinha, que até à data do inicio desta tese ainda não tinham sido estudados.

Na presente dissertação são descritos quatro genes *hes* de galinha que são expressos no sistema nervoso: três genes *hes5* (*hes5-1, hes5-2 e hes5-3*) e um *hes6 (hes6-2*). Os quatro genes são expressos na zona ventricular do neuroepitélio embrionário, onde se encontram os progenitores neurais e onde o receptor *Notch1* é expresso. Neste trabalho é demonstrado que a via de sinalização Notch regula positivamente os genes *hes5* mas reduz a expressão do gene *hes6-2*: quando a via Notch é activada os três genes *hes5* são activados e o gene *hes6-2* é reprimido. Pelo contrário, quando a via Notch é inibida, os genes *hes5* são reprimidos e o gene *hes6-2* é activado.

A expressão ectópica das proteínas HES5 no neuroepitélio do embrião de galinha conduz a um fenótipo semelhante ao que ocorre quando se induz a activação constitutiva da via Notch: a neurogénese é inibida, indicando que os genes *hes5* são verdadeiros efectores da via de sinalização Notch. Por outro lado, o gene *hes6-2* inibe os efectores da via Notch, cooperando assim com os genes proneurais na progressão da via de diferenciação neural. Mais: este trabalho revela ainda a existência de um novo circuito de inter- e auto- regulação transcricional entre estes genes. Os quatro genes *hes* de galinha regulam-se uns aos outros: as proteínas HES5 têm capacidade de reprimir a expressão do gene *hes6-2* e, por outro lado, os genes *hes5* são reprimidos pela actividade de HES6-2. As proteínas HES5-1 e HES5-2 têm também a capacidade de regular negativamente a expressão dos seus respectivos genes.

Este trabalho propõe que a função do circuito HES5/HES6 seja uma característica conservada da via de sinalização Notch, modulando a resposta das células neuroepiteliais a esta via de sinalização, em diferentes fases do desenvolvimento embrionário. Os progenitores neuronais deverão atravessar eventos sucessivos de activação da via Notch até que se diferenciem totalmente. Neste trabalho sugere-se um modelo em que o circuito HES5/HES6 de inter- e auto- regulação transcricional negativa contribui para terminar a actividade da via Notch após cada evento de activação. Isto permitirá aos progenitores voltarem para um estado "neither-ON-nor-

OFF ", onde estarão prontos para interagir outra vez através da Inibição Lateral ou, por outro lado, na ausência de um sinal inibitório Delta, entrarem no programa de diferenciação neural. Este modelo, ao ter em conta a cinética da via de sinalização Notch, de uma forma dinâmica, sugere uma nova perspectiva para a lógica que controla a produção dos neurónios e ainda sugere um mecanismo para regular a duração da sinalização Notch – o circuito de auto- e inter-regulação HES5/HES6.

De modo a testar este modelo e determinar se os referidos pulsos de actividade Notch realmente ocorrem, foi desenvolvido um sistema para monitorizar a actividade da via Notch em tempo real. Para tal, foi construído um plasmídeo repórter composto pela região promotora do gene *hes5-1,* ligado a uma proteína fluorescente instável–VENUS. Este repórter é expresso na região ventricular do neuroepitélio, recapitulando o padrão de expressão do gene endógeno *hes5-1.* Mais: este plasmídeo reporter é induzido pela via de sinalização Notch, constituindo assim, uma ferramenta eficaz para monitorizar a actividade da via Notch em tempo real. A análise da actividade do repórter a nível celular revelou um padrão dinâmico da expressão do gene *hes5*, sugerindo a existência de pulsos de actividade da via Notch. A possível relevância funcional destas oscilações para controlar o processo do neurogénese é amplamente debatida na discussão geral da presente dissertação.

ABBREVIATIONS

aa • amino acid

AC/VU • Anchor cell / Ventral uterine in *C. elegans* gonodal development

AP • Alcaline Phosphatase

AS • Achaete–Scute

ASH • AS vertebrate homologues

Ato • Atonal

BBR • Boeringer Blocking Reagent

bHLH • basic Helix Loop Helix

BMP • Bone Morphogenetic Protein

C. elegans • *Caenorhabditis elegans*

CADASIL • Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy

cDNA • complementary DNA

CNS • Central Nervous System

CSL • mammalian CBF-1, *Drosophila* Supressor of Hairless and *C. elegans* Lag-1

DNA • Deoxyribonucleic Acid

DTS • Downregulation Targeting Signal

E(spl) • Enhancer of Split

EGF repeats • Epidermal Growth Factor repeats – small protein domains of approximate 40 amino acids, defined by the presence of 6 conserved cysteines that form 3 conserved disulfide bonds.

EGFR • EGF Receptor

EGFR • Epidermal Growth Factor

ER • Endoplasmic Reticulum

EST • Expressed Sequence Tag

F • Fungizone

FGF • Fibroblast Growth Factor

G • Glutamine

GFP • Green Fluorescent Protein

GlcNAc • N-acetylglucosamine

GSK–3β • Glicogen Synthase Kinase-3β

HAM'S F12 SUP • HAM'S F12 medium supplemented with 1% Fungizone (F) 2%Glutamine(G) + 1% piruvateNa (P) + 1% Penicillin/streptomycin (P/S)(Gibco)

HAM'S F12 SUP AG • HAM'S F12 medium supplemented with 1% Fungizone (F) 2%Glutamine(G) + 1% piruvateNa (P) + 1% Penicillin/streptomycin (P/S)(Gibco) + 1%agarose

HAT • Histone Acetylase

HDAC1 • Histone Deacetylase 1

her • Zebrafish *hes*-related genes

hes • *Hairy Enhancer of Split* genes

HH • Hamburguer and Hamilton stage

hpe • hours post electroporation

hpt • hours post transfection

HRP • Horseradish Peroxidase

INM • Interkinetic Nuclear Migration

IRES • Internal Ribosome Entry Site

LB • Luria Bertani bacterial medium

LI • Lateral Inhibition

MAM • Mastermind

MAPK • Mitogen–Activated Protein Kinase

Min • minutes

ML • Mantle Layer

mRNA • messenger RNA

NICD • Notch Intra-Cellular Domain

NLS • Nuclear Localization Sequence

nRFP • nuclear Red Fluorescent Protein

Nuf • Nuclear fallout

o.n • over night

OD • Optical Density

ORF • Open Reading Frame

P • Na-piruvate

P/S • Penicillin/Streptomycin

PEST • region of a protein enriched in proline (P), glutamate (E), serine (S) and threonine (T) residues

PSM • Pre-Somitic Mesoderm

R • Guanidine or Adenine–deoxiribonucleotides

RA • Retinoic Acid

Raldh2 • Retinaldehyde dehydrogenase 2–RA synthesizing enzime

RIP • Regulated Intramembrane Proteolysis

RNA • Ribonucleic Acid

ROI • Region of Interest

S sites • CSL binding sites

sec • seconds

SPS • CSL paired sites

Su(Dx) • Supressor of Deltex

Su(H) • Supressor of Hairless

T-ALL • T cell acute lymphoblastic leukemia

VNP • Venus–NLS–PEST

VPC • Vulval Precursor Cells

Vz • Ventricular zone

Y • Timidine or Cisteine–deoxiribonucleotides

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Chapter I

General Introduction

General Introduction

THE NERVOUS SYSTEM REGULATES all features of body function and is astonishing in its complexity and diversity of cells. Millions of specialized neurons sense the internal and external environment and send information to other neurons to store and process the information. The human brain the control centre that stores, computes, integrates and transmits information, contains about 10^{11} neurons, each forming thousands of connections with other neurons, thereby assembling complex and intricate circuitries.

The building of the nervous system involves the production of a huge array of different neuronal and glial cell types that must be generated in the correct numbers and appropriate positions, so that ultimately cells send projections to the right place at the right time, to assemble a functional network.

An important feature of vertebrate nervous system development is that neurogenesis starts in different regions, at different times and, more strikingly, within each region neurons do not differentiate simultaneously (Edlund and Jessel, 1999; Hollyday, 2001). Neurogenesis occurs over a long period of time, allowing progenitor cells to be exposed to various cues that change during development, instructing progenitors to produce the different types of neurons and glia. Therefore, mechanisms must exist to control the precise production of neurons over this long period and ensure that a pool of progenitors is maintained until all neurons and glia are formed (Edlund and Jessel, 1999).

The Notch signalling pathway is one of the major mechanisms that regulates this controlled production of neurons throughout development, and the main aim of this work was to study the molecular events downstream of Notch signalling used to control the neurogenesis process.

In this General Introduction, I will first present a general overview on Notch signalling, which is the central theme of this thesis. I will begin by giving a brief historic perspective of this ancient signalling pathway, describe its components, its regulation and discuss its operational logic. Then, I will give a brief survey of the major landmarks of vertebrate neurogenesis, from neural induction to neuronal differentiation. Next, I will address the known roles of Notch signalling during embryonic neural development in vertebrates. Finally, I will discuss how the Notch pathway is tightly connected to the proneural circuitry, forming one of the 'syntagms' or developmental 'cassettes' that regulates neurogenesis from flies to humans.

I.1 THE NOTCH SIGNALLING PATHWAY

The building of a multicellular organism relies on the ability to form organized reproducible biological patterns - and this is only possible if cells talk to each other and influence one another's fate and behaviour. One of the most important cell-cell communication mechanisms is the Notch signalling pathway. This pathway is implicated in probably all developmental programs, not just neural development; it is implicated in vascular and kidney development, body segmentation, intestine, skin and hematopoetic development and many other developmental processes (reviewed in Artavanis-Tsakonas *et al.*, 1999; Lai, 2004, see table).

Not surprisingly, perturbations in the Notch signalling pathway result in several human pathologies, as Alagille syndrome, Spondylocostal Dysostosis and CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy), which affect multiple organ systems (reviewed in Gridley, 2003). In addition, defects in Notch signalling may also result in several types of cancer, like T-cell leukaemia and breast cancer. A link between Notch signalling and multiple sclerosis has been also revealed (reviewed in Gridley, 2004; Mastronardi and Moscarello, 2005; Radke, 2006-see table).

Table 1 Notch signalling in Development, Adulthood and Disease: a non-exhaustive list, new roles for Notch continue to be discovered (adapted from Lai, 2004).

	Drosophila	Vertebrates
Development	Inhibition of neurogenesis	Inhibition of neurogenesis
	Regulation of gliogenesis	Regulation of gliogenesis
	Neural lineages fates	Regulation of cell fate choices in the inner ear
	Inhibition of myogenesis	Inhibition of myogenesis
	Regulation of hematopoesis	Regulation of hematopoesis
	Inhibition of cardiogenesis	Inhibition of cardiogenesis
	Inhibition of wing venation	Regulation of somitogenesis
	Inhibition of midgut precursors	Hindbrain boundaries
	Induction of mesectoderm	Regulation of kidney development
	Induction of eye cone cells	Regulation of vascular development
	Induction of D/V eye polarity	Regulation of intestine development
	Induction of wing margin	Regulation of limb development
		Establishment Left-Right asymmetry
Adulthood		Mammary development during pregnancy
		Skin turnover
		Intestine turnover
		Neural stem cell regulation
		Hematopoetic system
Disease		CADASIL
		Allagile syndrome
		Spondylocostal Dysostosis
		T-cell leukaemia
		Breast cancer

I.1.1 Brief historical overview of the Notch pathway

Notch was identified genetically by a mutant hypomorph allele with "Notches" on its wings, indicating its requirement for wing outgrowth. But it wasn't until 1930-1940 that Donald Poulson characterized the function of *Notch* in embryonic neural development, proposing that Notch activity was associated with a cell fate choice – the decision between epidermal and neural cell fate (reviewed in Lai, 2004).

Complete *Notch* deletion results in hypertrophy of the neural tissue at the expense of the epidermis, giving rise to a neurogenic phenotype. This was the beginning of the discovery of one of the milestones of developmental biology: "inhibit thy neighbour" or Lateral Inhibition (www.nature.com /milestones/ development).

The concept of Lateral Inhibition arose from the early work of Wigglesworth (1940), who carefully drew the spatial patterns of bristles and concluded that new bristles appeared in the largest spaces between the pre-existing bristles. Wigglesworth suggested that a substance produced by the already formed bristles inhibits the

epidermal cells from also becoming bristles, and when the concentration of this substance becomes reduced to a certain threshold, the epidermal cell may become a bristle mother cell (in *Making of a Fly*, Lawrence, 1992).

Only 50 years later, the *Notch* locus was cloned and sequenced, revealing that *Notch* encodes a transmembrane receptor (Artavanis-Tsakonas *et al.*, 1983, Wharton *et al.*, 1985). This pinpointed Notch for a role in cell-cell interactions, which was compatible with Doe and Goodman's laser ablation experiments (Doe and Goodman, 1985). They have shown that laser ablation of a neuroblast (neural progenitor) resulted in the production of a new one from an adjacent cell, implying that the neuroblasts prevent neighbouring cells from adopting the same fate (Doe and Goodman, 1985). Then, it was the seminal paper by Heitzler and Simpson that showed, through mosaic analysis, that there is competition between cells to adopt the neural fate and that Notch signalling governs this process of lateral inhibition, with Notch acting as a receptor and Delta as a ligand (Heitzler and Simpson, 1991, see ahead).

Since then, Notch signalling has been shown to play critical roles in the development of the nervous system, both in invertebrates and vertebrates, as well as in other developmental programs (see Table1 for a description of the known Notch pleiotropic roles during development, adulthood and disease).

I.1.2 Notch pathway core architecture

At the core of the Notch signalling pathway is the transmembrane Notch receptor in one cell, interacting with the transmembrane ligand in a neighbouring cell. Both receptor and ligand are characterized by having in their interacting extracellular domains several EGF-like repeats.

Notch is a large type-I transmembrane receptor that accumulates at the plasma membrane as a heterodimer, composed of the Notch Extracellular Domain (NECD) and a membrane bound intracellular domain (NTM). These two polypeptides are formed in the trans-golgi as the result of proteolytic activity by a Furin protease that constituitively cleaves Notch molecules at the S_1 site (Fig.1). The Notch receptor heterodimer is then formed trough a non-covalent Ca2+ dependent bound (Mumm and Kopan, 2000; Shweisguth, 2004). However, it is noteworthy to mention that

Drosophila Notch receptor is not an heterodimer as in vertebrates, being instead composed of just one single polipeptide (Artavanis-Tsakonas, 1999).

The best known Notch ligands belong to Delta-Serrate-Lag2 (DSL) family and are also type-I transmembrane receptors. However, in contrast with the Notch receptor, the ligands contain a much smaller intracellular domain (Fleming, 1998).

Upon ligand-receptor interaction, the Notch receptor undergoes successive proteolytic cleavages that lead to the release of the Notch intra cellular domain (NICD). Cleavage at the S2 site is triggered by ligand binding to NECD and is carried out by the ADAM/TACE/Kuzbanian family of metaloproteases. This S2 cleavage generates an activated membrane-bound form of Notch, NEXT (Notch Extracellular Truncation).

Subsequently, NEXT is further processed at two more cleavages sites – S3 and S4, releasing the NICD into the cytoplasm and a small peptide (N□) to the extracellular space (the fate and possible signalling activity of the N peptide is unknown). These S3 and S4 cleavages sites are located within the transmembrane domain and are catalyzed by the □-secretase activity of the Presenilin-Nicastrin-Aph1-Pen2 protein complex (reviewed in Mumm and Kopan, 2000; Schweisguth, 2004) (Fig.1).

The NICD fragment is the active form of the receptor, acting in the nucleus as a transcription co-activator. NICD translocates to the nucleus (through its nuclear localization signals) and binds to the CSL transcription factor (mammalian CBF-1, *Drosophila* Supressor of Hairless and *C. elegans* Lag-1) and to the Mastermind (MAM and *C. elegans* Lag-3) co-activator, forming a ternary complex (Fig.1).

In the absence of NICD, the CSL transcription factor promotes the assembly of a repressor complex at the cis-regulatory regions of the CSL/NICD target genes (named Su(H) or S binding boxes), which are therefore transcriptionally inactive (Bailey and Posakony, 1995; Nellesen, 1999; Cave *et al.*, 2005; Lamar and Kintner, 2005; Ong *et al.*, 2005). When NICD translocates to the nucleus and binds to CSL, it is able to recruit HAT (Histone Acetylase) and displace the co-repressor complexes, relieving repression. But it is only when MAM binds to NICD/CSL, forming the ternary complex, that transcription is activated (reviewed in Mumm and Kopan, 2000). Therefore, in the absence of Notch activity, the Notch target genes are repressed by CSL. When Notch signalling is initiated, NICD makes the switch from CSL-mediated

repression to NICD/CSL/MAM activation, triggering transcription of the Notch target genes (Bray, 1998; Castro *et al.*, 2005).

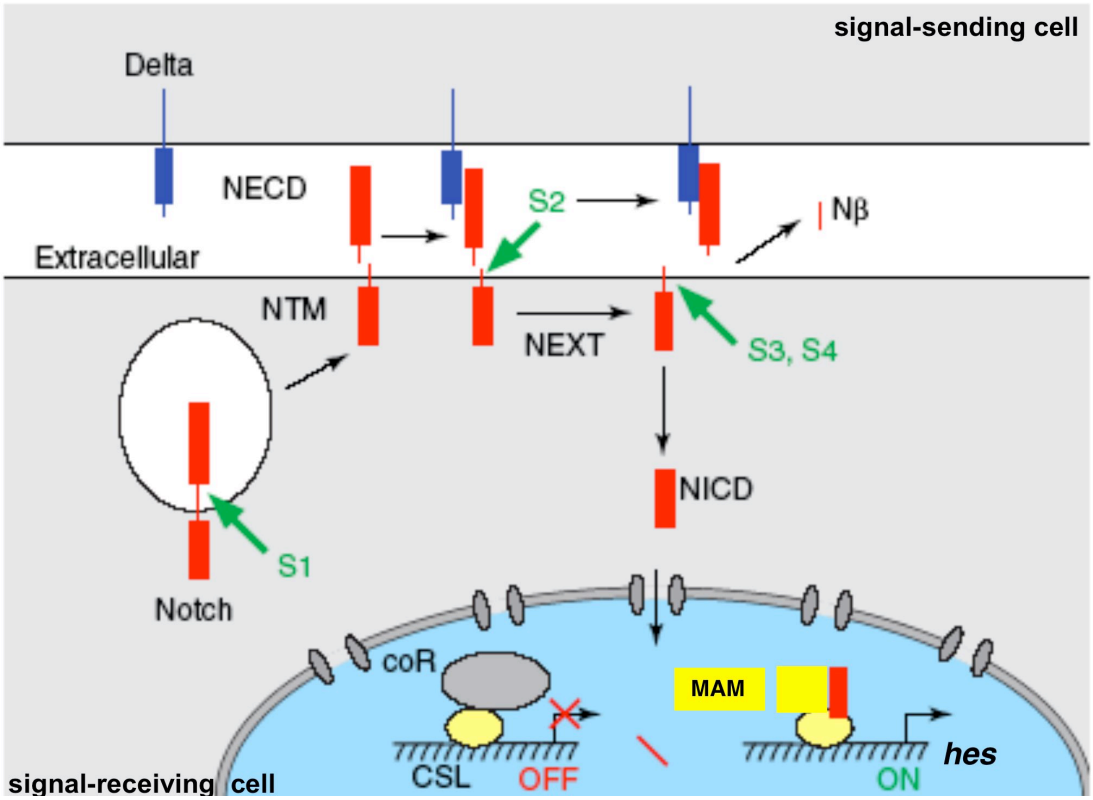


Fig.1 Notch pathway core architecture. Delta at the surface of the signal-sending cell binds the Notch extracellular domain. Upon ligand-receptor interaction, S2 proteolytical cleavage occurs releasing NEXT, NTM is then further processed at the S3 and S4 sites, releasing NICD. NICD then translocates into the nucleus where it associates with CSL and MAM, displacing the co-repressor (CoR) and thereby triggering a switch from repression to activation. The best studied targets of the Notch pathway are the *hes* family genes. Adapted from Schweisguth, 2004.

There are many binding sites for the CSL transcription factor throughout the genome (Rebeiz *et al.*, 2002), and it is not clear which actually represent Notch targets. The best-characterized Notch targets are the bHLH (basic Helix Loop Helix) transcription repressors of the *hes* (Hairy-Enhancer of Split) and *hrt* (*hes* related type) family genes (see ahead).

This core signalling pathway is evolutionary conserved in the metazoan *phyla*. However the number of paralogues of each element of the core pathway differs in the different animal models studied: whereas *Drosophila* has only one *Notch* gene, mouse has four different genes coding for Notch receptors (see Table 2 for comparison).

In addition to this core CSL-dependent Notch pathway, in which the key signalling molecule is NICD and the ultimate output is transcription, there is also evidence for a CSL-independent Notch signalling (reviewed in Martinez Arias *et al.*, 2002). This CSL-independent Notch signalling seems to rely on a Deltex dependent activity and, in some cases, it relies on different ligands that do not belong to the DSL family, like Contactin and DNER (Eiraku *et al.*, 2005).

Table 2 Components of the Notch signalling pathway are evolutionarily conserved.

	Drosophila	*C. elegans*	Chick	Mammals
Notch Receptor	*Notch*	*lin-12* *glp-1*	*Notch1* *Notch2*	*Notch1* *Notch2* *Notch3* *Notch4*
LIGAND	*Delta* *Serrate*	*lag-2* *apx-1* *arg-2* *f16b12.2*	*Delta1* *Delta4*	*Delta-1* *Delta-3* *Delta-4* *Jagged 1* *Jagged 2*
CSL	*Su(H)*	*Lag-1*	*CBF1/RBPJK*	*CBF1/RBPJK*
MAM	*Mam*	*Lag-3*	?	*Mam1* *Mam2* *Mam3*

I.1.3 Notch transduction design

The Notch receptor can be viewed as a membrane bound transcription co-factor (Schweisguth, 2004) that integrates signalling events at the membrane and transduces directly to the nucleus without any second messengers.

NICD release to the nucleus involves a two-step regulated intramembrane proteolysis (RIP) triggered by ligand binding. It has been pointed out that signalling via RIP imposes several features on the way the pathway is designed to signal (Schweisguth, 2004):

- Proteolytic cleavage is irreversible, each receptor can signal only once.
- Signal is direct and does not rely on second messengers. This precludes signal amplification and limits the possibilities for cross-talk with other signalling pathways.
- Finally, receptor processing releases extracellular by-products that may have signalling activities, although not yet addressed.

These features imply that the number and availability of Notch receptors at the membrane may be a limiting step to control the strength of the signal. Therefore, although the Notch RIP design predicts a reduction in the possibility of cross-talk with other signalling pathways, the fact that the availability of the Notch receptors at the membrane may be a limiting step, provides a check-point in the pathway that can be subjected to regulation. In fact, during the last years, increasing evidence is emerging that endocytosis plays a major role in regulating the pathway, controlling not only the availability but also the "quality" of receptor and ligands, thus providing an entry point to modulate the pathway (see ahead).

I.1.4 Modulation of Notch pathway activity

One would think that the spatial and temporal activation of Notch signalling would basically depend on the presence of its activating ligands, however this is not the case. In many contexts, the pattern of Notch activity is not coincident with the broad distribution of ligands and receptors, implying that Notch signalling is subjected to regulatory mechanisms that fine-tune the pathway. Actually, Notch activity must be under strict control and several mechanisms exist to regulate when, where and for how long the signal is ON or OFF. Also, the directionality of the signal is of extreme importance in a pathway that regulates cell-cell interactions.

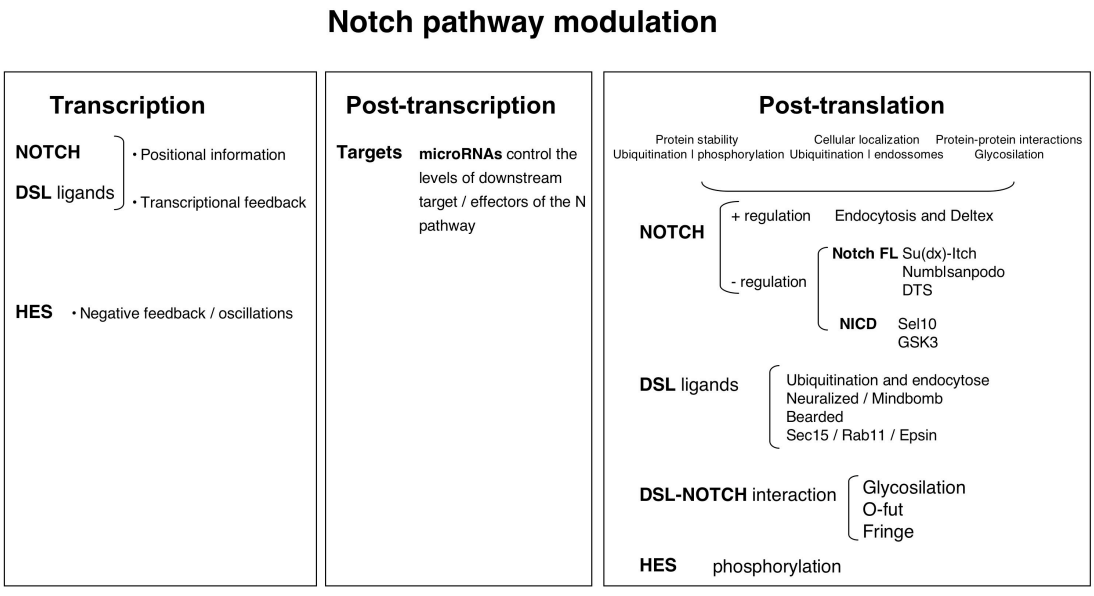


Fig.2 Summary of possible mechanisms of regulation of the Notch pathway.

All these features of the Notch pathway are tightly controlled through various mechanisms that act at different levels (transcriptionally, post-transcriptionally and post-translationally) to regulate the Notch receptor, Ligands, Receptor-Ligand interactions and downstream target/effectors genes i.e: the pathway is regulated from the cell surface of the signal-sending and signal-receiving cell to the cytoplasmic compartments, all the way to the nucleus (Fig. 2).

I.1.4.1 Modulation of the Notch pathway by transcriptional regulation

At the level of Notch receptor and Ligands

Spatial, temporal and directional control of Notch signalling can be achieved by spatially patterned expression of ligands and receptors. This imposes a strong bias on the direction of the signal, resulting in highly stereotyped cell-fate decisions. For instance, in the *C. elegans* gonad, distal tip cells express only the ligand *LAG-2* and signal in a unidirectional way to the adjacent germ line cells, which only express the Notch receptor *GLP-1*, thereby inducing their division (Henderson *et al.*, 1994).

Another way to impose directionality through transcriptional regulation is by transcriptional feedback, which can amplify small differences in the signalling capacities of the interacting cells: signalling activity can regulate receptor and/or ligand expression, in a way that the level of Notch activation in one cell will directly impinge on how this cell will receive and send back the signal.

During AC/VU decision in *C. elegans* (which will be described in more detail below), Notch/Lin12 activation in one cell can induce its own expression (positive feedback) and inhibit the expression of the ligand *Delta/Lag2* (negative feedback). In this way, a cell that receives more Delta signal will progressively have more *Notch* transcripts and less *Delta* expression, so that in the end this cell becomes the receiving-cell, whereas the other cell will become the signal-sending cell (reviewed in Greenwald, 1998).

However, the combination of these two feedbacks, positive and negative, is not the most commonly used strategy. And in most of the cases, the tactic is to regulate *Delta* expression. In addition, it has been shown that during boundary formation, Notch activation leads to transcriptional upregulation of the DSL ligands, providing yet another type of feedback (reviewed in Lewis, 1998).

At the level of Notch target genes

The best characterized Notch targets belong to the Hairy-Enhancer of Split (HES) family of bHLH transcriptional repressors. These proteins are able to negatively regulate the expression of their respective genes (Kramatscheck and Campos-Ortega, 1994; Cooper *et al.*, 2000; Hirata *et al.*, 2002; Bessho *et al.*, 2003), as well as negatively cross-regulate each other (Gajewski, 2003). This capacity of negative auto- and cross-regulation permits the generation of oscillatory behaviours (Monk, 2003, Lewis, 2003).

A good example where these negative feedback-loops play a crucial role is the process of somitogenesis, during which somites form sequentially in a rhythmic fashion from the presomitic mesoderm (PSM) (reviewed in Giudicelli and Lewis, 2004). This process is controlled by a molecular clock, in the form of a transcriptional oscillator that operates in the PSM with the same periodicity as somite formation (Palmeirim *et al.*, 1997). Most of the genes implicated in the oscillator belong to the Notch pathway, including the *hes* genes, whose expression oscillates with the same period as somite formation. And it has been proposed that the generation of the cyclic gene oscillations is based on the negative feedback-loop established by the HES transcription factors on their own promoters (Hirata *et al.*, 2002; Bessho *et al.*, 2003; Lewis, 2003; Hirata *et al.*, 2004;).

However, there are many contexts where all the cells involved in Notch signalling express both ligand and receptor and other mechanisms besides transcriptional feedback are required to ensure and further bias directionality of the pathway.

I.1.4.2 **Modulation of the Notch pathway by post-transcriptional regulation**

At the level of the Notch target genes

In *Drosophila*, it has been recently shown that two families of Notch target genes – the E(spl)-C bHLH repressors and the *Bearded* family genes, are negatively regulated by microRNAs (Lai *et al.*, 2005). These microRNAs inhibit translation or promote mRNA degradation by targeting conserved sites in the 3'UTRs of the Notch target genes. Ectopic overexpression of the Notch target-regulating microRNA, phenocopies many features of Notch loss-of-function (Lai *et al.*, 2005). In addition, genomic

transgenes mutated in the microRNA binding sites are hyperactive and disturb normal development of the PNS (Lai and Posakony, 1997; Lai *et al.*, 1998). Lai *et al.* (2005) suggest a model whereby these microRNAs help dampen or "tune" the expression of Notch target genes.

I.1.4.3 **Modulation of the Notch pathway by post-translational regulation**

Recently, post-translational mechanisms are emerging as a major way to control Notch signalling. Post-translational regulation includes mechanisms to control protein stability/ degradation, cellular localization of pathway components, and post-translational modifications that directly impinge on receptor-ligand interactions (reviewed in Schweisguth, 2004; Le Borgne *et al.*, 2005; Wilkin and Baron 2005; Le Borgne, 2006).

At the level of the Notch receptor

Post-translational mechanisms may negatively regulate the Notch receptor

Mechanisms that downregulate the Notch receptor may control the levels of the membrane-bound pool of the Notch receptors, or the stability/degradation of the nuclear NICD. The regulation of 'quantity and quality' of the membrane-bound pool of Notch available in the cell, or at the cell surface, relies on the endocytic pathway. The endocytic pathway can sort the membrane-bound Notch receptor to degradation at the lysosomes or deviate it to other endocytic compartments, away from the cell surface, avoiding the interaction of Notch with the ligands in neighbouring cells.
Several studies indicate now numerous mechanisms and players that may contribute to the down-regulation of the Notch receptor.

Membrane-bound Notch receptor downregulation and cell surface availability

Ubiquitin-lysosome pathway & E3 ubiquitin ligases Membrane-bound Notch can be ubiquitinated and targeted to the endocytic pathway through the action of several E3 ubiquitin-ligases, like the Nedd4 family members (*Drosophila* Nedd4, *Drosophila* Su(Dx)-Supressor of Deltex and its mammalian ortolog Itch and mammalian Cbl) (reviewed in Baron, 2003; Schweisguth, 2004; Le Borgne *et al.*, 2005; Le Borgne, 2006;). The outcome of this ubiquitination is the targeting of Notch to late endosomes and subsequent degradation by the lysosome.

Numb/Sanpodo Numb is a membrane associated protein that antagonizes Notch signalling during asymmetric divisions in the *Drosophila* nervous system (the asymmetric divisions are described in more detail below). Numb antagonizes Notch function by downregulating the activity of the positive regulator of Notch- Sanpodo. Numb triggers the α-adaptin-dependent endocytosis of Sanpodo, targeting it to late endosomes, thereby inhibiting its positive interaction with Notch at the membrane (Berdnick *et al.*, 2002; Hutterer and Knoblich, 2005).

In vertebrates, however, the role of Numb in regulating Notch activity is still controversial. Although some studies point to Numb as a negative regulator of Notch, targeting Notch for endocytosis and subsequent degradation (MacGil and MacGlade, 2003), other studies suggest that Numb is most probably a component of the Adherens Junctions (Afonso and Henrique, 2006; Kuo *et al.*, 2006) – a cellular structure, which is now being considered as an important cellular organizing and signalling centre.

NICD down-regulation: stability and degradation

Ubiquitin-proteosome pathway: MAM and Sel-10 Termination of Notch signalling must involve the removal of NICD from the nucleus of responding cells. This may be achieved by the ubiquitin-proteosome pathway and seems to involve another component of the NICD/CSL complex- MAM. MAM is able to recruit the CycC-CDK8 kinase that directly phosphorylates NICD, targeting it to poly-ubiquitination and proteosome degradation in a PEST- dependent manner by Sel-10 (Hubbard *et al.*, 1997; Oberg *et al.*, 2001; Gupta-Rossi at al, 2001; Wu *et al.*, 2001; Fryer *et al.*, 2004). This implies that MAM coordinates NICD mediated transcription with NICD turnover at the target genes, possibly explaining why it is so difficult to detect NICD in the nucleus.

Phosphorylation: GSK-3β kinase NICD can also be regulated by phosphorylation by another kinase – GSK-3β (Glicogen Synthase Kinase-3β). However, the outcome of this phosphorylation is not clear and may have a positive or negative effect on Notch signalling depending on the cellular context (Foltz *et al.*, 2002; Espinosa *et al.*, 2003).

Post-translational mechanism may positively regulate the Notch receptor

Endocytosis Endocytosis of the Notch receptor may also positively regulate the Notch signalling pathway. The initial observations with the *Drosophila shibire (shi*) mutant revealed that endocytosis is required for Notch signalling. *shibire* encodes Dynamin, which is a protein with GTPase activity involved in "pinching off" endocytic vesicles from the plasma membrane (Seugnet *et al.*, 1997). Mosaic analysis showed that Dynamin mediated endocytosis plays a positive role in Notch signalling both in the signal-sending and signal-receiving cell (Seugnet *et al.*, 1997). However, it seems that the requirement in the signal-receiving cell is at the level of Receptor-Ligand interaction, since *shi* mutant cells are unable to receive the Delta signal properly, whereas mutant *shi* cells that have active Notch independent of ligand activation can transduce the Notch signal (Seugnet *et al.*, 1997). The function of this Dynamin requirement in the Receptor-Ligand interaction is not yet understood.

Deltex The Notch pathway can also be positively modulated by the activity of Deltex in a cell autonomous manner. Deltex is another E3 ubiquitin ligase that binds to the intracellular domain of Notch (Diederich *et al.*, 1994; Matsuno *et al.*, 1995; Takeyama *et al.*, 2003) and positively regulates Notch signalling in *Drosophila* (Xu and Artavanis-Tsakonas, 1990; Busseau *et al.*, 1994; Matsuno *et al.*, 1995;) and vertebrates (Kishi *et al.*, 2001; Izon *et al.*, 2002). It has been suggested that Deltex may function by deviating Notch from the lysosomal degrading route, thus stabilizing the Notch receptor (Hori *et al.*, 2004; Sakata *et al.*, 2004). Alternatively, or additionally, it has also been reported that Deltex may act in the nucleus, preventing the recruitment of co-activators by NICD, downregulating the canonical pathway (Izon *et al.*, 2002). Indeed several authors suggest that Deltex mediates CSL and DSL-ligand independent Notch signalling in *Drosophila* and mouse (Ramain *et al.*, 2001; Hori *et al.*, 2004; Sakata *et al.*, 2004; Wilkin *et al.*, 2004).

At the level of the DSL ligands

Endocytosis is also required in the signal-sending cell to control signal activity. At least three E3 ubiquitin-ligases regulate the endocytosis of the DSL ligands in *Drosophila* and vertebrates: Neur (Neuralized); Mib-1 (Mindbomb-1, mindbomb in *Drosophila*

and vertebrates) and Mib-2 (mindbomb-2 in *Drosophila* and skelotrophin or mindbomb-like in vertebrates) (reviewed in Le Borgne, 2006).
It has been suggested that these E3 ubiquitin ligases are able to mono-ubiquitinate Delta and Serrate, targeting them to endocytosis and epsin-mediated sorting. The requirement of endocytosis for DSL ligands to signal seems to be a constituitive pre-requisite for Notch activation. But what is the mechanism by which endocytosis promotes ligand signalling activity? Two models have been put forward: one argues that endocytosis may induce a pulling force on the NECD to help receptor dissociation and activation (Parks *et al.*, 2000). The other model proposes that DSL ligands are produced in an inactive or poorly active state and endocytosis allows ligands to undergo post-translational modifications (Wang and Struhl, 2004) within these endocytic vesicles, which are them recycled to the membrane to expose the ligands, now in an active form (reviewed in Schweisguth, 2004; Le Borgne *et al.*, 2005; Le Borgne, 2006).

Recently, it has been shown that one of these E3 ubiquitin ligases, Neur, is subjected to regulation by the Bearded family of proteins. These proteins are able to interact with Neur and inhibit Neur-mediated endocytosis of Delta, presumably by preventing the interaction of Delta with Neur, thus antagonizing Notch function (Bardin and Schweisguth, 2006; De Renzis *et al.*, 2006). Bearded family members are activated by Notch signalling (Lai *et al.*, 2000) and may participate in a negative feedback loop that amplifies the differences in the signalling capacities between two interacting cells (Bardin and Schweisguth, 2006). In vertebrates, no Bearded family members have yet been identified (Bardin and Schweisguth, 2006). However, this does not exclude the possibility that other Neur-interacting inhibitors exist to regulate Notch signalling at this level in vertebrates (Bardin and Schweisguth, 2006).

During Sensory Organ Precursor (SOP) lineage selection (described ahead), another redundant mechanism of Delta regulation has been revealed, involving the controlled recycling of Delta to the plasma membrane specifically in the pIIb cell. It has been shown that Rab11 endosomes and the Sec15-containing exocyst participates in Delta recycling and is necessary to its activity (Emery *et al.*, 2005; Jafar-Nejad *et al.*, 2005).

At the level of Receptor-Ligand interaction: Glycosylation

Sugar modifications are found both in the Notch receptor and in its ligands, however, genetic studies in *Drosophila* have shown that glycosylation is essential in the signal receiving cell, indicating that these sugar modifications are more important for the biological activity of the receptor and not of the ligands (reviewed in Haines and Irvine, 2003; Haltiwanger and Lowe, 2004).

In addition to the standard N-linked glycosylation that is commonly found in extracellular/membrane bound proteins, Notch receptors are further subjected to post-translational sugar modifications in their Epidermal Growth Factor (EGF)-like repeats. Notch is subjected to two types of O-glycosylation: the addition of O-linked glucose and O-linked fucose. Whereas the role for O-glucosylation remains unknown, the role of O-fucosylation is implicated in modulating the receptor-ligand interaction (reviewed in Haines and Irvine, 2003).

O-fucose modifications O-fucosylation of Notch is mediated by an O-fucosyltranferase, encoded by the *O-fut1* gene. *O-fut1* loss of function in *Drosophila* and mammals resembles the complete absence of Notch signalling, arguing that O-fucosylation of Notch is an essential step in Notch signalling and that *O-fut1* is a member of the core pathway (reviewed in Haines and Irvine, 2003; Haltiwanger and Lowe, 2004;). High levels of O-fucosylation result in an increased affinity for Delta and, conversely a low level of O-fucosylation results in a decrease of Delta-Notch affinity. Levels of O-fucosylation can be regulated by controlling the levels of *O-fut1* expression: although *O-fut1* is broadly expressed, the mRNA levels are regulated spatially and temporally (Haines and Irvine, 2003; Scheiwsguth, 2004), thus O-fucosylation may be used as a regulatory mechanism to control Notch-Ligand interaction, modulating the Notch pathway.

However, it has been recently shown that O-fut1 has an additional role besides O-fucosylation. O-Fut1 has a chaperone activity independent of its enzymatic activity: it is able to bind to Notch in the Endoplasmic Reticulum (ER) and facilitate the correct folding of Notch, allowing the subsequent O-fucosylation and the normal trafficking of Notch to the membrane (Okajima *et al.*, 2005). These authors suggest that glycosyltransferases that are substrate specific, like O-Fut1, may have a specific role in protein quality control in the ER (Okajima *et al.*, 2005).

N-acetilglucosamine modifications Nevertheless, the Notch receptor is even further glycosylated: N-acetilglucosamine is added to the previously added O-fucose. This reaction is catalyzed by the N-acetilglucosaminyl transferase Fringe, which interacts with the properly folded Notch protein and modifies the O-linked fucose by adding N-acetilglucosamine. In general, glycosylation by Fringe makes cells more sensitive to Delta than to Serrate, presumably by increasing Notch affinity for Delta and decreasing its affinity to Serrate (Panin *et al.*, 1997; Bruckner *et al.*, 2000). In vertebrates, due to the diversity of Receptors, Ligands and Fringe proteins, the influence of Fringe on Notch may depend on which molecules are interacting in a given specific context (reviewed in Haines and Irvine, 2003).

Two non-exclusive models have been put forward to explain the role of Fringe modifications (reviewed in Shweisguth, 2004): one argues that Fringe modulates the affinity of Ligand-Receptor interaction between the two interacting cells (Bruckner *et al.*, 2000; Lei *et al.*, 2003), while the other model suggests that Fringe does not modulate the capacity of Delta or Serrate to activate Notch in trans, but instead, modulates the ability of Notch to be inhibited in cis by its ligands. This means that the ligands would act cell-autonomously in the signal-receiving cell to block Notch activity. This model suggests that Fringe inhibits the formation of Receptor-Ligand complexes in the Golgi, thereby preventing inhibition of Notch by its ligand in the signal-receiving cell (Sakamoto *et al.*, 2002).

Modifications by O-Fut1 seem to be a constituitive essential step in Notch signalling, whereas glycosylation by Fringe proteins are only required in a subset of Notch functions, most of them involving differential activation by Delta and Serrate during boundary formation (reviewed in Haines and Irvine, 2003; Schweisguth, 2004).

At the level of the Notch target genes

The Notch target/effectors bHLH repressors of the *hes* family can be post-translationally modified by phosphorylation. However, phosphorylation can either have a positive or negative effect on their activity. Phosphorylation has been shown to potentiate the repressor activity of E(spl)m8 and HES6, presumably by enhancing protein-protein interactions with their targets Atonal (Karandikar *et al.*, 2004) and HES1 (Gratton *et al.*, 2003), respectively. By contrast, it has been shown that

phosphorylation of HES1 inhibits its capacity to bind DNA and repress transcription (Strom *et al.*, 1997; Fujimori *et al.*, 2005).

Overall, all these mechanisms that modulate the Notch pathway contribute to the temporal, spatial, durational and directional nature of the signal. And some of these mechanisms work together in a redundant fashion to reinforce the pathway and, specially, to impose a bias on the directionality of signal transduction, which is essential in a cell-cell interaction mechanism (see ahead in *Drosophila* SOP lineage).

I.1.5 Notch operational logic

Notch pleiotropic effects were already implicit in the description by Donald Poulson of the *Notch* mutant fly: "All in all, a kind of hopeless monster is produced which can not develop beyond the embryonic stage" (reviewed in Lai, 2004). The Notch pathway is probably involved in the development of all tissues, regulating cell fate specification, patterning, proliferation, cell death, cell morphology and so on. However, all these pleiotropic effects tend to fall in two types of operational logic: Lateral Inhibition- that mostly mediates binary cell fate decisions, and Lateral Induction – that in most of the cases is involved in boundary formation (reviewed in Bray, 1998; Schweisguth, 2004).

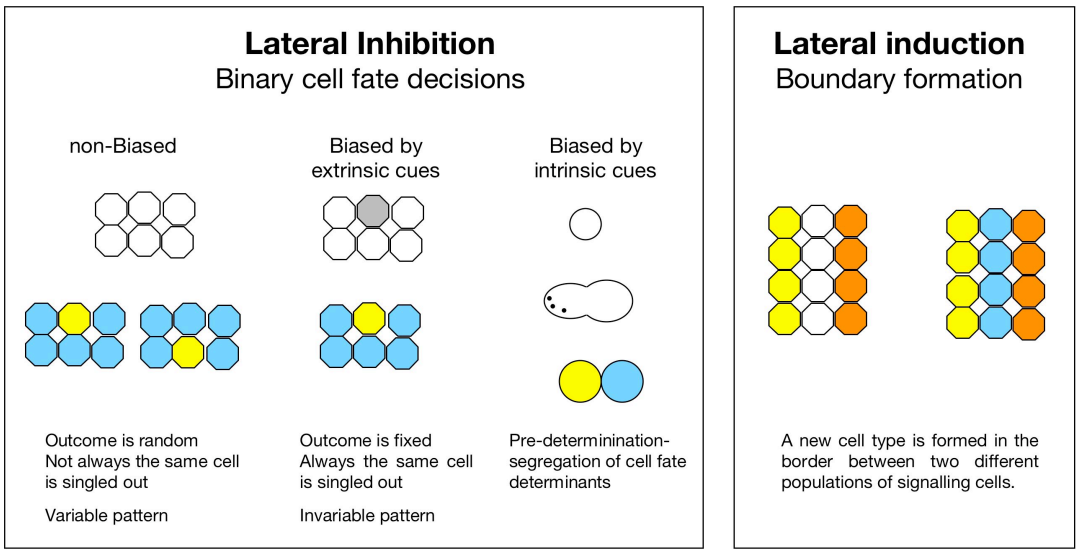


Fig.3 Notch operational logic tends to fall in two types of operation: Lateral Inhibition, mostly mediating binary cell fate decisions, and lateral Induction, mainly involved in boundary formation.

I.1.5.1 Lateral Inhibition: binary cell fate decisions

Cell-cell interactions mediated by Notch signalling regulate binary cell fate decisions, ensuring simultaneously that two interacting cells do not take the same fate and thereby guarantee that the two fates are taken in the end.

These binary cell-fate decisions have been shown to involve cells with similar or different developmental potential, either within a field of cells or between sibling cells.

The classical example of a binary cell fate decision mediated by Lateral Inhibition is the *Drosophila* neural-epidermal choice. This decision has been well studied during the singling out of neural precursors (neuroblasts) of the embryonic Central Nervous System (CNS) and during formation of the adult Peripheral Nervous System (PNS) sensory bristles.

The areas of the embryonic ectoderm and of the imaginal discs from which neural precursors arise are known as proneural clusters, whose cells have a neural potential due to the expression of proneural genes of the *Achaete-Scute* Complex (AS-C). Absence of *achaete* (*ac*) and *scute* *(sc)* in the imaginal discs leads to the loss of sensory bristles, whereas ectopic expression of these genes results in ectopic differentiation of bristles (Garcia-Bellido, 1979; Ghysen and Dambly-Chaudiere, 1988).

In the neuroectoderm, proneural genes are both necessary and sufficient to initiate and drive the neural differentiation program. Nevertheless, only one cell in these proneural clusters fulfils its neural potential and is chosen to be the neural precursor – the neuroblast.

However, if this cell is eliminated by laser ablation, a neighbouring cell will take up the job (Doe and Goodman, 1985). This implies two things: one, that all the cells in the cluster have the potential to be a neuroblast, meaning that cells within the proneural cluster are equipotential or at least have similar developmental potential; and two, neuroblasts prevent their neighbours from adopting the same fate. This last phenomenon is known as Lateral Inhibition and is mediated by Notch signalling.

Notch signalling restricts neural fate by repressing the expression of the proneural genes (Parks *et al.*, 1997). Absence of Notch signalling results in the so called "neurogenic" phenotype, where all cells in the ventral neuroectoderm develop as neuroblasts and no ventral epidermis is formed (Lehman *et al.*, 1983; Campos-Ortega, 1988). In the PNS, additional bristles are formed at the expense of epidermis (Shellenbarger and Mohler, 1978; Dietrich and Campos-Ortega, 1984; Simpson and

Carteret, 1989; Hartenstein and Posakony, 1990). By contrast, constituitive Notch signalling has the opposite phenotype – suppresses neuronal differentiation (Lieber *et al.*, 1993; Rebay *et al.*, 1993; Struhl *et al.*, 1993)

However, Notch is not necessary for the acquisition of the epidermal fate, it is only required to inhibit the neural fate, since double mutant cells for *Notch* and *ac-sc* differentiate into epidermis (Simpson and Carteret, 1989; Heitzler and Simpson, 1993). Therefore, Notch does not play an instructive role to induce the epidermal fate. Instead, it inhibits the neuronal fate and allows cells to adopt the alternative epidermal fate.

As already mentioned, it was the seminal work by Heitzler and Simpson (1991) which showed that *Notch* mutant cells develop cell autonomously as bristle precursors while, at the same time, neighbouring wild-type cells consistently adopt the epidermal cell fate and never the neural fate (Heitzler and Simpson, 1991) (Fig. 4D).

Moreover, inhibition of the neural fate by Notch activity depends on the ligand Delta, as double mutant *Notch/Delta* cells are no longer able to inhibit their neighbours (Heitzler and Simpson, 1993). In addition, *Delta* mutant cells, when adjacent to wild-type cells, are able to differentiate as epidermis, implying that Delta is not required for the reception of the inhibitory signal. In parallel, wild-type cells when adjacent to *Delta* mutant cells, become neural precursors in the majority of cases, implying that wild type cells are not receiving the inhibitory signal (Heitzler and Simpson, 1991).

Furthermore, dosage experiments showed that wild-type cells will always adopt the epidermal fate if they are adjacent to cells expressing lower levels of Notch than themselves, but will become neural precursors if they are next to cells that are expressing a higher level of Notch (Heitzler and Simpson, 1991) (Fig.4G).

These experiments show that cells measure the presence or absence of Notch protein/activity prior to their choice of fates and also show that there is competition between cells, since cells with less Notch protein than their neighbours have an increased ability to signal than their neighbours (Heitzler and Simpson, 1993). These experiments also suggested the existence of a feedback mechanism where cells that have less Notch activity relative to their neighbours have better signal-sending ability (Heitzler and Simpson, 1993). It was suggested that stochastic fluctuations in the

expression of the neurogenic or proneural genes can provide a small difference in Notch activity between neighbouring cells that would be amplified by the negative feedback on *Delta* expression (Heitzler and Simpson, 1991). Therefore, a cell that activates Notch will presumably have progressively less signal-sending capacity, biasing directionality of the signal and the singling out of the adjacent signalling cell.

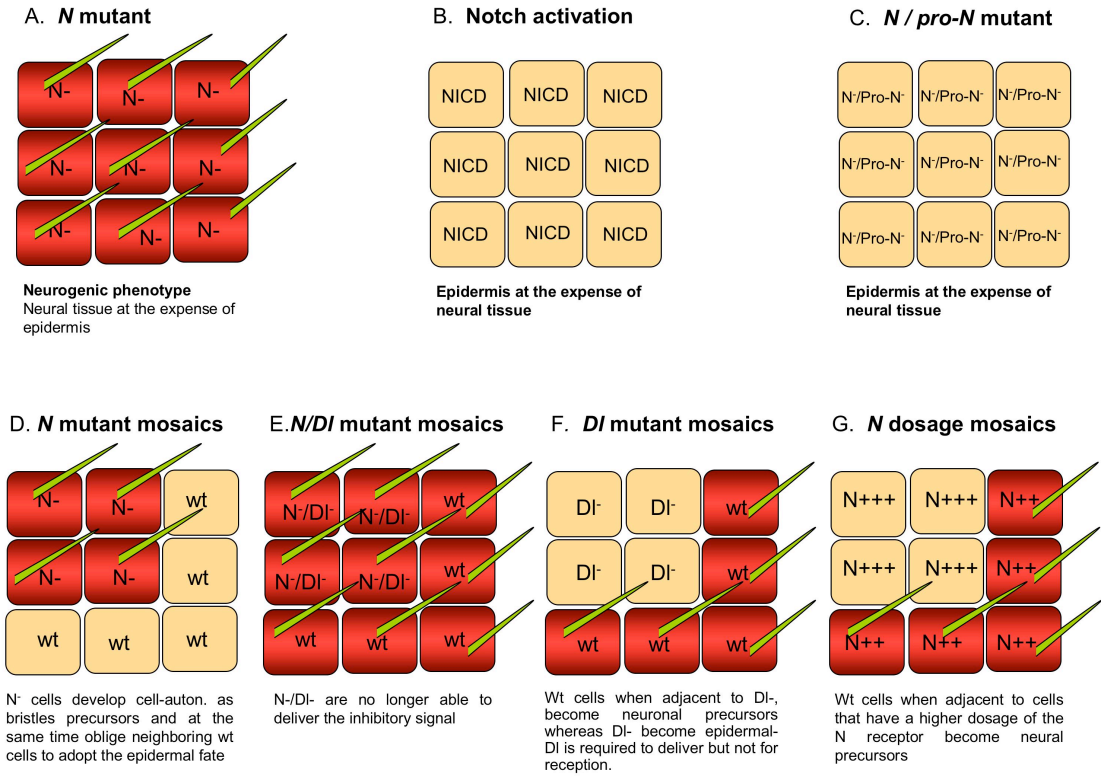


Fig.4 *Drosophila* neural / epidermal cell fate decision. A. Absence of Notch signalling results in the "neurogenic" phenotype- *Notch* mutant (N^-) cells develop as neural precursors (red) and additional bristles (green) are formed at the expense of epidermis. **B**. Constitutive Notch signalling (NICD) suppresses neuronal differentiation and all cells adopt the epidermal fate (light pink). **C.** Double mutant cells for *Notch/proneurals* (*N-/pro-N-*) differentiate into epidermis, implying that Notch is not necessary for the epidermal fate, is only necessary to suppress proneural activity and therefore the neural fate. **D.** *Notch* mutant cells develop cell autonomously as bristle precursors but at the same time force neighbouring wild-type (wt) cells to adopt the epidermal cell fate and never the neural fate. **E.** Wt cells adjacent to *Notch/Delta* double mutant (*N-/Dl-*) cells are able to acquire the neural fate, implying that *N-/Dl-* cells are no longer able to inhibit their neighbours. **F.** *Delta* mutant cells (*Dl-*) when adjacent to wt cells, are able to differentiate as epidermis, implying that Delta is not required for the reception of the inhibitory signal. In parallel, wt cells when adjacent to *Dl* mutant cells, in the majority of cases, become neural precursors, implying that the wt cells are not receiving the inhibitory signal. **G.** Dosage experiments showed that wt cells will always become neural precursors if they are next to cells that are expressing a higher dosage of the *N* receptor. Diagram of some experiments performed by Heitzler and Simpson (1991).

This was confirmed later by the discovery that Notch activity leads to the upregulation of genes encoding the E(spl) transcriptional repressors, which then inhibit transcription of proneural genes (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Proneural proteins are bHLH transcriptional activators that positively regulate expression of the *Delta* gene (Kunisch *et al.*, 1994), thus establishing a self-amplifying feedback loop. The mosaic analysis performed by Heitzler and Simpson provided a basis for this model: cells mutant for the *E(spl)* complex cell-autonomously differentiate as bristles even when adjacent to wild-type cells. However they have the ability of influencing their wild-type neighbouring cells- *E(spl)* mutant cells prevent wild-type neighbouring cells from adopting a neural fate (Heitzler *et al.*, 1996). These authors also showed that this capacity of *Notch* or *E(spl)* mutant cells to influence the neighbour's fate is dependent on the proneural proteins, which regulate the inhibitory signal – Delta.

These experiments, together with others (Wilkinson *et al.*, 1994; reviewed in Lewis, 1996; Greenwald, 1998), have provided a solid model for the process of Lateral Inhibition (LI), where Delta-Notch signalling between cells with similar developmental potential is resolved over time into unidirectional signalling, with one cell becoming the signalling cell and inhibiting its neighbours from adopting the same fate.

I.1.5.1.1 Bias or not Bias, that's the Notch

The progression from a group of equivalent, cells where all cells both signal and receive, to a state where just one cell sends the signal may be a random event or biased by intrinsic or extrinsic cues (reviewed by Simpson, 1997).

It is thought that the random choice of the signal-sending cell arises from stochastic fluctuations of the expression levels of the neurogenic genes or proneural genes, which will then be amplified by the feedback loop (Simpson, 1997). In contrast, the biased decision is based on active mechanisms that regulate the relative levels of expression/activity of the neurogenic or proneural genes and proteins and therefore determine which cell will be the signal-sending cell.

The singling out of the Sensory Organ Precursor (SOP) during bristle development in the *Drosophila* PNS provides examples of both situations (Simpson, 1997):

• Small sensory bristles- microchaetae, are formed in rows uniformly spaced, however the number and precise location of each bristle is variable from fly to fly, implying a random decision mediated by feedback.

• In contrast, the large bristles- macrochaetae, develop always in the same position and their number is fixed within each species, implying that the singling out of the sending cells is a biased decision. Although the precise mechanism of selection is still unknown, several intrinsic and extrinsic factors have been shown to influence the number and position of the macrochaetes. These include genes that regulate the expression/activity of proneural genes and neurogenic genes (*pannier, wingless, senseless, dpp*) (reviewed in Simpson, 1997; Gibert and Simpson, 2003).

The *C. elegans* AC/VU decision and the subsequent specification of Vulval Precursor cells (VPC) also provide excellent, yet more detailed examples, of random and biased binary cell fate decisions, respectively, mediated by Lateral Inhibition, which I will describe below.

Random selection of the signalling-sending cell

During AC/VU cell decision in *C. elegans,* two feedback regulatory loops are used to reinforce directionality and, consequently, the fate of the cells. Notch signalling (mediated by the *Notch* homologue *Lin12*) occurs between two interacting cells (Z1.ppp and Z4.aaa) but only one of these cells will become the AC (terminally differentiated cell that organizes vulval development), while the other will become the VU (cell that divides to produce uterus cells). Until very recently, which of the two cells becomes AC or VU was thought to be a random, non-biased decision (Kimble and Kirch, 1979). However, Karp and Greenwald showed recently that the decision is biased by birth date (Karp and Greenwald, 2003). Nevertheless, birth order may be viewed as the stochastic event that biases the AC/VU decision, so it can still be considered a random choice.

The first-born cell is usually the presumptive VU (pre-VU), which presumably has an advantage in activating Notch/Lin12. This advantage is then amplified by positive and negative feedback-loops: Notch/Lin12 activation leads to upregulation of its own transcription and to downregulation of the *Delta* homologue *Lag-2* (Wilkinson *et al.*, 1994) so that in the end the pre-VU only expresses the receptor, becoming the receiving cell. Down-regulation of *Delta/Lag*-2 expression is the result of the post-

translational repression of the *Delta/Lag-2* transcriptional activator HLH-2, by an unknown factor X activated by Notch (Karp and Greenwald, 2003) (Fig.5).

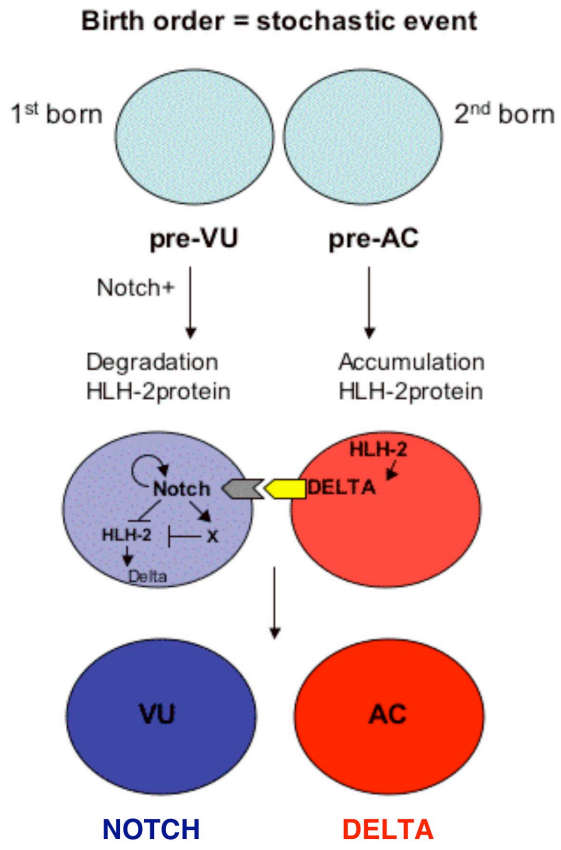


Fig.5 The AC/VU decision.
The first-born cell is usually the pre-VU, which presumably has an advantage in activating Notch. Activation of Notch leads to upregulation of its own transcription and to downregulation of the *Delta /Lag-2* transcription activator HLH-2 by an unknown factor X activated by Notch. By contrast, in the pre-AC Notch is progressively less activated, leading to the accumulation of HLH-2 protein that activates *Delta/Lag-2* expression, so that in the end this cell will only express *Delta/Lag-2* and becomes the signalling-sending cell. To simplify the *C. elegans* nomenclature was not used in the diagram but Delta=Lag2; Notch=Lin12.

By contrast, in the pre-AC, Notch/Lin12 is progressively less activated, leading to the accumulation of HLH-2 protein that activates *Delta/Lag-2* expression, so that in the end this cell will only express *Delta/Lag-2* and becomes the signal-sending cell (Fig.5) (Karp and Greenwald, 2003).

Biased selection of the signal-sending cell by extrinsic cues

Vulval precursor specification in *C. elegans* is a very well studied system which provides an excellent example of a binary cell fate decision mediated by Notch biased by extrinsic cues.

All six vulval precursor cells (VPCs) have the potential to adopt one of the three fates (named 1^0, 2^0 and 3^0). However, an invariant pattern of fates is always present in wild-type animals: 3^0 3^0 2^0 1^0 2^0 3^0 (Fig.6). The descendents of the 1^0 and 2^0 fates form the

vulva, whereas the daughter cells of the 3^0 fates fuse with the hypodermal syncytium, which constitutes the major epidermis of the worm (Greenwald, 1998).

Vulval development is triggered by an extrinsic cue provided by the AC (previously singled out by the random Lateral Inhibition event described above). The AC has high levels of HLH-2 expression that activates the production of EGF/LIN-3 signalling molecule (Hwang and Sternberg, 2004). The EGF released by the AC leads to activation of the EGFR/LIN-23-canonical Ras-MAPK cascade in the VPC cells in a graded manner: maximally in the nearest VPC, which is the presumptive 1^0 fate (pre-1^0 fate), and less in the flanking pre-2^0 fate cells (Yoo *et al.*, 2004) (Fig.6).

Maximal activation of the Ras-MAPK cascade in the pre-1^0 fate induces the 1^0 fate and leads to the up-regulation of DSL Notch ligands (Chen and Greenwald, 2004). Concomitantly, Ras-MAPK cascade leads to the downregulation of the Notch receptor in the pre-1^0 fate, by endocytosis through the DTS (di-leucine sorting motif and serine/threonine residues) targeting it for degradation by ubiquitination, possibly mediated by the Su(dx)-Itch E3 ubiquitin-ligase homologue ALX-1 (Shaye and Greenwald, 2002; Shaye and Greenwald, 2005).

Internalization and degradation of Notch in the pre-1^0 fate is an essential step to activate Notch in the flanking pre-2^0 fate cells. However, this requirement is not to prevent Notch activation in the pre-1^0 fate, but to allow clearance of Notch from the cell surface in order to prevent cis inhibition of the DSL ligands by Notch (Shaye and Greenwald, 2005). This implies that the Ras-MAPK cascade activates or cooperates with Notch lateral signalling and insures directionality at least by two ways: by activating expression of the DSL ligands and by inhibiting cis-inhibition of the same ligands by Notch.

Expression of the DSL ligands in the pre-1^0 fate activates Notch in the neighbouring pre-2^0 fate cells, inhibiting the 1^0 fate and presumably inducing the 2^0 fate. Inhibition of the 1^0 fate involves the activation of several downstream targets that act redundantly to inhibit all residual EGF/MAPK activity (Berset *et al.*, 2001; Yoo *et al.*, 2005).

Recently, it has also been shown that Notch promotes the 2^0 fate by activating the expression of a micro-RNA, mir61, which potentiates Notch activity through post-transcriptional downregulation of a negative regulator of Notch (Yoo *et al.*, 2005).

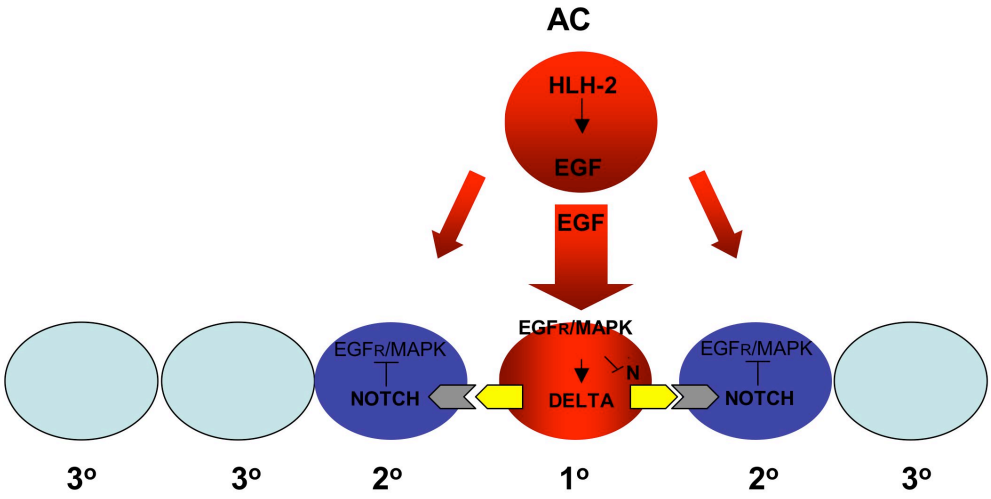


Fig.6 VPC specification. The AC has high levels of HLH-2 expression that activates the production of EGF/LIN-3 signal, which will lead to activation of the EGFR/LIN-23-canonical Ras-MAPK cascade in a graded manner: maximal in the nearest VPC – the pre-1^0 fate, and less in the flanking pre-2^0 fate cells. Maximal activation of the Ras-MAPK cascade in the pre-1^0 fate induces the 1^0 fate and leads to the up-regulation of DSL Notch ligands. In parallel the Ras-MAPK cascade also leads to downregulation of the Notch receptor. DSL ligands in the pre-1^0 fate activate Notch in the neighbouring pre-2^0 fate cells to inhibit the 1^0 fate. To simplify the *C. elegans* nomenclature was not used in the diagram but Delta=Lag2; Notch=Lin12; EGF=LIN-3; EGFR=LIN-23.

In conclusion, Notch activation in the pre-2^0 fate cells leads to two positive feedback-loops: by inhibiting EGF/MAPK activity, it prevents its own internalization and degradation and, by activating mir61, is also increasing its own activity through inhibition of a negative regulator.

Thus, with the help of an external cue, two feedback loops are triggered in each interacting cell to further bias and amplify the differences between interacting cells.

Biased selection of the signal-sending cell by intrinsic cues

Another example of a biased binary cell fate decision mediated by Notch is the sensory organ lineage specification during bristle development in *Drosophila*. In this case, however, Notch is used in a repeated manner and directionality is biased by several redundant intrinsic mechanisms, not by an extrinsic signal.

The *Drosophila* adult sensory organ is composed of five different cells: the socket, the shaft, the sheath, the neuron and a glial cell, which all arise from the same mother cell-the Sensory Organ Precursor (SOP). The SOPs are singled out from the neuroectoderm proneural clusters by Notch mediated Lateral Inhibition (previously described in the experiments by Heitzler and Simpson, 1991). After this selection, each

SOP undergoes a series of asymmetric divisions to produce all the five cells that form the sensory bristle.

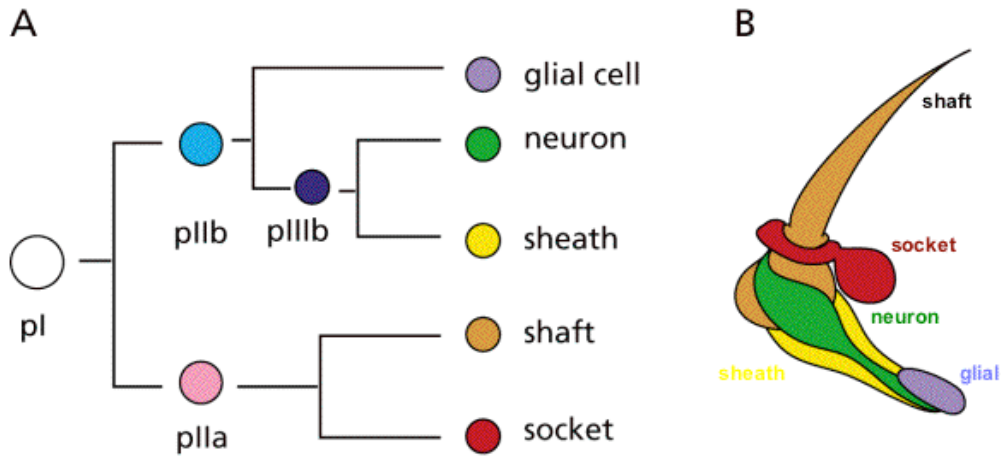


Fig.7 *Drosophila* adult sensory organ specification. **A**. The first division of the SOP (pI), generates a more anterior cell (pIIb) and a posterior cell (pIIa). The pIIa then divides to form the external cells, the shaft and the socket. The pIIb divides to produce the internal cells, which are generated from two successive divisions. The 1st divisions produces the glial cell and a precursor cell- the pIIIb cell, which undergoes the second division to produce the neuron and the sheath. In each step of this lineage, Notch is activated in one cell (pIIa/glia) by its sibling (pIIb/pIIIb) in a unidirectional way. **B.** The *Drosophila* adult sensory organ is composed of five different cell types: the socket, the shaft, the sheath, the neuron and a glial cell. (Adapted from Martinez Arias and Stewart, *Molecular Principles of Animal Development*).

The first division of the SOP occurs within the plane of the epithelium along their anterior-posterior axis, generating a more anterior cell, the pIIb, and a posterior cell, the pIIa. The pIIa then divides to form the external cells, the shaft and the socket, whereas the pIIb divides to produce the internal cells. The pIIb cell divides once to produce the glial cell that will be eliminated by apoptosis, and a precursor cell- the pIIIb cell, which undergoes one more division to produce the neuron and the sheath cell (Fig.7) (Shweisguth, 2004).

In each step of this lineage, Notch is activated in one cell (pIIa/glia) by its sibling (pIIb/pIIIb) in a unidirectional fashion.

The directionality of Notch signalling from pIIb to pIIa is assured by several redundant and complementary mechanisms, which mainly act by increasing the signal-sending capacities of pIIb and by inhibiting Notch activation in this cell. These mechanisms are (Fig.8):

- Asymmetric localization of the Notch negative regulator Numb to the anterior cortex of the dividing SOP, which results in its exclusive segregation to the anterior pIIb cell. In pIIb, Numb interacts with -adaptin to induce endocytosis of the Notch positive regulator Sanpodo, thus preventing Notch activity in this cell (Berdnick *et al.*, 2002; Hutterer and Knoblich, 2005).
- Concomitantly, the E3 ubiquitin ligase Neuralized is also segregated to the pIIb cell. Neuralized promotes Delta monoubiquitination and endocytosis (via the adaptor protein Epsin), which is necessary for Notch activation in the interacting sister cell (pIIa and glia) (reviewed in Le Borgne, 2006).
- To ensure these asymmetries, yet another mechanism is used: the asymmetric segregation of Rab11-recycling endosomes. These only form in the pIIb cell and their formation is inhibited in the pIIa cell. Rab11-recycling endosomes, together with sec15 containing exocyst machinery, regulate Delta traffick back to the membrane, thus activating Notch in the sister pIIa cell (Jafar-Nejad *et al.*, 2005). In pIIa, the recycling endosome does not form because Nuf (Nuclear fallout), a factor essential for endosome formation, is inhibited from accumulating in pIIa, accumulating only in the pIIb cell (Emery *et al.*, 2005).

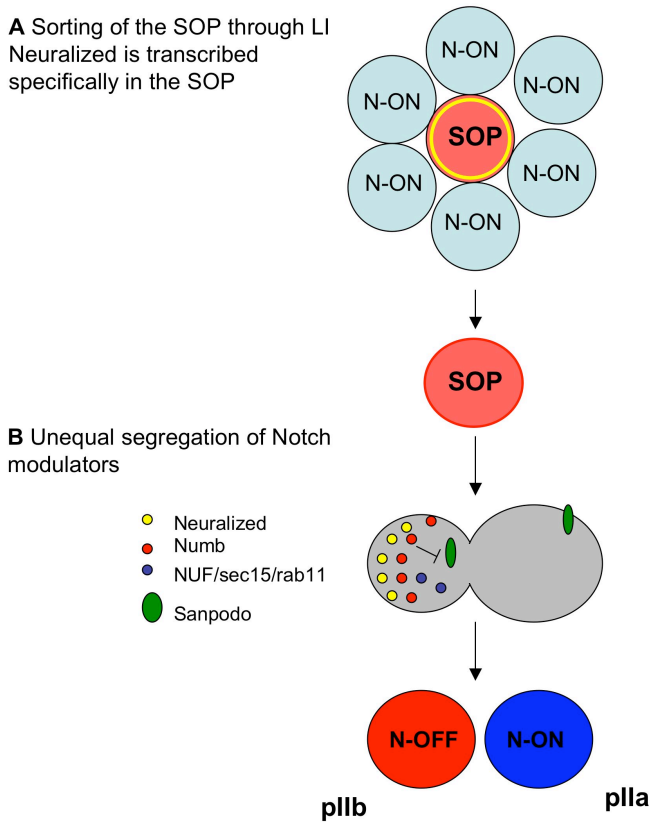


Fig.8 The directionality of Notch signalling from pIIb to pIIa is assured by the asymmetric segregation of Notch modulators.
A. The E3 ubiquitin ligase Neuralized is already specifically expressed in the SOP selected cell. **B**. Numb, Neuralized, Rab11-recycling endossomes and the sec15 exocyst machinery are all asymmetrically segregated to the anterior pIIb cell. N-OFF-Notch signalling is inhibited and N-ON-Notch signalling is activated (see text for details).

Overall, in the case of the random-non-biased selection, feedback is instrumental in making two cells to adopt two alternative cell fates. In the case of the biased selection, feedback seems to act only to reinforce and reassure the directionality of the signal.

I.1.5.2 **Lateral Induction - Boundary Formation**

A second type of operational logic of Notch signalling is Lateral Induction which promotes a new cell type between two different fields of cells. By inducing a new cell type between the different cell populations, Notch signalling creates a boundary between the different fields of cells.

Boundary formation is not an exclusive function of the Notch pathway. Other signalling pathways, like the Ephrin pathway, can promote the same operation. Nevertheless, Notch signalling is a conserved mechanism to generate boundaries in many contexts and species: the D/V boundary of the *Drosophila* wing, *Drosophila* leg segmentation, vertebrate somitogenesis, and zebrafish hindbrain segmentation are some examples of processes that require boundary formation mediated by Notch (Bray, 1998; Irvine and Rauskolb, 2001; Haines and Irvine, 2003; and Amoyel *et al.*, 2004; Cheng *et al.*, 2004).

One of the best-studied examples of boundary formation mediated by Notch is the D/V compartmentalization of the *Drosophila* wing. Notch is activated at the interface between the dorsal and the ventral field of cells, and is involved in maintaining the separation of these two cell populations.

Activation of Notch at this interface results in the formation of a new cell type - the border cells, which have characteristics of an organizer- these cells are able to coordinate the growth and patterning of the wing. Notch activates expression of specific targets, including *wingless*, which is then able to organize and induce wing outgrowth.

Although *Notch* is expressed in the entire wing disc, it is only activated at the D/V border. This is achieved by interactions between the cells in the dorsal compartment and the cells in the ventral compartment (reviewed in Bray, 1998; Irvine and Rauskolb, 2001; Haines and Irvine, 2003):

- The DSL ligand *Serrate* is expressed in the dorsal compartment and signals to cells in the ventral side of the D/V border. Cells in the dorsal compartment are

inhibited to signal to each other by the presence of Fringe, which is specifically expressed in the dorsal field. Fringe modifies Notch by glycosylation (see before) inhibiting Notch activation in the rest of the field, either by promoting cis-inhibition of Notch by Serrate or/and by inhibiting trans-activation of Notch by Serrate. Therefore, Serrate is only able to activate Notch in cells that do not express Fringe- the cells in the ventral side of the D/V border.

- The DSL ligand *Delta* is expressed in the ventral compartment and signals to cells in the dorsal side of the D/V border. Cells in the ventral compartment are inhibited to signal to each other by Delta-Notch cis-inhibition, and Delta is only able to activate Notch in the dorsal side of the D/V border- where Fringe is expressed. Here, Fringe acts to enhance Delta-Notch trans-activation.

An important hallmark in Notch operational logic during boundary formation is that it has a positive outcome, i.e. the cells acquire different characters. Thus, this type of signalling has also been called inductive (Artavanis-Tsakonas *et al.*, 1995). However, recently it has been shown that a major role of Notch signalling in the wing D/V boundary formation is to relieve Su(H) mediated repression of the Notch target genes, since loss of Su(H) is able to rescue Notch loss of function (Koelzer and Klein, 2006). This suggests that Notch activity, rather than being instructive plays a more permissive role (reviewed in Herranz and Milán, 2006).

In addition, it has been proposed that Notch signalling prevents intermingling of the dorsal and ventral cell populations by making a fence-the border cells, rather than establishing distinct dorsal or ventral cell type affinities (Major an Irvine, 2005). These border cells have a distinct actin organization that is promoted by Notch through a transcription independent mechanism (Major an Irvine, 2005).

Another striking feature of boundary formation is that Notch activates production of the ligands, so this mode of Notch signalling can be called lateral induction, irrespectively of whether activation of Notch induces or not a new cell fate (Lewis, 1998). Cells that receive the Delta signal in the dorsal side of the D/V border activate expression of *Serrate*, which will activate Notch in the opposite side- the ventral side of the D/V border. Cells that are activated by Serrate in the ventral side of the D/V border activate *Delta* expression, which in turn will activate Notch in the dorsal side. Therefore, in contrast to the negative-feedback loop that represses ligand expression

during Lateral Inhibition, a positive-feedback on ligand expression operates during boundary formation (reviewed in Bray, 1998; Irvine and Rauskolb, 2001; Haines and Irvine, 2003).

Another example of the role of Notch/Fringe in establishing boundaries occurs during vertebrate somitogenesis, where blocks of mesodermal cells are formed in a rhythmic fashion. However, it is not clear whether Notch is involved in making the boundary between the somites per se, or/and is just synchronizing the oscillatory behaviour of adjacent cells (Jiang *et al.*, 2000; Lewis, 2003). By synchronizing oscillations, Notch would be tuning cells for the same behaviour as opposed to its role during Lateral Inhibition, where it insures and reinforces the differences between neighbouring cells. Thus, this could be yet another way for Notch signalling to operate: tune the cells for the same behaviour, thereby attenuating the differences between neighbouring cells.

Overall, whereas lineage decisions and boundary formation are processes that can rely on other signalling paradigms, Lateral Inhibition seems to be mediated exclusively by the Notch signalling pathway.

I.2 NEUROGENESIS OVERVIEW

I.2.1 Neural induction

The primordium of the nervous system consists of a uniform epithelial sheath - the neural plate, which has the potential to generate all the cells that build up the nervous system. The neural plate consists of the ectoderm lying along the dorsal midline of the embryo and will form subsequently the neural tube, which will give rise to the main subdivisions of the Central Nervous System (CNS): the forebrain, midbrain, hindbrain and spinal cord (reviewed in Wilson and Edlund, 2001; Stern, 2005)(Fig.9).

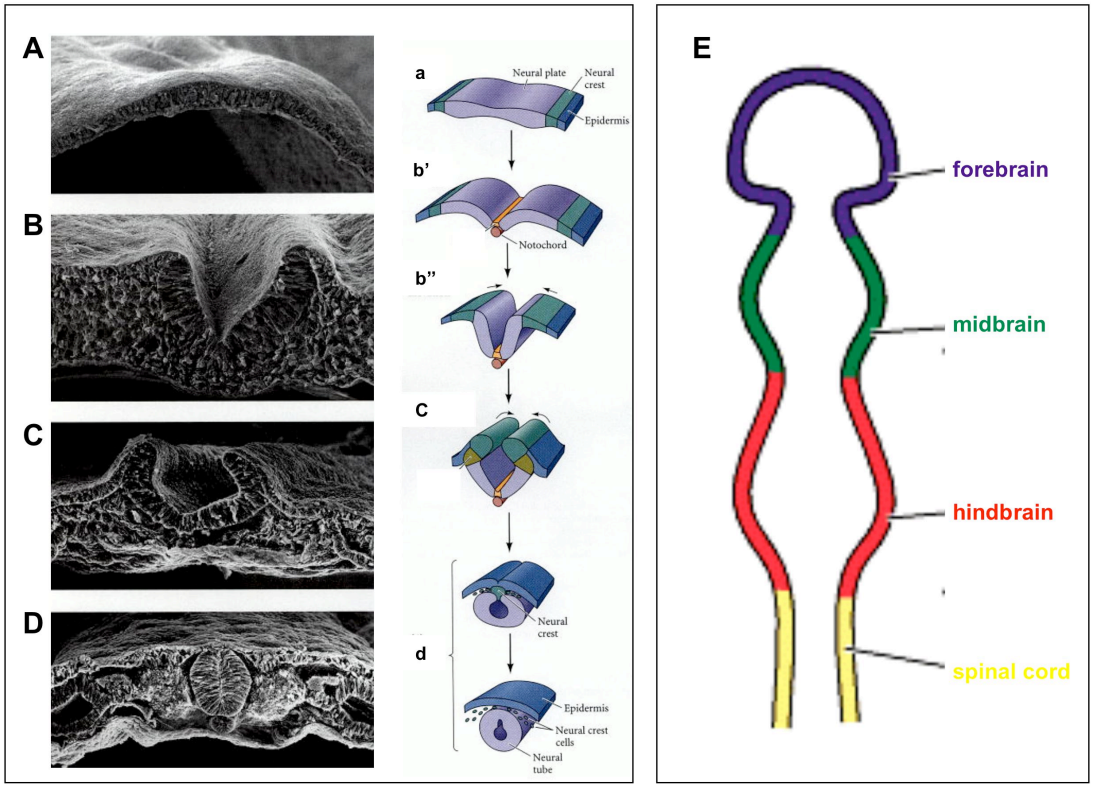


Fig.9 Neural tube formation and regionalization of the CNS. A-D. Neural tube formation in the chick embryo. (A, a) Neural plate. B, b'. Folding begins as the medial neural plate on top of the Notchord, while the presumptive epidermal cells move towards the centre. b'. The neural folds are elevated. (C, c) Convergence of the neural folds and epidermal cells push toward the centre. (D, d) The neural folds are brought into contact with one another and the neural tube closes. Adapted from Gilbert, Scott F, 6the edition *Developmental Biology*, 2000. **E**. The embryonic CNS is grouped in four main subdivisions: forebrain, midbrain, hindbrain and spinal cord. Adapted from Purves, Dale; Augustine, George.J.; Fitzpatrick, David; Katz, Lawrence.C.; LaMantia, Anthony-Samuel., *Neuroscience*. 2nd ed.,2001.

The Peripheral Nervous System (PNS) arises from the lateral neural plate- a border region between the neural plate and the lateral ectoderm, which gives rise to the neural crest and ectodermal placodes (reviewed in Crane and Trainor, 2006).

It was the key experiment performed by Mangold and Spemann in 1924 that showed that neural character is specified through the interaction between different tissues. Mangold and Spemann transplanted a group of mesodermal cells - the organizer, from one amphibian embryo to another at the gastrula stage, generating a second body axis with almost all of the CNS derived from the host ectoderm, not from the graft (reviewed in Wilson and Edlund, 2001; Stern, 2005).

This experiment established the concept of neural induction as an instructive interaction between a group of mesodermal cells - the organizer (Spemann organizer of amphibians, the shield in zebrafish, Hensen's node in the chick and the equivalent region of the node in mouse) and the neighbouring ectoderm. This instructive interaction leads to the induction of the nervous system (reviewed in Wilson and Edlund, 2001; Stern, 2005).

However, several lines of evidence now indicate that neural induction starts before mesoderm formation, at the blastula stage, not during gastrulation as earlier proposed, since the neural plate is formed in the absence of a functional organizer (reviewed in Wilson and Edlund, 2001). Instead, neural induction starts at the blastula stage, through the interaction between the medial and lateral epiblast cells and then later, during gastrulation, the neural character is further reinforced and maintained by signals from the organizer.

The embryonic ectoderm gives rise both to neural tissue and epidermis, and the specification of either lineage is achieved by the concerted action of three pathways: Wnt, Fibroblast Growth Factor (FGF) and Bone Morphogenetic Protein (BMP) signalling pathways.

The epiblast of blastula stage embryos is regionalized in a middle central region and a lateral region. Medial epiblast cells from blastula embryos form the neural plate, which generates neural progenitor cells that express pan-neuronal markers (such as *Sox1-3* genes and *Pax6*), whereas lateral epiblast cells differentiate as epidermal cells (Wilson *et al.*, 2000) (Fig.10.A).

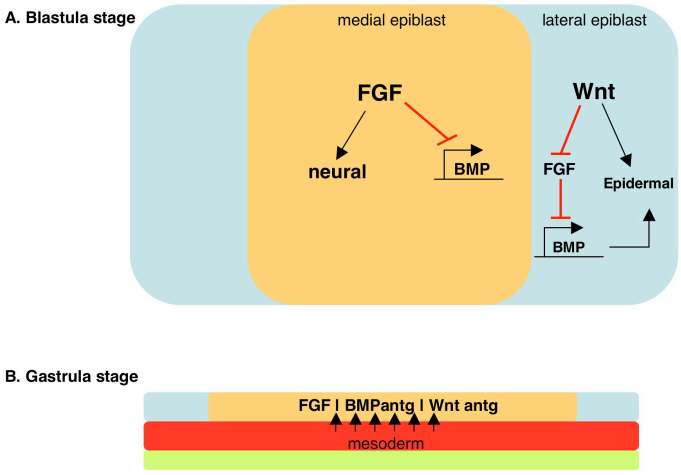


Fig.10 Neural induction. **A**. Gene interactions at the blastula stage that instruct the neural and epidermal fates **B.** Gene interactions at the gastrula stage that maintain the neural specification program.

In the medial epiblast, FGF has a dual function: it inhibits *BMP* transcription, preventing BMPs from instructing an epidermal fate at the expense of the neural fate and directly activates neural specification by an independent pathway.

In the lateral epiblast, Wnt signalling has also a dual role: it instructs the epidermal decision directly and is responsible for attenuating FGF signalling, allowing BMP activity in these cells, which also instructs the epidermal cell fate decision (Fig.10) (reviewed in Wilson and Edlund, 2001).

At the neural plate border, intermediate levels of BMPs are thought to specify the neural crest territory (reviewed in Meulemans and Bonner-Fraser, 2004; Crane and Trainor, 2006).

Subsequently, in gastrula stages the mesodermal organizer emits signals that maintain the neural specification program: FGFs, BMP antagonists as Noggin, Chordin and Follistatin, and Wnt antagonists like Dickkopf (reviewed in Wilson and Edlund, 2001).

The building of the nervous system is laid down in a rostral to caudal order, where each cell that builds up the CNS has to have positional information as to its position in the A/P (anterior/posterior) and D/V axis, enabling the formation of the appropriate connections between the CNS and peripheral targets.

I.2.2 Anterior-Posterior patterning

The brain and spinal cord must develop in complete synchrony with other body structures, particularly the mesodermally derived skeleto-muscular system. Thus, patterning of the nervous system must be absolutely linked to the developing mesoderm.

Neural progenitor cells derived from the neural plate initially contain a rostral "forebrain-like" character. Later, the signals provided by the newly formed mesodermal tissues (caudalizing signals) reprogram the rostral character into caudal-like character giving rise to the midbrain, hindbrain and spinal cord (Muhr *et al.*, 1999; Wilson and Edlund, 2001; Diez del Corral and Storey, 2004).

Caudalization of the neural tissue is the result of the joint action of three signalling pathways: Wnt, FGF and RA (Retinoic Acid).

Wnt signals provided by the paraxial mesoderm and from the neuroectoderm itself act in a graded manner on neural cells with rostral forebrain character to induce their progressive differentiation into caudal forebrain, midbrain and hindbrain character (Nordstrom *et al.*, 2002).

The spinal cord is generated over a long period of time in this rostral-to-caudal sequence as the body axis extends. Thus, this "caudalization" process requires the existence and maintenance of a stem zone that gradually provides neural progenitors, which will later differentiate into neurons. FGF is responsible for the maintenance of this stem zone and the differentiation driving force is RA. This caudal stem zone is composed by the Caudal Neural Plate (CNP) ectoderm, localized in the region adjacent to the regressing primitive streak. This region is considered a stem zone because it contains a resident pool of cells that give rise to neural progenitors that then leave this region (Mathis *et al.*, 2001).

It is thought that FGF signals, derived from the paraxial mesoderm and the ectoderm itself, play several roles in the regulation of the neural stem cell population by:

- Maintaining their proliferative capacity;
- Inhibiting migration away from the stem zone
- Repressing neuronal differentiation and ventral patterning.

Thus, FGF ensures the maintenance of a pool of undifferentiated neural precursors in the stem zone, which are able to give rise to the complete spinal cord (reviewed by Diaz del Corral and Storey, 2004). Recently, it has been shown that the Notch pathway

is also implicated in the maintenance of this stem zone by co-operating with FGF to maintain the proliferative capacity of these cells (Akai *et al.*, 2005).

Cells in the stem zone receive FGF signals both from the adjacent paraxial mesoderm and from the neuroectoderm in an autocrine manner. And when cells leave the stem zone, not only the cues that they receive start changing, but their own paracrine levels of FGF start decreasing, since FGF is only transcribed in the stem zone (Dubrulle and Pourquie, 2004).

When neural progenitor cells leave the stem zone, they enter a transition region where they are now in contact with the PSM. However, neuronal differentiation only starts when progenitors reach the forming neural tube flanked by the somites and are no longer in contact with the PSM. Indeed, removal of the PSM results in precocious neuronal differentiation, while later blockage of signalling between somites and neural tube results in the impairment of neuronal differentiation. This implies the involvement of a repressor provided by the PSM and an activator provided by the somites. The repressor derived from the axial and presomitic mesoderm is FGF, and the signal derived from the somites that drives neuronal differentiation is RA (Diaz del Corral *et al.*, 2003).

FGF is expressed in a graded manner from caudal to rostral (in paraxial and caudal PSM), whereas the expression of the enzyme that produces RA (Raldh2) is expressed in an opposite fashion - in rostral PSM and somites, but absent in more caudal regions. Thus, while FGF in the stem zone itself, and from paraxial and presomitic mesoderm, maintains proliferation and inhibits neuronal differentiation, RA provided by the somites activates the neuronal differentiation program.

FGF and RA opposing gradient is the result of their mutual inhibition. Caudally expressed FGF8 repress the transcription of Raldh2 and activates transcription of the RA degrading enzyme Cyp26, therefore restricting RA signalling to the most rostral regions. Conversely, RA decreases the expression of FGF8 by repressing transcription or/and by modulating the FGF mRNA stability. In addition, RA activates expression of MAPK phosphatase, inhibiting the activity of the FGF downstream target/effector MAPK (reviewed by Diez del Coral and Storey, 2004).

However, the levels of FGF signalling are also crucial for the process of somitogenesis. Levels of FGF below a threshold in the PSM define the position of the future somite boundary -the “determination front” (Dubrulle *et al.*, 2001). Thus, the counteracting

activity of RA on FGF signalling provides a link between the maturation of the mesoderm-segmentation and neuronal differentiation, coordinating the two processes in time and space.

I.2.3 Anterior-Posterior identity: the Hox code

Ultimately, the positional identity in the rostral-caudal axis of the neural progenitors is conferred by the set of *Hox* genes that they express. These transcription factors are organized in clusters in four chromosomes, and 3'genes are activated first in the rostral CNS, while more 5' genes are expressed progressively later in caudal regions, as they are forming- this mechanism is known as colinearity (reviewed in Kmita and Duboule, 2003).

It has been shown that RA is required for the expression of 3' genes, whereas FGF is necessary for 5'genes. In this way, the first neural precursors to escape from the stem zone are subjected to less FGF exposure and initiate transcription of 3' *Hox* genes that confer a rostral character. Neural precursors that remain in the stem zone and only escape later have thus been subjected to a longer period of exposure to FGF, and activate 5' *Hox* genes that confer caudal character. In concert, RA expressed more rostrally attenuates FGF signalling, permitting the expression of more 3'*Hox* genes of rostrally character. In addition, RA may also stabilize/activate *Hox* gene expression by promoting a relaxed form of the chromatin that facilitates transcription (reviewed in by Diaz de Coral and Storey, 2004).

I.2.4 Dorsal-Ventral patterning

Once provided the rostro-caudal coordinates, cells also require positional information on the Dorsal-Ventral (D/V) axis, which is reflected by the appearance of distinct cell types at defined positions along the D/V axis of the neural tube.

The patterning mechanisms of the forebrain are not so well known, which is partially due to the complexity of the rostral CNS. However some general principles of D/V organization seem to be conserved from spinal cord to forebrain (Lee and Jessel, 1999).

Spinal cord progenitors give rise to two types of neurons, which are basically segregated to different positions in the D/V axis (Lee and Jessell, 1999; Jessell, 2000):

- Interneurons - neurons that are involved in the processing of cutaneous sensory input to the brain and are located mostly in the dorsal half of the neural tube.
- Motoneurons - neurons that coordinate the motor output and are located in the ventral half of the spinal cord.

The D/V coordinates are essentially provided by ventral Sonic hedgehog (Shh) secreted from the floor plate and underlying notocord, which induces the ventral neuronal subtypes: motor neurons and some ventral interneurons. Dorsal cell types, such as neural crest cells, glial roof-plate cells and dorsal interneurons are mainly induced by BMP signalling. The source of BMPs firstly comes from the epidermal ectoderm that induces neural crest cells and roof-plate cells, then, after neural tube closure, the roof-plate cells become the only source of BMPs that will specify the dorsal interneurons (Jessel, 2000).

In addition, BMP antagonists (e.g Follistatin, Chordin and Noggin) are produced by the somites and/or the notochord, to further sensitize cells to Shh signalling.

The ventral Shh signalling and the dorsal source of BMPs antagonize each other, thus establishing a gradient of Shh from ventral to dorsal and a BMP gradient from dorsal to ventral (Lee and Jessell, 1999; Jessell, 2000) that then direct the expression of the right combination of transcription factors, many of the Homeodomain family, that determine neuronal subtype.

1.2.5 Neurogenesis, cell cycle and histogenesis

Neuronal differentiation, neuronal subtype specification and also neuronal migration are processes tightly related to the timing of cell cycle withdrawal of neural progenitors.

The withdraw from the cell cycle is an essential step for differentiation to occur, since proliferation and differentiation are generally incompatible states in the CNS - cells that initiate neuronal differentiation but maintain their mitotic potential often die (Lee *et al.*, 1992). This crucial step of arresting the cell cycle has been proposed to be a mechanism for protecting the specified progenitor cells from the influence of extrinsic determinant cues (Edlund and Jessell, 1999). Therefore, the time of the final cell

division is considered the "birth date", which is thought to correlate with the time when the cell fate is specified (reviewed in Hollyday, 2001; Ohnuma and Harris, 2003). Accordingly, the first neural progenitor cells to exit the cell cycle take up early fates, whereas cells that withdraw later from the cycle take on different fates. In addition, CNS progenitors generate neurons before glial cells (Ohnuma and Harris, 2003). For instance, in the ventral spinal cord, progenitors cells first give rise to motoneurons and later to oligodendrocytes, whereas more dorsal progenitors generate first interneurons and later astrocytes.

In the developing CNS, cell proliferation and cell cycle exit are patterned in space, leading to the regionalization of the neural tube: dividing neural progenitors are located in the Ventricular zone (Vz)- the interior region of the neural tube, and post-mitotic neurons accumulate in the outer region of the neural tube- the Mantle Layer (ML) (Fig.11A).

Neural progenitor cells are bipolar cells with a radial membrane process that contacts the basal lamina in one end and another radial process that attaches the apical lumen of the neural tube. Cells are anchored to one another by specialized apical junctions- the adherens junctions (reviewed in Hollyday *et al.*, 2001). When a neural progenitor undergoes its last division and withdraws from the cell cycle to start the differentiation program, it looses the apical attachment and migrates out of this region accumulating in the ML (Fig.11A).

Moreover, the progenitors' cell cycle phases are also patterned in space in the developing neuralepithelium: the nuclei of neural progenitors move within the cytoplasm back and forth across the wall of the neural tube in a process called Interkinetic Nuclear Migration (INM), and these nuclear positions vary in relation to the phases of the cell cycle (Fig.11.B)(reviewed in Hollyday, 2001):

- Nuclei in Mitosis are only found near the lumen of the neural tube (apical);
- Nuclei in G1 are found in the inner half of the Vz and then continue to move toward the basal side;
- S phase nuclei are found in the outer-basal half of the Vz. Then, as cells leave S phase and enter G2, nuclei move back towards the lumen of the neural tube;
- Finally, G2 nuclei are located in the inner half of the Vz.

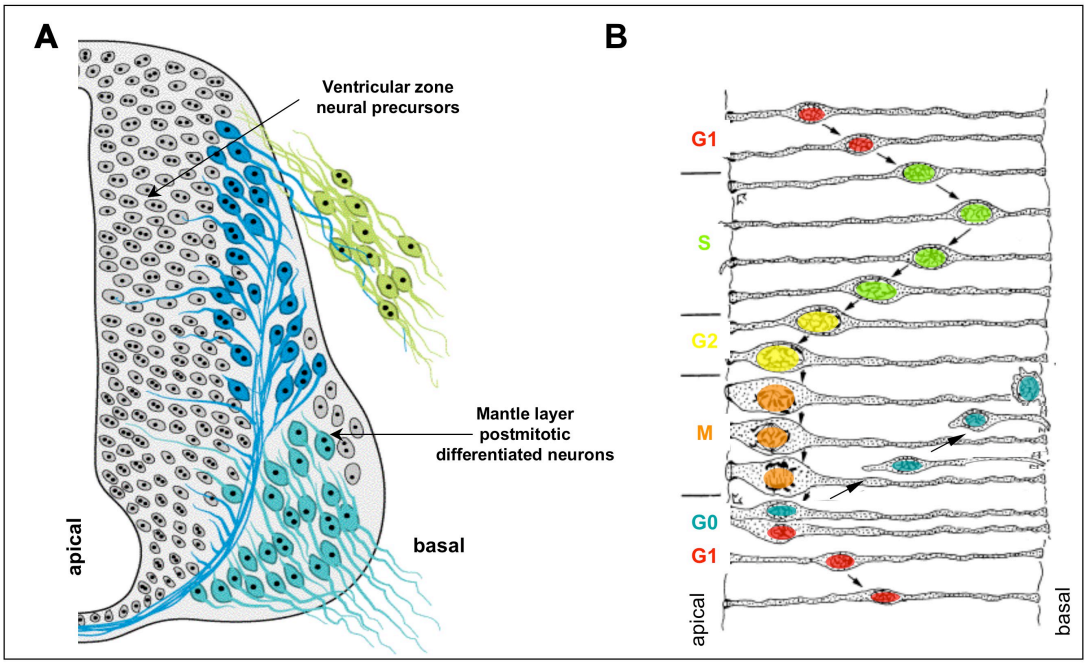


Fig.11 The neural tube is spatially regionalized and nuclei of the neural progenitors are also spatially organized according to their cell-cycle phase. A. The spinal cord is regionalized: dividing neural progenitors are located in the Ventricular zone (Vz)- the interior region of the neural tube and post-mitotic neurons accumulate in the outer region of the neural tube- Mantle layer (ML). Adapted from Wolpert, *Principles of Development*. **B**. INM. Nuclei in Mitosis (M-orange) are only found near the lumen of the neural tube (apical): nuclei in G1 (red) are found in the inner half of the Vz and then continue to move toward the basal side; S phase nuclei (green) are found in the outer-basal half of the Vz. Then, as cells leave S phase and enter G2 (yellow), nuclei move back towards the lumen of the neural tube and are preferentially located in the inner half of the Vz. Cells that become committed to neuronal differentiation, exit the cell cycle, enter G_0 (light blue) and loose their apical attachment and accumulate in the basal side-the ML. Adapted from Hollyday, 2001.

The functional relevance of the INM remains elusive. Frade and colleagues (Murciano *et al.*, 2002) suggested that the INM permits the segregation of two regions within the neuroepithelium: an apical neurogenic zone (G2, M and G1) where the neurogenic (*Notch/hes*) and proneural genes are expressed, and a basal pre-neurogenic zone where cells are undergoing S phase and do not express (or express low levels) of neurogenic or proneural genes. Frade and colleagues suggested that this segregation decreases the possibility of interaction between cells that express proneural and neurogenic genes with other cells that have a reduced capacity to express these genes (Murciano *et al.*, 2002). However this model seems to account only for the somas of these cells and does not seem to consider that cells which have their nuclei apically located may interact with cells with soma located basally, either through their long

membrane radial process that extend throughout the width of the neural tube or/and probably through the adherens junctions located apically.

I.2.6 Neuronal differentiation: the proneural cascade

The combinatorial code of A/P and D/V positional information not only determines subtype identity but also serves to regulate the expression of the genes that drive the general neuronal differentiation path- the proneural genes. Proneural genes are key regulators of neurogenesis as they coordinate all features inherent to the process of neuronal differentiation:

- Proneural proteins coordinate the transition from a proliferating neural progenitor to a post-mitotic neuron, generally by activating the expression of Cyclin-dependent kinase (CdK) inhibitors, which will promote cell cycle exit (Farah *et al.*, 2000; Ohuma *et al.*, 2001; Bertrand *et al.*, 2002; Nguyen *et al.*, 2006).
- Proneural proteins also coordinate the acquisition of both generic and specific neuronal characters, as they trigger the expression of cascades of other transcription factors that regulate pan-neuronal and subtype specific characters (reviewed in Bertrand *et al.*, 2002).
- Proneural proteins trigger the process of Lateral Inhibition by activating expression of the Notch ligand *Delta* (Kunisch *et al.*, 1994; Castro *et al.*, 2006). By triggering the process of Lateral Inhibition, proneural genes inhibit their own expression in neighbouring neural progenitors. In this way, besides being the driving force for neuronal differentiation, proneural proteins also trigger the process that enables the maintenance of the progenitor pool, thus contributing to the homeostasis of the developing nervous system (see ahead).
- Finally, recently was shown that proneural proteins also contribute to the onset of migration of nascent neurons from the Vz to the Mantle layer. Proneural proteins regulate neuronal migration by inhibiting *RhoA* expression and activating *Doublecortin* and *p35* expression, molecules that directly regulate actin and microtubule dynamics (Bielas *et al.*, 2004; Ge *et al.*, 2006).

I.3 NOTCH IN VERTEBRATE CNS DEVELOPMENT

Notch signalling most probably has a crucial word to say in all neurons and glia that are produced, both in the CNS as well in the PNS. Moreover, Notch signalling is not only used during different steps of the embryonic development of the nervous system, but is also active in adulthood. However, I will limit this introduction to the roles of Notch signalling during embryonic CNS development in vertebrates.

I.3.1 Maintenance of neural progenitors

Neurogenesis occurs over a long period of time (for instance, in the mouse, it takes from embryonic day E8.5 up until postnatal day P21), starting at different regions at different times. Also within each region neurons do not differentiate simultaneously. Thus, mechanisms must exist to regulate the prolonged and controlled production of neurons ensuring that a pool of progenitors is maintained until all neurons and glia are generated. This long-lasting process of differentiation allows progenitor cells to be exposed to different changing cues that instruct progenitors to generate different types of neurons and glia. This implies that neural progenitors have to be maintained throughout the course of development, and one of the most important mechanisms to ensure the maintenance of progenitors is Notch signalling.

Neural progenitors have to make a choice: either remain as progenitors or embark on neuronal differentiation. If they choose to remain as progenitors, they retain their dividing capacity and stay within the Vz of the neuroepithelium, whereas if they decide to differentiate as neurons, they withdraw from the cell cycle and migrate out of the Vz to the ML, where they differentiate and accumulate (Lewis, 1998; Holliday, 2001).

This crucial choice is dictated by the balance between two different sets of basic-Helix-Loop-Helix (bHLH) transcription factors: positive factors- the bHLH proneural proteins which drive progenitors to neuronal differentiation, and negative factors- the HES proteins which, by inhibiting the positive factors, repress neuronal differentiation and maintain the progenitors in an uncommitted state (reviewed in Campos-Ortega, 1994).

However, this choice is not a self-centred and deaf choice: progenitors choose their fates taking into account what their neighbours have chosen. This cell 'talking' is

mediated by Notch, in a remarkably similar manner to the choice between the epidermal and neural decision in the *Drosophila* neuroectoderm.

Over the past decade, several lines of evidence, from experiments in *Xenopus*, chick, zebrafish and mouse, in different parts of the CNS (retina, spinal cord, cortex) support a conserved and crucial role for Notch in maintaining the neural progenitor population. *Notch1* (Weinmaster *et al.*, 1991; Myat *et al.*, 1996) and *hes* genes (Sasai *et al.*, 1992; Takebayashi *et al.*, 1995) are expressed in the Vz, where the progenitors are located, whereas *Delta1* and proneural genes are preferentially expressed in cells that have undergone or are undergoing their last division- mentioned as nascent neurons (Henrique *et al.*, 1995; Chitnis *et al.*, 1995; Gradwohl *et al.*, 1996; Ma *et al.*, 1996; Bertrand *et al.*, 2002; Murciano *et al.*, 2002).

Nascent neurons, which have high levels of proneural proteins, trigger the process of Lateral Inhibition by activating expression of the Notch ligand *Delta-1* (Castro *et al.*, 2006), which then activates Notch in neighbouring progenitor cells that express *Notch* receptors. Activation of the pathway in progenitor cells leads to upregulation of downstream target /effectors genes- the *hes* genes, which will then suppress proneural activity in progenitors, thereby preventing these cells from differentiating prematurely into neurons and from expressing the ligand *Delta* (Fode *et al.*, 1998; Ma *et al.*, 1998; Casarosa *et al.*, 1999; Cau *et al.*, 2002; Bertrand *et al.*, 2002; Castro *et al.*, 2006). In this way, cells that express the ligand *Delta1* undergo neuronal differentiation, becoming neurons, but simultaneously ensure that the neighbouring cells do not make the same choice.

Thus, whereas in the *Drosophila* neuroectoderm Lateral Inhibition controls the decision between becoming a neural precursor (neuroblast) *versus* epidermis, during vertebrate neurogenesis it controls the decision between becoming a neuron *versus* remaining as a neural progenitor.

Lateral Inhibition (LI) works like a homeostat providing a feedback mechanism to control the production of neurons: excessive production of neurons will result in an excess of inhibitory signal, thus preventing further production of neurons, whereas a low production of neurons will result in a reduction of inhibitory signal, relieving inhibition of neuronal differentiation. In this way, any perturbations on the balance of

production of neurons will be self-correcting, and will tend back to the equilibrium point (De la Pompa *et al.*, 1997; Henrique *et al.*, 1997).

This model was well established from several experiments, namely in the vertebrate retina. Here, over-activation of the Notch pathway by overexpressing Delta1 or NICD (Austin *et al.*, 1995; Henrique *et al.*, 1997; Dorsky *et al.*, 1997) resulted in the failure of neurogenesis and all cells remaining as progenitors. In contrast, blockage of the Notch pathway by overexpression of a dominant-negative form of Delta resulted in premature differentiation of neurons and no progenitors being left (Henrique *et al.*, 1997) (Fig.12).

Moreover, cells that prematurely differentiate in the absence of Notch signalling adopt the earlier cell fates (ganglion and amacrine cell types) and later cell fates are not formed (Dorsky *et al.*, 1997; Henrique *et al.*, 1997).

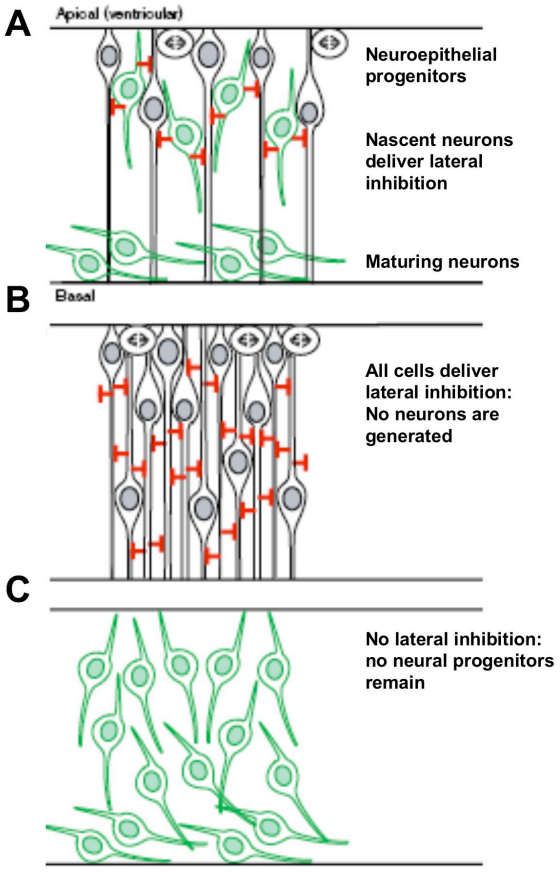


Fig.12 Lateral Inhibition controls the balance between neuronal progenitors and neurons. A. Nascent neurons inhibit neighbouring progenitor cells from adopting the neural fate. **B.** When all cells deliver LI, by ectopic expression of Delta1, all cells inhibit one another from embarking on neuronal differentiation therefore, no neurons are generated. **C**. When all Delta-Notch signalling is impaired, no LI occurs and all progenitors differentiate as neurons and no progenitors remain. Adapted from Henrique *et al.*, 1997.

These experiments showed that Delta-Notch signalling controls the choice between remaining as a progenitor or embarking in neuronal differentiation and, moreover, that Delta-Notch does not seem to be directly involved in generating neuronal diversity (Henrique *et al.*, 1997; Lewis, 1998).

Although Notch maintains the uncommitted-progenitor state it does not necessarily induce cell proliferation, as it could be expected by the dictomy between differentiation and proliferation. The role of Notch in regulating the cell cycle is not clear. In some context it seems to promote proliferation, like in the *Drosophila* eye (Solecki *et al.*, 2001; Baonza and Freeman, 2005; Firth and Baker, 2005) whereas in others it appears to promote cell cycle exit, as in the vertebrate retina (Bao and Cepko, 1997; Dorsky *et al.*, 1997; Scheer *et al.*, 2001; Sriuranpong *et al.*, 2001; Ohnuma *et al.*, 2002).

Nevertheless, what is consensual is that during neurogenesis Notch signalling inhibits neuronal differentiation and maintains neural progenitors in an undifferentiated state. Therefore, Notch activity is crucial to preserve a pool of neural progenitors throughout the prolonged period of neurogenesis, so that these cells can be exposed to various environmental cues and produce all the different types of neurons/glia during development.

I.3.2 Regulation of gliogenesis

The human brain is composed of 90% of glial cells whereas the *Drosophila* nervous system only contains 10-20% of glial cells. This suggests that glial function is critical for the exponential complexity of neurological functions that has emerged during evolution.

The major glia subtypes are astrocytes and oligodendrocytes. Astrocytes have many crucial roles: they provide structural support, regulate water balance and ion distribution, maintain the blood-brain barrier, participate in cell-cell signalling through calcium flux, neuropeptide production and modulation of synaptic transmission. Oligodendrocytes produce myelin, which provides insulation for axons and form the nodes of Ranvier, allowing rapid impulse conduction. In addition, Oligodendrocytes precursor cells in the hippocampus were shown to form synapses with some interneurons, modulating their activity (reviewed in Rowitch, 2004).

Neuron-glial switch

Neuroepithelial progenitors first give rise to neurons and then to glial progenitors, both in the CNS and PNS. This neuron-glial switch is mainly achieved by the downregulation of the proneural genes. Proneural genes, in addition to drive neuronal differentiation, also inhibit gliogenesis. Mutation in proneural genes not only leads to a loss of certain types of neurons but also to premature gliogenesis (Tomita *et al.*, 2000; Nieto *et al.*, 2001).

It has been shown that a proneural gene, *Neurogenin-1* (*Ngn-1*) acts as a transcriptional activator to induce neuronal differentiation, but at the same time inhibits glial differentiation by titrating and inhibiting transcription complexes necessary for gliogenesis, as the STAT3-CBP-Smad1 complexes (Sun *et al.*, 2001). Thus, downregulation of proneural genes seems mandatory for glial differentiation to occur.

Given that the *hes* genes are bonafide inhibitors of proneural activity, it is not surprising that numerous studies point out that Notch signalling has an instructive role in inducing gliogenesis. Indeed, it has been shown that Notch signalling may promote most of the glial cell types- radial glia, astrocytes, oligodendrocytes and Muller glia in the retina (reviewed in Gaiano and Fishel, 2002), depending on the cellular contex and timing of Notch activation.

However, the definition of glial cells has been challenged- many studies now indicate that cells that in the past were defined as radial glia, are now believed to be neural progenitors, which first give rise to neurons and later to glia (reviewed in Ever and Gaiano, 2005). In addition, astrocytes (another type of glia that Ramon y Cajal described as "nothing more than displaced and modified neuroepithelial cells") are now thought to be neural stem cells, which in the adult give rise to neurons and glia (reviewed in Buylla and Lim, 2004). Although there is increasing evidence that some glial cells are progenitors capable of giving rise to glia and neurons, not all glia are believed to have this capacity (Gaiano and Fishel, 2002).

Therefore, many studies which argued that Notch has an instructive role in promoting the glial fate are now being re-interpretated (Ever and Gaiano, 2005) to the simplest model in which Notch, by inhibiting proneurals genes, is at the same time inhibiting neurogenesis and allowing gliogenesis. In this way, Notch can maintain the undifferentiated state characteristic of that time window and context. Then, if the

right cues are present, cells may go through the glial differentiation path, a path specific of that developmental context.

I.3.3 Establishing borders in the CNS

In addition to controlling the production of neurons and glia, Notch is also involved in patterning the CNS and establishing borders in the nervous system.

During CNS development, the neuroepithelium is partitioned along the A-P axis into different regional domains, which are specified by positional cues and maintained by sharp interfaces between compartments- the boundaries.

The role of Notch signalling in boundary formation in the nervous system is well illustrated during segmentation of the zebrafish hindbrain, being remarkably reminiscent to what occurs in the *Drosophila* wing margin. It seems that Notch activation regulates cell affinity properties that segregate cells to the boundaries of the hindbrain and, in addition, it activates *wnt1* expression at these boundaries in a Fringe dependent manner (Amoyel *et al.*, 2004; Cheng *et al.*, 2004). Wnt-1 from the boundary cells is thought to activate neurogenesis in non-boundary cells by inducing proneural genes and consequently *Delta* expression. Then, cells expressing Delta in the non-boundary region are thought to activate Notch in the boundary cells to both inhibit proneural genes and, thereby, inhibit neurogenesis and regulate the cell affinity properties that characterize the boundary cells (Amoyel *et al.*, 2004).

Although reminiscent to the D/V boundary of the *Drosophila* wing, the exact roles of the different Notch players are still unclear.

I.4 PRONEURAL-NOTCH-HES SIGNALLING SYNTAGM

From this review of the Notch signalling pathway, its mode of operation and its general role during neural development, a major feature emerges: the existence of conserved interconnected circuitry between the proneural genes and the Notch pathway genes of the *hes* family. Actually, neurogenesis is based on the antagonistic relationship between these two different sets of bHLH proteins: proneural proteins play a positive role in promoting the commitment to a neural fate and HES proteins repress this cell fate decision.

Therefore this conserved circuitry could be considered as a 'syntagm' or a developmental cassette, a concept developed by A. Garcia-Bellido to describe "a group of genes that interact to perform a discrete developmental operation" (Huang, 1998; Garcia-Bellido, 1981). Here, I propose that the proneural genes, together with the *Notch/Delta/hes* pathway, could constitute the "syntagm" that regulates neurogenesis, controlling the balanced production of neurons and progenitors from flies to vertebrates.

I will first describe each family of transcription factors and then discuss the interlocked circuitry that underlies this 'syntagm'.

I.4.1. Proneural genes

The concept of 'proneural' gene was first defined in *Drosophila* to characterize genes that are both required and sufficient to drive neuronal commitment in the context of the neuroectoderm. *Drosophila* mutants for these genes loose neural structures, which instead are converted to epidermis, whereas ectopic expression leads to the development of ectopic neurons at the expense of epidermis (Ghysen *et al.*, 1989; Romani *et al.*, 1989; Ghysen *et al.*, 1993).

I.4.1.1 Structural characters and mode of action

Proneural proteins are bHLH transcription factors characterized by two major domains:

- The basic region, which is comprised by 8-13 highly basic amino-acids. This domain is required for DNA binding to specific DNA sequences of six nucleotides- the

E boxes (CANNTG). However, different sub-families of proneural proteins, with distinct basic domains, bind to different variations of this E box sequence (Bertrand *et al.*, 2002). Moreover, it has also been shown that the basic domain can recruit co-activators to the vicinity of the proneural regulated promoters (Chien *et al.*, 1996). Therefore, selected transcriptional activation may be achieved by the basic domain, which selects not only the precise E box but also the right co-factors.

- The HLH domain, which is characterized by two □-helices separated by a variable loop. This domain is mostly responsible for protein dimerization. *In vivo*, proneural proteins bind DNA as heterodimers complexes with the widely expressed bHLH E proteins (*Drosophila* Daughterless (*Da*) or one of the three mammalian *E2A* genes) (Bertrand *et al.*, 2002).

Proneural proteins generally act as transcription activators, however, their ability to interact with other transcription factors can also obstruct transcription of genes that might need the co-factors recruited by proneural proteins. This is well illustrated in vertebrates, where it has been shown that NGN-1 recruits co-factors as p300/CBP (CREB binding protein) and PCAF (p300/CBP associated factor) to induce transcription of genes of the neuronal lineage. However, by recruiting theses co-factors to NGN responsive promoters, NGN-1 is also preventing the binding of these co-factors to other transcription factors (STAT3) necessary for activation of glial specific promoters (GFAP-specific of astrocytes) (Nakagawa *et al.*, 1999; Sun *et al.*, 2001). Therefore, NGN-1 simultaneously activates the neuronal differentiation path and inhibits the glial program.

I.4.1.2 **Proneural subfamilies**

Proneural genes are conserved from flies to vertebrates and can be classified in two subfamilies according to their protein similarities with the two *Drosophila* proneural subfamilies: the *achaete-scute* (*AS*) and the *Atonal* (*Ato*) genes (Bertrand *et al.*, 2002; Gilbert and Simpson, 2003):

Achaete-Scute subfamily

Drosophila The AS subfamily contains four *Drosophila* genes and three vertebrate representatives involved in neural development. The *Drosophila* AS complex (AS-C) comprises *achaete* (*ac*), *scute* (*sc), lethal of scute (l'Sc*) and *asense* (*ase*), all of them required for the development of the peripheral nervous system and most of the CNS (Ghysen and Dambly-Chadiere, 1988; Bertrand *et al.*, 2002). These genes are organized in a cluster within ~100 kilobases of DNA, with a number of independently acting regulatory enhancers, which are scattered along the complex, many being shared between members of the complex (Gibert and Simpson, 2003).

Vertebrates The AS vertebrate homologues (ASH) include *Ash1* (mouse *Mash1*, chick *Cash1*, zebrafish *Zash1*, *Xenopus* Xash1), mouse *Mash2*, mouse *Mash3*, *Xenopus Xash3* and chick *Cash4* (see table*).*

Table 3 Non exhaustive list of the Proneural family members in *Drosophila* and vertebrates.

PRONEURAL	***Drosophila***	**Vertebrates**
achaete-scute	*achaete (ac), scute(sc), lethal of scute(l'sc), asense(ase)*	*Mash1, Cash1, Zash1, Xash1 Xash3* *Cash4* *Mash2*
***atonal*-related**	*atonal, amos, cato, tap*	*Atonal* *Math1, Cath1,* *Math5, Cath5, Xath5* *Neurogenin* *Ngn1, Ngn2, Ngn3* *NeuroD* *NeuroD, NeuroD2, NeuroM* *Math3* *Olig* *Olig1, Olig2, Olig3*

Atonal subfamily

Drosophila The *Atonal*-related family includes also four genes in *Drosophila, ato, cato, tap* and *amos*, which specify subtypes of sense organs. Expression of *ato* is also regulated by modular arrangements of enhancers located upstream and downstream of the coding region (Gibert and Simpson, 2003).

Vertebrates In contrast to the reduced number of AS vertebrate representatives, the Atonal family is greatly expanded in vertebrates and can be roughly subdivided into four groups according to sequence similarities within the bHLH domain (reviewed in Bertrand *et al.*, 2002): Atonal (Ath), Neurogenin (Ngn), NeuroD (NeuD) and the Olig subgroups (see table).

I.4.1.3 Auto-regulation of proneural genes

One crucial characteristic of this family of genes encoding bHLH transcription factors is its capacity to positively regulate their own expression. This auto-regulative capacity may provide a crucial mechanism to reinforce and up-regulate proneural expression in the selected progenitors that will undergo neuronal differentiation and, ultimately, maintain expression even after the inductive signal is OFF. This has been shown for some of the AS complex members and for *ato* in *Drosophila* (reviewed in Gibert and Simpson, 2003). In vertebrates, *Math1* also positively regulates its own expression (Helms *et al.*, 2000).

This can be achieved through a direct mechanism, by binding to E boxes on their own promoters (reviewed in Gibert and Simpson, 2003).

In addition, proneural proteins have also the capacity to activate the transcription of genes that increase their own proneural activity, therefore activating indirect auto-regulatory loops. For example, *Drosophila* proneural proteins are able to activate expression of the zinc-finger transcription factor Senseless which, when expressed at high levels, is able to promote proneural expression (Nolo *et al.*, 2000; Jafar-Nejad *et al.*, 2003) and at the same time inhibit the E(spl) proneural inhibitors (Nolo *et al.*, 2000).

In vertebrates, proneural proteins activate expression of *hes6* which prevents, by protein-protein interactions, the inhibitory activity of HES1 on proneural genes (Bae *et al.*, 2000; Koyano-Nakagawa *et al.*, 2000), establishing also an indirect auto-regulatory loop.

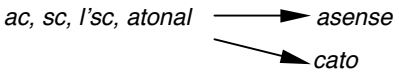
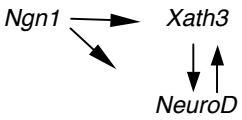
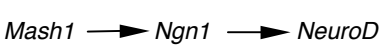
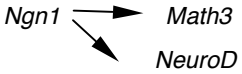
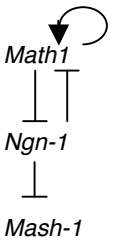
I.4.1.4 Cross-regulation between proneural genes

Another central characteristic of proneural genes is the cross-regulatory interactions that occur between members of this family, leading to cascades of proneural activity.

In *Drosophila* the *ac, sc, l'sc* or *ato* genes are expressed first in proneural domains, while *ase* and *cato* are expressed later in the selected neural precursors. Similarly, in vertebrates, *Mash1* and *Neurogenins* are expressed earlier in neural progenitors and the *NeuroD* family members are expressed later in immature neurons (Helms and Johnson, 1998; Bertrand *et al.*, 2002).

Direct cross-regulation has been demonstrated for *ac* and *sc* in *Drosophila*. In vertebrates, several examples exist. For instance, it has been shown that NGN1 activates both *Xath3* and *NeuroD*, which both cross-activate each other (Martinez and Modollel, 1991; Van Doren *et al.*, 1992; Ma *et al.*, 1996; Perron *et al.*, 1999; reviewed in Bertrand *et al.*, 2002) (see Table 4 for non exhaustive summary of some reported cross-interactions).

Table 4 Examples of auto- and cross-regulation between members of the Proneural family.

	Cross-regulatory interactions	References
Drosophila		Van Doren *et al.*, 1992;
		
Vertebrates		Perron *et al.*, 1999
		Cau *et al.*, 1997
		Ma *et al.*, 1998
		Fode *et al.*, 2000 Helms *et al.*, 2001 Gowan *et al.*, 2001

Moreover, in vertebrates, it has been shown that cross-inhibition may also occur to define discrete non-overlapping expression domains in progenitor populations of the developing neural tube: *Ngn-1* and *Math-1* mutually repress one another and *Ngn-1* represses *Mash-1* (Gowan *et al.*, 2001). However, the molecular mechanism underlying this cross-inhibition is still unknown (Gowan *et al.*, 2001).

I.4.2. *hes* genes

As already described, the major proteins that counteract the activity of the proneural genes are the bHLH-Orange (bHLH-O) transcriptional repressors that belong to the *hes* family (Hairy and the Enhancer of Split) (Davis and Turner, 2001).

I.4.2.1 Structural characteristics and mode of action

HES proteins are characterized by four major structural domains (reviewed in Davis and Turner, 2001) (Fig.13A):

- The basic domain, which is responsible for DNA binding to specific target sequences. It has been shown that HES repressors are able to bind E boxes and to N-boxes (CACNAG), with different affinities according to the protein (Sasai *et al.*, 1992; Takebayashi *et al.*, 1994; Van Doren *et al.*, 1994; Jennings *et al.*, 1999; Hirata *et al.*, 2000). The ability to bind E-boxes enables these proteins to compete with the bHLH activators for the same box, thus inhibiting transcription of bHLH activators targets (Jennings *et al.*, 1999).

- The HLH domain mediates homo and heterodimerization. Heterodimerization has been reported to occur between members of different subfamilies of repressors but also with bHLH activators. However, it has recently been shown that heterodimerization between bHLH repressors and bHLH activators is not mediated by the HLH domain, being instead mediated by the first 80aa of the HES repressor protein (E(spl)) and the C-terminal domain of the activator (Sc) (Giagtzolou *et al.*, 2005). In *Drosophila*, this capacity to form heterodimers with bHLH activators seems to be exclusive for the E(spl) family, as Hairy is not able to interact with any of the bHLH activators (Alifragis *et al.*, 1997). Members of the E(spl) family are able to form homo and heterodimers between them and also with the proneural bHLH proteins Ac, Sc, Atonal, Asense and their partner Da (Oellers *et al.*, 1994; Gigliani *et al.*, 1996;

Alifragis *et al.*, 1997). In vertebrates, in contrast, heterodimerization between the two subfamilies Hairy and E(spl) (HES1-HES6) and with bHLH proneural activators has been reported (HES5-MASH1, HES5-E47 and HES1-E47) (reviewed in Davis and Turner, 2001).

- The orange domain confers subfamily specificity. Swapping the orange domain of E(spl)m8 with that of Hairy conferred to E(spl)m8 Hairy-like functions (Dawson *et al.*, 1995), presumably by mediating protein-protein interactions with other transcription factors. For example, E(spl)m8 binds to the zinc-finger transcription factor Senseless (Sens*)* through the orange domain, inhibiting its positive and synergistic action with Ac/Da heterodimers (Jafar-Nejad *et al.*, 2003).
- Finally, HES proteins contain a conserved C-terminal tetrapeptide: the WRPW (Trp-Arg-Pro-Trp sequence) motif. This WRPW motif is able to recruit the co-repressor Groucho/TLE to inhibit transcription (Paroush *et al.*, 1994; Grbavec *et al.*, 1996; Jimenez and Ish-Horowicz, 1997) (Fig.13). Groucho/TLE proteins are transcriptional co-repressors that do not bind directly to DNA but are recruited to target genes by DNA bound repressors and are able to inhibit transcription, at least in part, by recruiting Histone Deacetylases (HDAC).

Overall, HES proteins may inhibit transcription by two different mechanisms: either by direct DNA binding or by protein-protein interactions:

- They may directly bind DNA (N and E boxes) and recruit co-repressor complexes like Groucho, or they may compete with bHLH activators for the same DNA box (Ohsako *et al.*, 1994; Van Doren *et al.*, 1994; Chen *et al.*, 1997);
- They are able through protein-protein interactions to form heterodimers with other transcription factors deviating or inactivating bHLH transcriptional activators (proneural), their partners (Da/E proteins) or co-activators (Senseless) from the proneural- responding promoters (Davis and Turner, 2001). Moreover, this capacity of protein-protein interaction with the proneural activators can also work as a bait for bringing the transcriptional repressors to the target genes of the activator. In this way E(spl) repressors are inhibiting transcription via DNA binding sites of the activators (Eboxes) (Giagtzolou *et al.*, 2003).

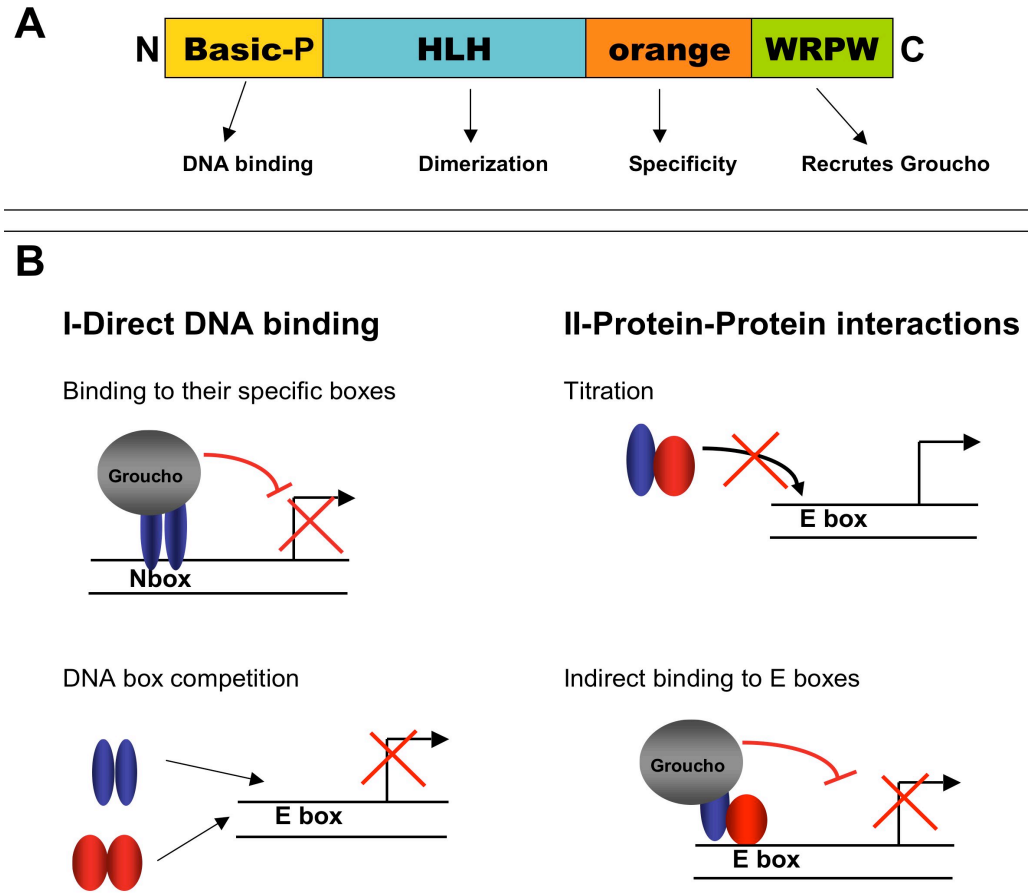


Fig.13 HES proteins structural domains and mode of action. A.HES family proteins are characterized by four major domains. **B**.HES proteins (blue) may inhibit transcription through direct DNA binding (I) or through protein-protein interactions (II). I. HES proteins (blue) may directly bind specific DNA boxes and recruit the co-repressor Groucho or they may compete with bHLH activators (red) for the same DNA box (E box). II. HES proteins can also inhibit transcription through protein-protein interactions. HES proteins may form heterodimers with other transcription activators like the proneurals proteins blocking their binding to the DNA. In addition, HES proteins may also bind indirectly to E boxes through the proneural activators, and then recruit Groucho and inhibit transcription.

I.4.2.2 *hes* subfamilies

The *hes* family comprises two distinct conserved sub-families: the Hairy and the Enhancer of Split-like sub-family, also named after their *Drosophila* counterparts. However, the nomenclature in vertebrates is rather confusing, since most authors did not make the distinction between the two sub-families and simply refer to these genes as *hes* genes.

Table 5 Members of the *hes* family of genes encoding transcription repressors in *Drosophila* and mammals.

bHLH-O proteins	***Drosophila***	**Mammals**
Enhancer of Split	E(spl) complex	*hes2*
	E(spl)m	*hes3*
	E(spl)m	*hes5*
	E(spl)m	*hes6*
	E(spl)m3	*hes7*
	E(spl)m5	
	E(spl)m7	
	E(spl)m8	
Hairy	*hairy*	*hes1*
		hes4 (human only)

Hairy subfamily

Drosophila In *Drosophila*, the *hairy* gene functions in two time windows: during embryogenesis, it works as a primary pair-rule gene in the establishment of segments, whereas in the larval stage functions by restricting proneural expression domains. However, *hairy* does not seem to be involved in Lateral Inhibition, working instead as a pre-patterning gene to establish the initial proneural domains (Ohsako *et al.*, 1994, Van Doren *et al.*, 1994; Davis and Turner, 2001). This last role of *hairy* in restricting proneural expression is responsible for the phenotype of excess of hairs in the *hairy* mutant flies, which gave the name to the gene.

Vertebrates Vertebrate *hairy*-like genes, (including mouse *hes1* and zebrafish *her5*), like the *Drosophila hairy*, have been shown to regulate midbrain-hindbrain boundary formation, working as pre-patterning genes, independently of Notch signalling, to create a neurogenesis-free zone with organizing capacities (Gelling *et al.*, 2004; Ninvock *et al.*, 2005; Takada *et al.*, 2005; Baek *et al.*, 2006).

However, these genes seem to have also acquired a role in mediating Notch signalling. In the context of somitogenesis, mouse *hes1* and chick *hairy*1/2 have been shown to act downstream of Notch signalling, where they are part of the molecular clock that regulates the rhythmic production of the somites (Guidicelli and Lewis, 2004).

In the context of neurogenesis, Hairy-like proteins besides working as pre-patterning factors also seem to work downstream of Notch signalling. In the mouse, *hes1* is

expressed before *Notch, Delta* or proneural genes, indicating that its expression is regulated by factors other than Notch signalling (Hatakeyama *et al.*, 2004). However, its expression is maintained and present later when Notch signalling is operating and neurogenesis starts (Hatakeyama *et al.*, 2004). Moreover, Notch1 has been shown to activate expression of *hes1* in a variety of cell lines and retinal explants (Jarriault *et al.*, 1995; Ohtsuaka *et al.*, 1999) and this activation is dependent on the binding of NICD/CSL to the CSL binding sites present in the *hes1* promoter (Jarriault *et al.*, 1995). Nevertheless, is still not clear whether *hes1* is downstream of Notch signalling in the neuronal context, since Notch1 and CBF-1 mutant mice do not present reduced levels of *hes1* expression (de la Pompa, 1997).

However, it is possible that *hes1* may be downstream of Notch signalling under the influence of other Notch receptors and ligands, as Notch2 (Solecki *et al.*, 2001). Alternatively, *hes1* may be indeed under Notch1 control but its regulation may have been masked by regulation by other *hes* genes.

A conciliatory view is to consider that *hes1* accumulates both functions: restricting proneural expression as a pre-patterning gene independent of Notch signalling and, later, mediating Lateral Inhibition downstream of Notch signalling. It has been shown that HES1 regulates the early expression of the proneural gene *Mash1,* thereby defining the olfactory placodal domain that undergoes neurogenesis. Later HES1 seems to control the density of neural progenitors in this domain, presumably downstream of Notch signalling, working in synergy with the other *hes* gene- *hes5* controlling the expression of the next proneural in the cascade- *Ngn-1* (Cau *et al.*, 2000).

Enhancer of Split subfamily

Drosophila The name 'Enhancer of Split' [E(spl)] was used originally to describe mutations that enhance the phenotypes of a *Notch* mutant allele called *Split* (Lehmann *et al.*, 1983). The *Drosophila* E(spl) complex contains seven genes coding for bHLH proteins, clustered in a 60kb complex (*m□, m□, m□, m3, m5, m7, m8*) together with the gene encoding *groucho* co-repressor (Knust *et al.*, 1992).

Mutations in these genes result in a neurogenic phenotype- an overproduction of neural tissue at the expense of epidermis (Knust *et al.*, 1987; Delidakis *et al.*, 1991; Delidakis and Artavanis-Tsakonas, 1992), with the extent of the phenotype being

dependent on the number of *E(spl)* genes deleted. When all seven bHLH-encoding genes are deleted, almost all cells choose the neural pathway and only a few epidermal cells differentiate, whereas weaker phenotypes are observed when fewer *E(spl)* genes are lost (Heitzler *et al.*, 1996), and mutations in individual *E(spl)* genes display no phenotypic defects (Delidakis *et al.*, 1991), indicating overlapping and redundant functions.

In contrast, overexpression of genes of this complex results in inhibition of neuroblast formation, as shown by CNS hypoplasia and lack of sensory organs (Tata and Hartley, 1995; Nakao and Campos-Ortega, 1996), a phenotype similar to the overexpression of a constituitively active Notch protein (Lieber *et al.*, 1993).

Genetic and molecular data place the *E(sp1)* genes downstream of Notch and upstream of the proneural genes, therefore acting as targets and effectors of the Notch signalling cascade and performing the major function of inhibiting the transcription and activity of the proneural transcription factors.

Other Notch target genes are located at the *E(spl)* locus, as the *bearded* genes, which encode negative regulators of Notch activity. However, these genes do not code for bHLH transcription factors. Bearded proteins have been shown to bind to the E3 ubiquitin-ligase Neuralized, preventing its binding to Delta and thereby impairing Notch activation by Delta (Bardin and Schweisguth, 2006, see before).

The expression of the seven related *E(spl)* genes is dependent on Notch activation (Jennings *et al.*, 1994; Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) and all contain binding sites for Su(H) (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Nelessen *et al.*,1999; Cooper *et al.*, 2000).

In contrast with the AS-C, which contain various enhancers that integrate different combinations of pre-patterning genes to convey expression in specific developmental domains, *E(spl)* genes are mostly regulated by small DNA fragments of ~400-500bp close to the transcription start site of each gene (Nelessen *et al.*, 1999; Cooper *et al.*, 2000). These small regulatory sequences contain Su(H) binding sites and are able to respond to Notch. However, Notch activation is not able to elicit transcription from the *E(spl)* enhancers in all locations *in vivo*, indicating that Notch activity must be integrated with other spatially restricted co-factors. One such class of co-factors that synergise with Notch are the proneural proteins, which have been shown *in vivo* and

in vitro to cooperate with Notch in activating transcription of *E(spl)* genes (Kramachek, 1994; Singson *et al.*, 1994; Bailey and Posakony, 1995; Nellesen *et al.*, 1999; Cooper *et al.*, 2000). Therefore, it seems that *E(spl)* regulatory sequences integrate Notch activity with spatially restricted factors, like the proneural genes, which themselves had been subjected to complex pre-patterning events (Cooper *et al.*, 2000; Gibert and Simpson, 2003).

Although each *E(spl)* gene is mostly regulated autonomously by independent cis-regulatory domains associated with each gene (Nelessen *et al.*, 1999; Cooper *et al.*, 2000), the nearest neighbours in the complex show the most similar and overlapping expression patterns, indicating that sharing of regulatory elements also occurs (Cooper *et al.*, 2000).

Vertebrates In mammals, in contrast to what occurs in *Drosophila*, *E(spl)*-like genes are not organized in a cluster. Also, not all *E(spl)*-like genes are Notch targets/effectors, neither are all implicated in neural development.

Three of these genes (*hes3*, *hes5* and *hes6*) have a role in neural development, but only one unequivocally responds to Notch signalling- *hes5*. Concerning the other two remaining genes, *hes2* and *hes7*, the first does not respond to Notch signalling (Nishimura *et al.*, 1998) and the second, *hes7*, functions downstream of Notch, together with *hes1,* to control the rhythmic production of somites (Bessho *et al.*, 2001).

hes3 does not seem to be not regulated by Notch signalling, since its expression is not affected in *Notch1* mutants and its promoter does not respond to Notch activation in *in vitro* transcription essays (Nishimura *et al.*, 1999). *hes3* performs mostly pre-patterning functions in the developing neural tube and regulates boundary formation together with the *hairy* homologue *hes1*. *hes3* and *hes1* regulate and maintain the midbrain-hindbrain boundary by inhibiting the expression of the proneural genes, creating a proneural free region with organizing capacities (Hirata *et al.*, 2001; Baek *et al.*, 2006). The midbrain-hindbrain boundary (or Isthmus organizer) expresses secreting factors as FGF8 and Wnt1, which induce the development of the midbrain and anterior hindbrain. In double mutants for *hes1* and *hes3*, cells in the isthmic organizer prematurely terminate expression of these secreting factors and ectopically

express proneural genes, leading to premature differentiation into neurons (Hirata *et al.*, 2001; Baek *et al.*, 2006).

In contrast with the uncertainties regarding *hes1* as being a direct target of Notch signalling in the neuronal context, several lines of evidence point for *hes5* as the bonafide target and effector of Notch signalling during neural development. First, the *hes5* promoter contains consensus binding sites for CSL and is activated by Notch signalling in cell lines and in retinal explants (Nishimura *et al.*, 1998; Ohtsuka *et al.*, 1999; Ong *et al.*, 2005). Second, *hes5* transcripts are almost absent from neural tissue in CSL mutant embryos and severely reduced in Notch1-deficient embryos (de la Pompa *et al.*, 1997). *hes5* mutants display premature neuronal differentiation but with no major morphological defects. However, the severity of premature neuronal differentiation is enhanced in the *hes1-hes5* double mutant, indicating redundancy of the two *hes* genes (Ohtsuka *et al.*, 1999).

Moreover, missexpressing the activated form of the Notch receptor (NICD) results in the up-regulation of both *hes1* and *hes5* and inhibition of neurogenesis (Ohtsuka *et al.*, 1999). This inhibition of neurogenesis by activated Notch occurs in wild type, *hes1*-null and *hes5*-null cells but not in absence of both *hes* genes, implying that *hes1* and *hes5* functionally compensate each other and place these genes as downstream effectors of the Notch pathway (Ohtsuka *et al.*, 1999). Although inhibition of neurogenesis by NICD is reduced in the double mutants *hes1/hes5*, activated Notch can still partially inhibit neuronal differentiation. In addition, *Notch1* mutant embryos display a more severe phenotype than *hes1/hes5* double mutants (de la Pompa *et al.*, 1997; Swiateck *et al.*, 1994; Conlon *et al.*, 1995). This implies that other genes are participating or compensating for the loss of the *hes1* and *hes5* (Ohtsuka *et al.*, 1999, Cau *et al.*, 2000). In fact, it seems that combining the *hes1/hes5* double mutants with *hes3* inactivation results in the complete loss of the progenitor population (Hatakeyama *et al.*, 2004), the phenotype expected for the complete loss of Notch signalling (Lewis, 1998). In these *hes1/3/5*-deficient embryos, the progenitor population is not maintained and massively differentiate into early-type neurons, depleting the late-born neuronal fates (Hatakeyama *et al.*, 2004).

In both mouse and *Xenopus, hes6* homologues are not regulated by Notch but instead are regulated by proneural proteins. Moreover, instead of inhibiting neurogenesis they were shown to promote it, but only in regions of the neural plate where the proneural

genes are already expressed (Koyano-Nakagawa *et al.*, 2000; Bae *et al.*, 2000). *hes6* has been shown to act in a positive feedback loop with the *Neurogenins* to promote neuronal differentiation by inhibiting the repressive activity of HES1 on proneural genes. HES6 inhibits HES1 activity through the formation of HES1:HES6 heterodimers. These heterodimers are unable to interact with the co-repressor Groucho rendering HES1 unable to repress transcription of the proneural genes (Koyano-Nakagawa, 2000; Bae, 2000). In addition, HES6 interaction with HES1 also seems to promote the proteolytic degradation of HES1 (Gratton *et al.*, 2003).

Table 6 Mammalian *hes* genes chromossome location, neural expression, DNA binding activity and protein interactions.

***hes* gene**	**Chr location**	**Neural expression**	**Notch dependent**	**DNA binding activity**	**Protein interactions**
hes1	Chr 16 4 exons	yes	yes	N and E boxes	HES1-HES6 HES1-E47
hes2	Chr 4 4 exons	no	no	N and E boxes	
hes3	Chr 4 4 exons alternative splicing of 1st exon	yes	no	N and E boxes (HES3b)	HES3-MASH1
hes5	Chr 4 3 exons	Yes	yes	N box	HES5-MASH1
hes6	Chr 4 4 exons	yes	no		HES1-HES6
hes7	Chr 11 4 exons	No	yes	N and E boxes	

I.4.2.3 Auto-regulation of *hes* genes

In *Drosophila,* it has been shown that *E(spl)* genes are able to negatively regulate their own expression (Kramatscheck and Campos-Ortega, 1994; Cooper *et al.*, 2000), since mutants for the *E(spl)-C* show increased expression of *E(spl)* transcripts.

In vertebrates, *hes1* and *hes7* have also been shown to negatively regulate their own expression (Hirata *et al.*, 2002; Bessho *et al.*, 2003). This negative feedback generates an oscillatory expression of these genes, with the same periodicity as somite formation- in the chick every 90min, 120min in the mouse and 30min in the zebrafish.

This oscillatory mRNA expression appears to play a central role in the core mechanism of the segmentation clock (Dubrulle and Pourquie, 2004; Guidicelli and Lewis, 2004). Therefore, this capacity of auto-regulation is essential for the process of somitogenesis and for the Notch pathway architecture used during somitogenesis. For instance, if the oscillatory expression of the Notch regulator *Lunatic fringe (Lfng*) is disrupted by constituitively expressing this gene in the PSM, defects in somite patterning and vertebral organization are induced similar to those displayed by *Lfng* null mutants (Serth *et al.*, 2003). Likewise, in mutant mice where *hes7* oscillations have been disrupted somite patterning and vertebral organization is also severely disorganized (Hirata *et al.*, 2004).

Moreover, it has been shown that *hes1* expression also oscillates *in vitro* in various cell lines (Hirata *et al.*, 2002), therefore opening the possibility that this oscillatory behaviour may also occur in other developmental processes.
Whether other *hes* genes have or not an auto-regulatory capacity and generate an oscillatory behaviour in other cellular contexts, namely during neurogenesis, remains unknown.

I.4.2.4 **Cross-Regulation between *hes* genes**

Transcriptional cross-regulation between members of the *Drosophila* E(spl) complex has been reported: *E(spl) m7* and *m*□ are able to negatively regulate the *m*□ promoter and, to a lesser extent, also the *m*□ and *m*□ promoters (Cooper *et al.*, 2000).
In vertebrates, when the work described in this thesis began, no transcription cross-regulations between *hes* family members had been reported. Later, it has been described that *hes5* expression is upregulated in *hes1*-/- deficient embryos. Equally, in *hes5*-/-deficient embryos, *hes1* transcription is increased (Hatakeyama *et al.*, 2004).
Post-transcriptional regulation between *hes* genes has been also reported: HES6 is able to form herodimers with HES1 blocking its ability to inhibit proneural activity, by preventing HES1-Groucho interaction and/or inducing HES1 protein degradation (Bae *et al.*, 2000; Gratton *et al.*, 2003).

I.4.3 The neurogenesis 'syntagm'

From *Drosophila* to vertebrates Proneural proteins trigger the process of Lateral Inhibition (LI) by activating the expression of the Notch ligand *Delta* (Hinz *et al.*, 1994; Kunich *et al.* 1994; Ma *et al.*, 1996, Cau *et al.*, 2002; Bertrand *et al.*, 2002; Castro *et al.*, 2006), which binds and activates Notch in adjacent cells, leading to activation of downstream target genes of the *hes* family. The HES proteins, in turn are able to repress proneural expression and/or activity in these cells (reviewed in Davis and Turner, 2001). Thus, the proneural genes activate a signalling process (LI) that inhibits its own activity in a non-cell-autonomous manner (in trans).

However, it has been shown also that proneural proteins are able to directly activate expression of the *hes* genes in cis (Kramacheck *et al.*, 1994; Singson *et al.*, 1994; Oellers *et al.*, 1994; Bailey and Posakony, 1995; Cooper *et al.*, 2000; Gazit *et al.*, 2004; Castro *et al.*, 2005). In *Drosophila* it was demonstrated that AS-C proteins bind *in vitro* the *E(spl)m8* and *m7* promoters and that activation of *E(spl)m8 in vivo* requires an intact E box (Singson, 1994; Kramatschek, 1994). In vertebrates, *mash1* mutant embryos show a loss in *hes5* expression and it was shown that the MASH1 protein is able to bind the *hes5* promoter (Gazit *et al.*, 2004). Another example comes from *Xenopus*, where the *Esr10* expression is dependent on the direct binding of NGN1 to the promoter of *esr10* (Lamar and Kintner, 2005).

Table 7 Proneural proteins may activate directly the expression of *hes* genes.

Proneurals directly activate *hes* genes	References
Ac-Sc → *E(spl) m7*, *E(spl) m8*, *E(spl) m□*	Kramatschek *et al.*, 1994; Singson *et al.*, 1994; Oellers *et al.*,1994; Cooper *et al.*, 2000; Castro *et al.*, 2005; Cave *et al.*, 2005
L'Sc/Da → *E(spl) m8*	
MASH-1 → *hes5*	Gazit *et al.*, 2004
NGN1 → *esr10*	Lamar and Kintner, 2005

These interactions between proneural and *hes* genes during neurogenesis reveal the existence of two conserved and interconnected circuits (Fig.14):

- An inter-cellular circuitry mediated by Notch signalling, where proneural proteins activate *hes* expression in a non-cell autonomous manner, thereby contributing to inhibit neighbouring cells from embarking on neuronal differentiation.
- An intra-cellular circuitry, where proneural proteins cell-autonomously activate *hes* expression. In addition, one should also consider the existence of positive auto and cross-regulation of proneural genes, within the same cell.

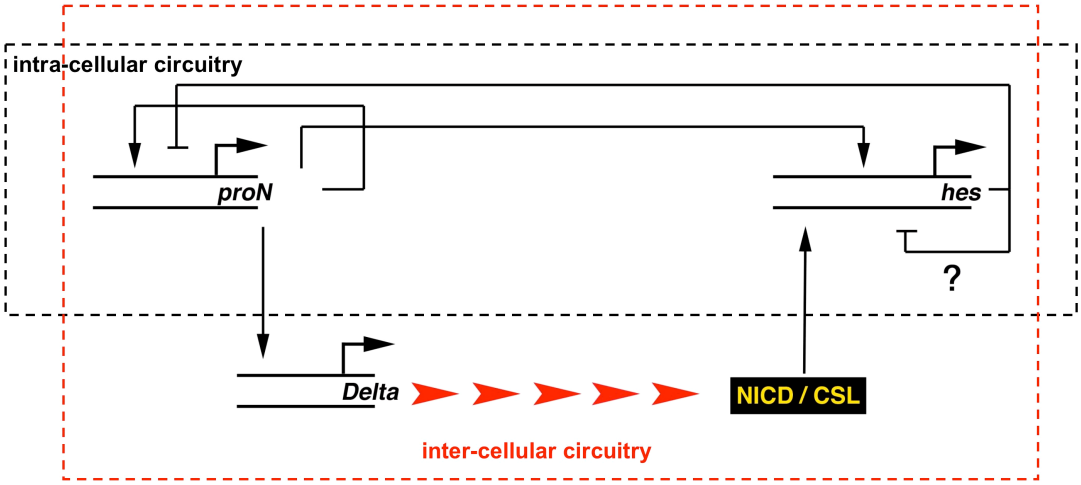


Fig.14 The Neurogenesis 'syntagm'. The controlled production of neurons relies on the balance between the activity of proneural bHLH activators and bHLH HES repressors, which is mediated by Notch signalling.

What is the logic of this circuitry with counteracting activities (proneural proteins activating expression of their own repressors) is still not known.

By making an analogy with an electrical circuit, Meir and colleagues proposed that the *proneural/hes* intra-cellular loop could be working as a homeostat, reducing proneurals levels and thereby working as a buffer and reducing the sensitivity to developmental noise (as stochastic changes in transcription or translation rates). This design would prevent the network from switching individual cells ON or OFF by noise, leading to a new state-a neither ON-nor-OFF steady state, which would delay the ON or OFF switch until some extrinsic cue forces the system to choose one of the states (Meir *et al.*, 2002).

In summary, during neurogenesis, a number of feedback-loops are operating to control the neural cell-fate decision, revealing a complex genetic circuitry that fits the definition for developmental 'syntagm' (Huang, 1998), which include both proneural and neurogenic genes.

1.5. AIMS AND SCOPE OF THIS THESIS

The aim of this work is to study the molecular events downstream of Notch signalling that are used to control the production of neurons during vertebrate embryonic development. I have concentrated on the *hes* genes, which are the best-characterized Notch targets and effectors, however many questions remain open regarding their general regulation and function.

In the mouse, four *hes* genes (*hes1, hes3, hes5* and *hes6*) are expressed in the neural tube, but only one – *hes5*, seems to be a Notch target *in vivo* (de la Pompa *et al.*, 1997; Lutolf *et al.*, 2002). Nevertheless, deletion of *hes5* does not phenocopy the loss of *Notch1* during neurogenesis, and only the combined inactivation of *hes5* with other two *hes* genes, *hes3* and *hes1,* results in a complete elimination of the progenitor pool (Hatayama, 2004) – a phenotype expected for the complete loss of Notch signalling (Lewis, 1998). This raises the possibility that other *hes* genes could also participate in Notch signalling during neurogenesis, and that this regulation by Notch might have been masked by cross-regulations between the *hes* genes. Supporting this idea, it has been shown that HES6 acts as a negative regulator of HES1 (Bae *et al.*, 2000; Koyano-Nakagawa *et al.*, 2000; Gratton *et al.*, 2003), but whether it interacts with the other *hes* genes, or if the other *hes* genes interact with each other, remains an open question.

Moreover, in the context of somitogenesis, it has been shown that *hes* genes have the capacity to negatively regulate their own expression (Hirata *et al.*, 2002; Bessho *et al.*, 2003). This negative feedback allows the generation of an oscillatory expression of these genes with the same periodicity as somite formation, being essential for the process of somitogenesis (reviewed in Dubrulle and Pourquie, 2004). However, during neurogenesis, it remains an open question whether the *hes* genes have this auto-regulatory and oscillatory behaviour.

To tackle these questions and provide a better understanding of the molecular events downstream of Notch signalling that control neuronal production, I started by characterizing the *hes*-like genes in the chick, which at the time of the beginning of this thesis had not been identified.

The chick embryo provides an amenable tool to study neurogenesis during embryonic development. Techniques like *in ovo* electroporation of the neural tube enable the

misexpression of full length/truncated proteins, as well as labelling and *in vivo* tracking of individual cells with fluorescent-tagged proteins. Moreover, several markers for the different steps of neuronal differentiation are also available.

This Thesis is organized in five Chapters:
In Chapter I, I present a general Introduction.

In Chapter II, I describe the methodology used in this work.

In Chapter III, I present the pattern of expression of four chick *hes* genes in the neural tube: three *hes5*-like genes (*hes5-1, hes5-2* and *hes5-3*) and one *hes6*-like (*hes6-2*). Then, I describe the studies aimed at investigating their function, specifically whether they are Notch targets and effectors during neurogenesis and how they regulate this process. Next, I describe the work done to analyse the regulatory relationships between the different *hes* genes, which revealed an intricate circuitry of negative auto and cross regulations among them. These regulatory interactions lead to the proposal that the HES5/HES6 circuitry of negative cross-regulations is a conserved feature of the Notch pathway, which would contribute to shut down the pathway after each Notch activation event. This work led to a model where neural progenitors go through successive Notch activation events until they finally differentiate.
Note that most of the results presented in this Chapter have been published in the paper: **Fior** and Henrique (2005). A novel *hes5/hes6* circuitry of negative regulation controls Notch activity during neurogenesis. *Dev Biol.* May 15;281(2):318-33 (Appendix).

Next, in Chapter IV, I describe the work done to determine if pulses of Notch activity indeed occur in neural progenitors and to study the dynamics of *hes5-1* gene expression. To achieve this, a real-time imaging system was designed to analyse *hes5-1* expression with single-cell resolution during neurogenesis, using the *hes5-1* promoter fused to a destabilized fluorescent protein. I show that the reporter recapitulates the endogenous pattern of *hes5-1* and responds to Notch signalling, providing a powerful tool to monitor Notch activity in real time. Analysis of time-lapse imaging of the

reporter revealed a dynamic gene expression pattern suggestive of pulses of Notch activity and oscillations of *hes5* expression.

Finally, in Chapter V, I present a general discussion of the whole work and discuss and explore the model that has been developed during the course of this work.

Chapter II

Materials and Methods

Materials and Methods

II.1 Preparation and transformation of competent *E. coli* bacteria

Preparation of chemically competent bacteria was based on Hanahan (1983). The bacterial strains of *Escherichia coli* used were DH5α and DH5T. Cultures of these bacterial strains were made competent for transformation with plasmid DNA by treatment with calcium chloride ($CaCl_2$). A single colony was placed in 10 ml of LB medium and shaken at 37 °C overnight. The overnight culture was inoculated in 400 ml of LB and shaken at 37 °C until an $OD_{600\ nm}$ of 0.6/0.8. After cooling to 4 °C, the culture was centrifuged for 15 minutes (min) at 4000 rpm. The pellet was re-suspended in 100 ml of a cold solution consisting of 30 mM KCH_3COO : 50 mM $MnCl_2$: 10 mM $CaCl_2$: 100 mM KCl : 15 % glycerol and then centrifuged at 4000 rpm for 8 min. The bacteria were again re-suspended in 20 ml of a second cold solution consisting of 10 mM NaMops (pH 7): 75 mM $CaCl_2$: 10 mM KCl : 15 % glycerol and then frozen as 0.5 ml aliquots in liquid nitrogen and stored at -80°C.

Plasmid transformation of competent bacteria

Frozen aliquots of competent cells were thawed on ice. Plasmid DNA (0.01-0.8 μg) was incubated with 150 μl of cells on ice for 30 min. The cells/DNA mix was heat-shocked for 120 seconds (sec) at 42 °C and then incubated on ice for 2 min. 600μl ml of SOB solution was added to the mix, which was then incubated with shaking at 37 °C for 45 min. The mix was centrifuged for 30 sec and 600 μl of solution were removed. The cells were re-suspended in the remaining volume and plated on appropriate selective LB agar media and incubated at 37°C overnight.

Plasmid DNA purification

For small scale preparation of plasmid DNA, 2 ml of a 3 ml overnight bacterial culture of transformed competent cells, in the appropriate selective LB medium, was

processed using the *Wizard® Plus SV Minipreps DNA Purification System* (Promega), according to the manufacturer's instructions.

For large scale preparation of plasmid DNA, 200 ml of the appropriate selective LB medium was inoculated with 0.5 ml of plasmid bacterial culture and shaken at 37 ºC overnight and processed using the *Concert High Purity Plasmid Midiprep System* (GibcoBRL) according to the instructions of the manufacturer.

DNA and RNA quantification

The concentration of DNA and RNA was determined by spectrophotometry using GeneQuant*pro* or Nanodrop spectrophotometer. One A_{260} unit corresponds to 50 μg/ml of double stranded DNA and to 35 μg/ml of single stranded RNA (Sambrook *et al.*, 1989). The purity of the nucleic acid preparation was estimated by the ratio between the readings obtained at 260 nm and 280 nm (pure preparations of DNA and RNA show ratio values of 1.8-2.0).

Restriction digestions and ligation reaction

Enzymatic restriction of DNA was preformed for approximately 1hour using 5-10U of commercially available restriction enzymes and respective buffers (Promega, Roche, Fermentas, New England Biolabs). The volume of enzyme used in each reaction never exceeded 10 % of the total reaction volume.

Ligation reactions were carried out in a final volume of 10 μl, for a total DNA amount of 0.5 μg. The ligation reactions were performed overnight, at 4 ºC for blunt-end ligations and at 15 ºC for cohesive-end ligations, using 1 U of T4 DNA ligase (Promega) and the suitable ligation buffer.

Analysis and isolation of DNA by agarose gel electrophoresis

To separate and estimate the size of DNA fragments, agarose gel electrophoresis was carried out. Gels were prepared by heating agarose (Invitrogen) until complete dissolution in 1x TAE buffer. The final agarose concentration depended on the size of the DNA fragments to be resolved: 1.2 % (w/v) for <1 kb and 1.0 % (w/v) for 1-10 kb. DNA was visualized by the addition of ethidium bromide (GibcoBRL) to the gel to a final concentration of 0.2 μg/ml. DNA samples were mixed with 1x DNA Loading buffer (15 % Ficoll (v/v): 10 mM EDTA with traces of OrangeG - Sigma) and

electrophoresis was carried in 1x TAE buffer at 5 to 10 V/cm of gel length until the desired resolution was achieved. DNA was visualized under an ultraviolet light at 340 nm and the size of the fragments was estimated by comparison with linear DNA strands of known molecular weight (*1 kb Plus DNA ladder* - Invitrogen). The region of the gel containing the DNA fragment of interest was excised under ultraviolet light at 365 nm and purified using the *NucleoSpin®* kit (Machery-Nagel), following the manufacturer's instructions.

II.2 Anti-sense RNA probe synthesis

During the course of this work several Digoxigenin and Fluorescein-labeled RNA anti-sense probes were used for *in situ* hybridization on whole mount or cryostat sections of chick embryos.

Digoxigenin and Fluorescein-labeled RNA anti-sense probes were synthesized by T3 or T7 RNA polymerase, from plasmid templates containing the cDNAs of several genes (Table 1). Antisense RNA probes complementary only to the 3'UTR of the four chick *hes* genes were also synthesized (Table 1).

DNA template preparation

DNA template preparation was performed as follows: 10 μg of plasmid DNA was linearized in a final volume of 100 μl, using 50 U of the restriction enzyme, for 2 hours at 37 ºC. After confirmation of complete digestion, the reaction was interrupted by adding 5 μl of 10 % SDS (w/v) and 1 μl Proteinase K (20 mg/ml - Boehringer), incubating the sample at 55 ºC for 15 min. To exclude proteins, the DNA template was subjected to two phenol-chloroform extractions (5' centrifugation). And finally the DNA template was precipitated using etanol (2.5V) + NaCl (3M-1:20) at –20ºC overnight or 30' at –70ºC, and re-suspended in 50 μl of RNAse-free water.

Probe synthesis

Anti-sense transcripts were produced by using 1 μg of linearized plasmid DNA and 20 U of the appropriate RNA polymerase in the presence of 30 mM DTT, 1x DIG-NTP mix (1 mM ATP, CTP, GTP, 0.65 mM UTP and 0.35 mM DIG/FLUO-UTP), 40 U RNAsin (Promega) and 1x Transcription Buffer (Stratagene), in a final volume of

25□l. After incubating at 37 °C for 3 hours, the sample was precipitated by adding 20.5 □l of RNAse-free water, 2 □l of 0.5 M EDTA (pH 0.8), 2.5 □l of 8 M LiCl and 150 □l of ethanol, incubating for 1 hour at -20 °C. After centrifugation, the RNA precipitate was washed with ethanol, re-suspended in 100 □l of 10 mM EDTA and, after brief denaturation at 70 °C for 15 min, checked by agarose gel electrophoresis.

The templates used in this thesis for *in vitro* transcription reactions are listed in Table1.

Table 1 Constructs used as templates for *in vitro* transcription reactions. EST clones were supplied by the MRC GeneService (Cambridge, UK) and RIKEN Institute (Japan).

Probe	Linearization site	RNA polymerase	References
hes5-1	*NotI*	T3	RIKEN ESTB6
hes5-2	*NotI*	T3	RIKEN EST F5
hes5-3	*NotI*	T3	RIKEN EST B7
hes6-2	*NotI*	T3	RIKEN EST
hes5-1-3'UTR	*stuI*	T3	
hes5-2-3'UTR	*AccI*	T7	RM
hes5-3-3'UTR	*XhoI*	T7	3'UTRhes5-3@pKS
hes6-2-3'UTR	*pstI*	T3	
hes5-1 cod.region	*NotI*	T3	hes5-1cr@pKs
Delta-1	*NotI*	T3	3kb template Henrique, 1995
Notch	*BamHI*	T3	Clone12
Ngn-1	*EcoRI*	T7	
Ngn-2	*HindIII*	T3	Gift from Nakafuku
NeuroD	*EcoRI*	T3	
NeuroM	*HindIII*	T3	
VNP	*BamHI*	T3	VNP@pKS
Delta4	*SstI*	T3	Barros *et al.*, unpublish

II.3 Oligonucleotides

The oligonucleotides used for PCR during the course of this work are listed Table 2. All the oligonucleotides (primers) were synthesized by Sigma Genosys.

II.4 Polymerase Chain Reaction (PCR) and related methods

Standard PCR

To produce inserts for cloning the several DNA vectors, PCR primers were designed for the specific target sequence on the insert DNA.

Reactions were prepared in a final volume of 25 μl (1 ng template plasmid DNA, 1x PCR Buffer with 1.5 mM $MgSO_4$, 0.3 mM dCTP, 0.3 mM dGTP, 0.3 mM dATP, 0.3 mM dTTP, 2.5 U PFU DNA polymerase – Stratagene – and 1 nmol of each primer). Amplification was performed with an initial heating at 94 ºC for 1 min, denaturation step at 94 ºC for 30 sec, followed by 25 cycles at 94 ºC for 30 sec, annealing 52 ºC for 120 sec, 72 ºC for 2 min. This general cycling program was adjusted for each primer and template set.

Table 2 Oligonucleotides used during the course of this work

Oligonucleotide	Oligonucleotide sequence
cHes5-1-D-EcoRI	5'CGGAATTCATAATGGCACCCAGCGCTCT3'/GGT3'
cHes5-1-R- EcoRV	5'CCGGATATCCTACCAAGGCCTCCAGAGGT3'
cHes5-2-D- EcoRI	5'CGGAATTCTGGAATGGCTCCCA3'
ches5-2-R- XbaI	5'GCTCTAGACTACCAGGGTCTCCA3'
cHes5-3-D- EcoRI	5'CGGAATTCGAGATGGCTCCCAGCTGGTT3'
cHes5-3-R- EcoRV	5'CCGGATATCCTA CCAGGGCCTCCAGAGA3'
cHes6-2 FL-ATG-RI	5'CGGAATTCGCCACAATGACGGCCGCAG3'
cHes6-2 FL-R-EcoRI	5'CGGAATTCCGCATTTTACCATGGT3'
cHes5-1 BamHI DN	5'CGGGATCCGGGCATTGCTTGTGACC3'
cHes5-2-2 R DN3	5'CGGGATCCGGTATGAGCTGCTG3'
cHes5-3 BglII DN	5'GGAAGATCTAGCTGATTGGCTCTTCTG3'
cHes6-2-R-DN-BamHI	5'CGGGATCCGGGCTGCAGGACCT3'
Pches5-1-pdR1	5'CGG GAT GCA CAC TAG GGA CAC TCC 3'
PcHes5-1-pRpstI	5' CCT GTG CCA GCT GCA GTC AGC CTG 3'
Pches5-1-pDpstI	5' CAG GCT GAC TGC AGC TGG CAC AG 3'
Pches5-1-pRNcoI	5' CGC TGG GTG CCA TGG TCC GAG AGC 3'
P1-Hes5DXhoI	5'CCGCTCGAG GCA CAC TAG GGA CAC TCC AGG G 3'
P2-Hes5R	5' CAT TAT CCG AGA GCT GCT GTC AGC 3'
P3-VNP-D tailP	5' GCT GAC AGC AGC TCT CGG ATA ATG GTG AGC AAG GGC GAG GAG CTG TTC
P4-VNP-RXbaI	5'CTAGTCTAGA GCG GCC GCA CTA GTG ATC TAC AC3'
P5-3'UTR-D-XbaI	5' CTAGTCTAGA GCC AAG AGC ACG CTC ACC ATC AC
P6-3'UTR-R-HindIII	5' GAT AAG CTT GAT ATC GAA TTC CTT CTC

Cycle sequencing

The DNA samples to be sequenced were processed according to a protocol provided by the Genomics Unit at Instituto Gulbenkian de Ciência. 4μl of Terminator Ready Reaction Mix (supplied by Genomics Unit), 750 ng of double stranded DNA and 5 pmol of the desired primer were mixed in a final volume of 10 μl and submitted to a PCR reaction. Cycle sequencing was performed with the following conditions: a denaturation step at 96 ºC for 1 min, followed by 25 cycles at 96 ºC for 10 sec, 50 ºC for 5 sec, 60 ºC for 4 min and a final step at 4 ºC until ready to precipitate. The samples were ethanol precipitated and the dry pellets sent to Genomics Unit (IGC) for

analysis with ABI PRISM 377 DNA Sequencer Applied Biosystem. Alternatively, samples were sequenced in Genomed at the Instituto de Medicina Molecular.

II.5 DNA constructs generated for functional studies

Several plasmids constructs were generated to do the functional studies performed during the course of this work (Table 3). Plasmid constructs to express the chick HES proteins and NICD were cloned in pCAGsIRES-GFP. The pCAGsIRES-GFP plamid is derived from the pCAGs vector (Niwa *et al.*, 1991) but contains an additional IRES (Internal-Ribossomal-Entry-Site)-GFP cassette downstream of the polylinker, which is followed by a stop codon in each frame (Bekman, E. and Henrique, D., unpublished) allowing coupled expression of the proteins of interest with GFP. The vectors pCIG and X-Su(H)DBM@pCIG were kindly provided by Dr. Andy McMahon (Megason and McMahon, 2002). The pCAGsMCS vector was also used- this is a pCAGS vector that was modified by E.Beckman to contain the MCS of pKS. The nRFP (nuclear Red Fluorescent Protein) used in this work is inserted in a pCAGs backbone and was kindly provided by Dr. Schordr. The commercially available bacterial cloning vectors pBluescript II KS (Stratagene) was used for sub-cloning procedures.

Table 3 Constructs generated for the functional studies.

construct	plasmid
HES5-1	pCAGsIRESGFP
HES5-2	pCAGsIRESGFP
HES5-3	pCAGsIRESGFP
HES6-2	pCAGsIRESGFP
hes5-3-3'UTR	pKS
HES5-1 coding region	pKS
HES6-2VP16	pCAGsIRESGFP
NICD	pCAGsIRESGFP
rNGN-1	pCIG
rNGN-2	pCIG
NICD	pCAGsMCS
VNP	pKS
VNP	pCAGs
P(2kb)hes5-1	pKS
3'UTRhes5-1	pKS
Phes5-1VNP	pKS
Phes5-1VNP3'UTR	pCAGs backbone
Phes5-1VNP3'UTR-polyA	pCAGs backbone

Molecular cloning strategies

HES5-1

The *hes5-1* full-length sequence was obtained by Pfu PCR using the primers cHes5-1D/cHes5-1-R (Table 2) from a template EST(B6). The 473bp PCR fragment was digested with *EcoRI/EcoRV* and cloned at *EcoRI/EcoRV* site of pCAGsIRES-GFP vector.

HES5-2

The *hes5-2* full-length sequence was obtained by Pfu PCR using the primers cEspl-2 ATG Eco/cEspl-2WXbA from a template EST(F5). The 500bp PCR fragment was digested with *EcoRI* and phosphorilated with a Kinase (Pfu makes blunt ends) and cloned at *EcoRI/EcoRV* site of dephosphorilated pCAGsIRES-GFP vector.

HES5-3

The *hes5-3* full-length sequence was obtained by Pfu PCR using the primers cHes5-3-D/cHes5-3-R from an EST(B7) template. The 490bp PCR fragment was digested with *EcoRI/EcoRV* and cloned at *EcoRI/EcoRV* site of pCAGsIRES-GFP vector.

HES6-2

The *hes6-2* full-length sequence was obtained by Pfu PCR using the primers cEspl-1-D-EcoRI/cEspl-1-R-EcoRI from a template EST cDNA. The 673 bp PCR fragment was cloned in *EcoRI* site of pKS vector. Finally the resulting vector was digested with *EcoRV/NotI* and cloned at *EcoRV/NotI* site of pCAGsIRES-GFP vector.

HES6-2-VP16

The WRPW domain of *hes6-2* was swapped by the VP16 activating domain. In order to obtain fragments lacking the WRPW domain reverse primers upstream of the WRPW domain were designed, which also had to be in frame with VP16 activating domain. A 640bp PCR fragment was obtained with the primers cEspl-1-D-EcoRI/cEspl-1-R-DN-BamHI that was cloned at *EcoRI/BamHI* site of pKS-. Then the cEspl-1 640bp *EcoRI/BamHI* fragment (from pKS) and the 240bp VP16 *BamHI/NotI* (obtained from m7VP16@pKS digested with *BamHI/NotI*) were cloned at the *EcoRI/NotI* site of pCAGsIRES-GFP vector.

hes5-3-3'UTR@pKS

A *HindIII* 1kb fragment that includes the 3'UTR of *hes5-3* was subcloned in the *HindIII* site of pKS vector, the resulting vector was linearized with *XhoI* and the probe was synthesized with T7 RNA polymerase.

hes5-1 coding region@pKS

A 500bp *NotI/StuI* fragment coding for the *hes-1* coding region (excluding the 3'UTR) was obtained by *NotI/StuI* digestion of the hes5-1 EST(B6) and cloned in *NotcI/EcoRV* site of pKS.

NICD@pCAGsIRESGFP

A *NotI* 2.2kb DNA fragment from cNicd-pYDF30 (Wakamatsu *et al.*, 2000) was subcloned in the pCAGsIRES-GFP vector.

NGN-1

The cDNA encoding the full-length rat NGN-1 protein *EcoRI* fragment (Mizuguchi *et al.*, 2001) was subcloned in the *EcoRI* site of pCIG.

NGN-2

The cDNA encoding the full-length rat NGN-2 protein *EcoRI* fragment (Mizuguchi *et al.*, 2001) was subcloned in the *EcoRI* site of pCIG.

NICD@pCAGs

A *NotI* 2.5kb DNAfragment from cNicd-pYDF30 (Wakamatsu *et al.*, 2000) was subcloned in the pCAGsMCS vector.

VNP@pKS

A 908bp PCR fragment coding for the VNP was obtained by PFU-PCR using as a template a plasmid kindly provided by Dr. Eli Shibler with the primers P3-VNP-D tailP and P4-VNP-RXbaI. This fragment was then inserted in the *EcoRV* site of pKS.

VNP@pCAGs

The 909bb PCR fragment coding for the VNP was obtained by *EcoRI/XbaI* digestion of the VNP@pKS, followed by a klenow treatment to blunt the ends and ligated to the pCAGsMCS digested with *EcoRV* and scored for correct orientation.

P(2kb)hes5-1@pKS

A 2kb DNA fragment containing the 2kb proximal promoter region of *hes5-1* was obtained by PFU PCR using as primers P1-Hes5DxhoI and P2-Hes5R and genomic DNA as template. This DNA fragment was then cloned in pKS digested with *EcoRV*, for sequencing.

***3'UTRhes5-1*@pKS**

A 1kb DNA fragment containing the 3'UTR of *hes5-1* was obtained by PFU PCR using as primers P5-3'UTR-D-XbaI and P6-3'UTR-R-HindIII and *hes5-1* EST as template. This DNA fragment was digested with *XbaI/HindIII* and cloned in *XbaI/HindIII* sites of pKS.

Phes5-1VNP@pKS

The 2Kb DNA fragment coding for the promoter region of *hes5-1* (Phes5-1) was ligated by PCR to the 1kb DNA fragment coding for VNP, in order to maintain the exact sequences that surround the ATG of *hes5-1*. For this, the two fragments used as templates were first obtained by PCR: the 2kb Phes5-1 fragment was obtained by PCR with the primers P1-Hes5DxhoI and P2-Hes5R using as template the P(2kb)hes5-1@pKS construct; and the VNP fragment was obtained by PCR using the primers P3-VNP-D tailP and P4-VNP-RxbaI and the template VNP@pKS. These two fragments were then purified and used as templates for the ligation PCR, using as primers- P1-Hes5DXhoI and P4-VNP-RXbaI. The 3kb fragment obtained was purified and digested with *XhoI/XbaI* and introduced in the corresponding sites in pKS.

Phes5-1VNP3'UTR

This vector was generated from a three-piece ligation: the *hes5-13'UTR* was ligated to Phes5-1VNP into the pCAGs backbone. The *hes5-13'UTR* DNA fragment was obtained by *XbaI/HindIII* digestion of the 3'UTRhes5-1@pKS. The Phes5-1VNP was

obtained by *XhoI/XbaI* digestion of the Phes5-1VNP@pKS vector. The pCAGs backbone was generated by *SalI/HindIII* digestion and the 2.5kb fragment, which excludes the CMV- β-actin promoter and the polyA was purified and used as the backbone vector. This backbone vector includes the replication origin ORI and the ampicilin resistance cassette.

Phes5-1VNP3'UTR-polyA

The 538bp DNA fragment coding for the rabbit β-globin polyA fragment was obtained by digestion of the pCAGsMCS vector with the restriction enzymes *EcoRV/HindIII*. This fragment was then inserted in the Phes5-1VNP3'UTR vector previously digested by the restriction enzymes *EcoRV/HindIII*.

II.6 In ovo chick embryo electroporation

Super-coiled plasmid DNA was injected into the neural tube of HH11–12 chicken embryos at a concentration of 2 μg/μl in PBS, with exception of the HES6-2, VNP @pCAGs and Phes5-1VNP constructs, which were used at 1 μg/μl and nRFP wich was used at 0,5μg/μl or 1μg/μl. Platinum electrodes (Nepagene CUY613G), distanced 4 mm between anode and cathode, were placed parallel to the neural tube, and embryos were pulsed 4 times (30 V/50 ms), using a Electro Square Poratori ECM830 (BTX). Embryos were incubated for 8h, 24 h or 48h and then harvested.

II.7 Preparation of cryostat sections

Chicken embryos were staged according to Hamburguer and Hamilton (1951), collected and fixed in 4 % paraformaldehyde:1x PBS, 2h at room temperature, or over-night at 4^0C. Embryos were then washed in 1x PBS, passed twice through a 15%sucrose solution for cryoprotection and finally embedded in 7.5 % gelatin/15%sucrose/PBS and frozen in cold isopenthane (-70^0C). Frozen embedded embryos were then cryosectioned in 12μm sections and were de-gelatinized and processed for immuno-histochemistry and *in situ* hybridization.

II.8 *In situ* hybridization

In situ hybridizations in whole-mount embryos

Chicken embryos were collected and fixed in 4% paraformaldehyde/PBS at 4^0C over night or for 2h at room temperature. Whole-mount *in situ* hybridizations were performed as described in Henrique *et al.*, 1995, with modifications.

In situ hybridizations on cryostat sections

For hybridization on cryostat sections, fixed embryos were cryoprotected in 15% sucrose in PBS, embedded in 7.5% gelatin/15% sucrose/PBS and cryosectioned (12μm). Hybridization on cryostat sections was done as previously described (Myat *et al.*, 1996), with modifications.

Double *in situ* hybridization on cryostat sections

Double in situ hybridization on cryostat sections was done with DIG- and fluorescein labeled RNA probes. The protocol was developed during the course of this thesis, and is as described:

1) Hybridisation with DIG + Fluo probes [use Fluo labelling for the weaker probe]

2) Post-hybridization washes | Blocking | Antibody anti-FluoAP 4^0C o.n as described in Myat *et al.*, 1996.

3) Staining reaction for AP with Fast Red

4) Wash with PBS 5x 5min

5) Blocking 2% BBR (Boeringer Blocking Reagent)

6) Antibody anti-DIGPOD 1:100 @blocking 2%BBR 4^0C o.n

7) Wash with TNT buffer [0,1M Tris-HCl, pH7,5; 0,15M NaCl; 0,05% Tween-20] 3x1h

8) Block Tyramide 30min

9) 1:50 Tyramide-FITC@ amplification buffer (kit), 5min

10) Wash with TNT pH=7.5,

11) Can repeat step 9)

12) DAPI staining

Kit TSA™-DIRECT (GREEN) NEN™ Life Science Products Perkin Elmer

II.9 Imunofluorescence

The cryostat sections of chick neural tubes were de-gelatinized and processed for immunohistochemistry as described previously (Ohno *et al.*, 2001).

Antibodies

Electroporated cells were visualized after in situ hybridization using a rabit-polyclonal antibody against GFP at 1:500 (AbCAM). The mouse anti-TUJ-1 antibody (Lee *et al.*, 1990) was kindly provided by A. Frankfurter (Univ. Virginia) at 1:500. The mouse anti-HU antibody is from molecular probes and was used 1:500.

Secondary antibodies used were Alexa 594-conjugated goat anti-rabbit (Molecular Probes), Alexa 594-conjugated donkey anti-mouse (Molecular Probes), Alexa 488-conjugated goat anti-rabbit (Molecular Probes).

II.10 BrdU labeling

24h after electroporation a 30 min pulse of 150□l of 12,5mg/ml BrdU was applied on top of the embryos. Four day (E4) or 48h after electroporation chick embryos were injected with 5□l of 12,5mg/ml BrdU into the veins and were collected 30min after injection. Embryos were fixed, criosectioned and taken through a 50%formamid 1xSSC incubation at 65^0C for 2 hours (when they were not subjected to *in situ* hybridization), rinsed, incubated in 2N HCl for 30min, 0,1M Tris pH8 2x10min, rinsed and stained using a mouse anti-BrdU antibody (1:1000; Sigma) and then as a secondary antibody a goat anti-mouse-IgG-Alexa594 (Molecular Probes) was used.

II.11 Fluorescence Imaging

Sections were analyzed using the fluorescence microscope Leica DMR, equiped with a Leica DC 350F digital camera or the laser confocal microscope Zeiss LSM510. Images were processed using the Adobe Photoshop software. For confocal imaging the sections were analyzed using the laser confocal microscope Zeiss LSM510. Images acquired in the confocal microscope were processed using the Zeiss LSM Image Browser and treated for noise reduction and color adjustments in Adobe Photoshop.

II.12 Live Imaging

Embryo slice preparation and culture

1) Embryos were electroporated with either Phes5-1VNP3'UTR-polyA alone (1□g/□l) or with VNP@pCAGs (1□g/□l) and incubated for an additional 4h or 24h.

2) Electroporated embryos were screened under a fluorescent dissecting stereo microscope (Leica MZFLIII) and selected the ones that showed fluorescence in the neural tube.

3) Embryos were then harvested and sectioned into 100 □m slices using a McILTwain Tissue Chopper (Mickle Laboratory Engineering). The slices were always handled with a pipette with medium HAM'S F12 SUP [HAM'S F12 supplemented with Fungizone (F) 1%+ Glutamine(G) 2%+ piruvateNa (P) 1%+ Penicillin/streptomycin (P/S)(Gibco)1%] and transferred to a Petri dish containing the same medium.

The slices that presented better fluorescent intensity and isolated cells were selected under the inverted microscope and then harvested in a Petri dish with medium HAM'S F12 SUP AG [HAM'S F12 SUP supplemented with agarose1% pre-heated at 40^0C]. Slices are embedded in this pre-heated medium with agarose and then transferred to a new Petri dish with a central hole surrounded by solidified medium (HAM'S F12 SUP AG). The slices must be well spread in the central bottom of the Petri dish. After solidification of the agarose ~5ml of medium HAM'S F12 SUP was added, followed by mineral oil until all surface of the Petri dish was covered in order to avoid evaporation of the medium.

Slice imaging

4) Slices were imaged in confocal Zeiss LSM510 microscope, with a 20x objective (Plan-NeoFluor; NA0.5) in a 37^0C humid chamber. Forty-two optical sections (z stacks) with 3□m step size were imaged at 10.4 min intervals up to 12h.

5) The fluorescent images were subjected to the data analysis as follows: The individual fluorescent cells were identified manually, and the stack with higher fluorescent signal was followed manually throughout the time-lapse series to create the movies. The movies were then assembled using Image J software. To quantify fluorescent intensity, the region of interest (ROI) was defined as the center of the nuclei and the fluorescent intensity signal was measured using Image J software. ImageJ was also used to convert image sequences to QuickTime movies.

II.13 Controls and statistical analysis

For each construct, a minimum of 5 electroporated embryos were analyzed by *in situ* hybridization and immunofluorescence, with at least 10 sections from each embryo scored for phenotypes. Images presented in figures are representative of each experiment. Controls were done by electroporating the pCAGsIRES-GFP vector alone and no alterations in gene expression were observed, with any of the probes here described. Statistical analysis of results presented ChapterIII-Fig.15 was done using the Student's *t* test (paired).

Chapter III

A novel *hes5/hes6* circuitry of negative regulation controls Notch activity during Neurogenesis

Most of the results presented in this chapter have been published in the paper:

Fior and Henrique (2005). A novel *hes5/hes6* circuitry of negative regulation controls Notch activity during neurogenesis. *Dev Biol.* May 15; 281(2):318-33

III.1 ABSTRACT

HES transcriptional repressors are important components of the Notch pathway regulating neurogenesis, from *Drosophila* to vertebrates. These proteins are normally induced by Notch activity and inhibit neural commitment by antagonizing the activity of proneural genes. In this work, four chick *hes* genes that are expressed during neurogenesis are described; three *hes5*-like genes (*hes5-1, hes5-2* and *hes5-3*) and one *hes6*-like (*hes6-2*). I show that HES6-2 represses transcription of the *hes5* genes, thus functioning as a negative regulator of Notch signalling. Conversely, *hes6-2* is repressed by HES5 activity. In cells committing to differentiation, *hes6-2* is up-regulated by proneural genes and contributes to the proneural program of neuronal commitment by preventing Notch activity in these cells. In neural progenitors, Notch signalling produces an initial burst of HES5 activity, which represses *hes6-2*. However, as *hes5* transcription declines due to negative auto-regulation, *hes6-2* may become active and inhibit the remaining *hes5* expression to end Notch signalling. These cells can then enter a new cycle of fate decisions and will be kept as progenitors if a new pulse of Notch activity occurs. Maintenance of progenitors during vertebrate neurogenesis therefore requires that these cells go through successive pulses of Notch activity. This work proposes that the *hes5/hes6* circuitry of negative cross-regulations is a conserved feature of the Notch pathway that underlies these cycles in neural progenitors.

III.2 INTRODUCTION

A conserved feature of the genetic circuitry regulating neurogenesis, in animals as different as flies and mammals, is the antagonism between two different sets of basic-Helix-Loop-Helix (bHLH) proteins. Proneural proteins of the Achaete-Scute and Atonal/Neurogenin families play a positive role in promoting the commitment of a cell to a neural fate, while bHLH-Orange (bHLH-O) proteins from the Hairy and Enhancer of Split (*hes*) family repress this cell fate decision. The balance between the activity of these two sets of bHLH proteins and, therefore, the final fate of the cell is dictated by a cell-cell communication system known as lateral inhibition, mediated by the Notch receptors and their ligands Delta/Serrate (reviewed by Campos-Ortega, 1994). In *Drosophila*, where this system was first studied, proneural proteins are expressed in groups of ectodermal cells, called proneural clusters, which thereby

acquire the potential to follow a neural fate. Although each cell in a group has an equivalent potential, only one of them becomes neural and inhibits its neighbours from adopting a similar fate, by activating the Notch pathway in the latter cells. Notch activation leads to transcriptional up-regulation of genes encoding HES proteins, which suppress the activity of the proneural genes and, thereby, keep these cells uncommitted. In this way, one cell in the equivalence group realizes its neural potential and ensures that other cells are prevented from doing so.

This basic mechanism has been well conserved during animal evolution and controls the development of a great variety of cell types, not only of neural cells (reviewed by (Artavanis-Tsakonas, 1999; Lewis, 1998). In recent years, the molecular details of the Notch signalling pathway has been the focus of intense research, and regulation at different levels of the pathway have been shown to have important contributions to its final outcome (reviewed by Schweisguth, 2004; Lai, 2004). Despite its complexity, a unique feature of the Notch cell-cell communication system is that it mediates a simple binary decision, ensuring that cells acquire one of two alternative fates, the nature of which depends on the embryonic context and developmental time. This unique feature is based on the robust design of the Notch pathway, at the core of which an inter-cellular feedback loop functions to amplify small differences in the potential of the cells, leading invariably to a distinct outcome in each of them.

In *Drosophila*, the bHLH-O proteins that mediate Notch activity in responding cells are encoded by the *Enhancer of split* Complex [*E(spl*-C], which contains seven genes clustered in a single 60 kb complex and seem to have mostly overlapping functions (Bray, 1997; Knust *et al.*, 1992). *Drosophila* contains other genes encoding bHLH-O proteins, like *hairy* and *deadpan*, which regulate neural development independently of Notch signalling.

The HES family of bHLH-O proteins are characterized by the presence of a conserved proline at the basic region and a WRPW tetrapeptide at the carboxy-terminus, which was shown to interact with the co-repressor encoded by the *groucho* gene (Paroush, 1994). Another domain, located after the bHLH region, was also found to be conserved among HES proteins and named Orange domain (Dawson *et al.,* 1995), being important for the specificity of protein-protein interactions between various

HES proteins. These proteins are known to be DNA-binding transcriptional repressors and the recognition sequences to which they bind have been characterized (N-boxes and E-boxes), being different from the E-boxes recognized by proneural proteins (Davis and Turner, 2001). The main mechanism of transcriptional repression by HES proteins is based on the WRPW-mediated recruitment of Groucho, which interacts with and inhibits the transcriptional machinery. HES proteins might also block the activity of the proneural bHLH proteins by direct protein-protein interaction, forming heterodimers that are unable to promote neural commitment. Therefore, different mechanisms can be used by the HES proteins to counteract the activity of the proneural proteins during neurogenesis.

The first *hes* genes described in vertebrates were a homologue of *hairy* and a homologue of the *E(spl)* genes, which were named *hes1* and *hes3*, respectively (Sasai *et al.,* 1992). Several other vertebrate genes of the *hes* family have since then been described, some of which were shown to participate in Notch signalling during neurogenesis (reviewed in (Davis, 2001). However, the general regulation of *hes* gene function during neural development is still poorly understood. In the mouse, for instance, four *hes* genes are expressed in the developing neural tube (*hes1*, *hes3*, *hes5* and *hes6*), with distinct but partially overlapping patterns. Only *hes5* has been shown to be directly regulated by the Notch pathway (de la Pompa *et al.*, 1997; Lutolf *et al.*, 2002), but its deletion does not phenocopy Notch inactivation during neurogenesis (Ohtsuka *et al.*, 1999). In addition to *hes5*, two other *hes* genes (*hes1*, *hes3*) have to be inactivated to cause complete elimination of the neural progenitor pool and premature neuronal differentiation (Hatakeyama *et al.*, 2004), as expected for a total absence of Notch signalling (Henrique *et al.*, 1997). Since neither *hes1* nor *hes3* are direct targets of Notch signalling in the neural tube, this apparent redundancy in *hes* function raises the question of whether *hes1* and *hes3* normally function as Notch effectors in the neural tube, and how they interact with *hes5* to control neurogenesis. The *hes6* gene might participate also in this network of *hes* genes, as it was shown to act as a negative regulator of *hes1* (Bae *et al.*, 2000; Koyano-Nakagawa *et al.*, 2000; Gratton *et al.*, 2003). Whether it interacts also with *hes3* and *hes5* and how these interactions contribute to the Notch pathway's function during neurogenesis remains to be known.

In this Chapter, I address the regulation and function of *hes* genes in the developing spinal cord of the chick embryo and how they participate in the cascade of events in the Notch pathway that regulate neuronal production. This work shows that a series of interactions between the *hes5* and *hes6* genes, and of those with the proneural genes, are important to control different steps along neural development. In particular, I show that the Notch targets and effectors *hes5* genes are transcriptionally repressed by the product of the *hes6-2* gene, which may function as a negative regulator of Notch activity, both in neural progenitors and nascent neurons. I propose that this *hes5/hes6* circuitry of negative regulation is a key mechanism to ensure a proper modulation of Notch activity throughout neurogenesis.

III.3 RESULTS

III.3.1 The chick genome contains three *hes5* and two *hes6* homologues

In this work 5 new members of the chick *hes* gene family were studied. Three of these genes encode highly related proteins with strong homology to the mammalian HES5 proteins and were named *hes5-1*, *hes5-2* and *hes5-3*. The other two genes encode proteins with homology to mammalian HES6 and were therefore named *hes6-1* and *hes6-2*, being *hes6-1* the one with higher homology to mammalian HES6 (Fig 1). However, the chick *hes6-1* gene is not expressed at early stages of neurogenesis and was not studied further.

Full-length cDNAs encoding the three chick *hes5* genes (*hes5-1*, *hes5-2* and *hes5-3)* and the two *hes6* genes (*hes6-1* and *hes6-2*) predict proteins of 157, 178, 154, 206 and 228 amino-acid residues, respectively, with all the structural features of the E(spl) subfamily of bHLH-O transcriptional repressors (Davis and Turner, 2001). The three chick HES5 proteins show a high degree of homology between each other in the bHLH region (around 80-95% identity), and to human HES5 (82 to 85% identity), but show more divergence in the Orange domain (48 to 66% identity between them and to hHES5) (see Table1). As the Orange domain confers specificity for protein-protein interaction (Dawson *et al.*, 1995), the three chick HES5 proteins may have slightly different properties.

The chick HES6-1 and HES6-2 proteins display only 56 and 52% identity with human HES6 in the bHLH domain, respectively, but have more than 60% identity at the

Orange domain, in which we could identify a signature sequence for the HES6 subfamily (GYIQCHEVH) (Fig. 1). The bHLH domain of the chick HES6 proteins, like those of mouse and human HES6, contains a shorter loop region when compared to the other HES proteins.

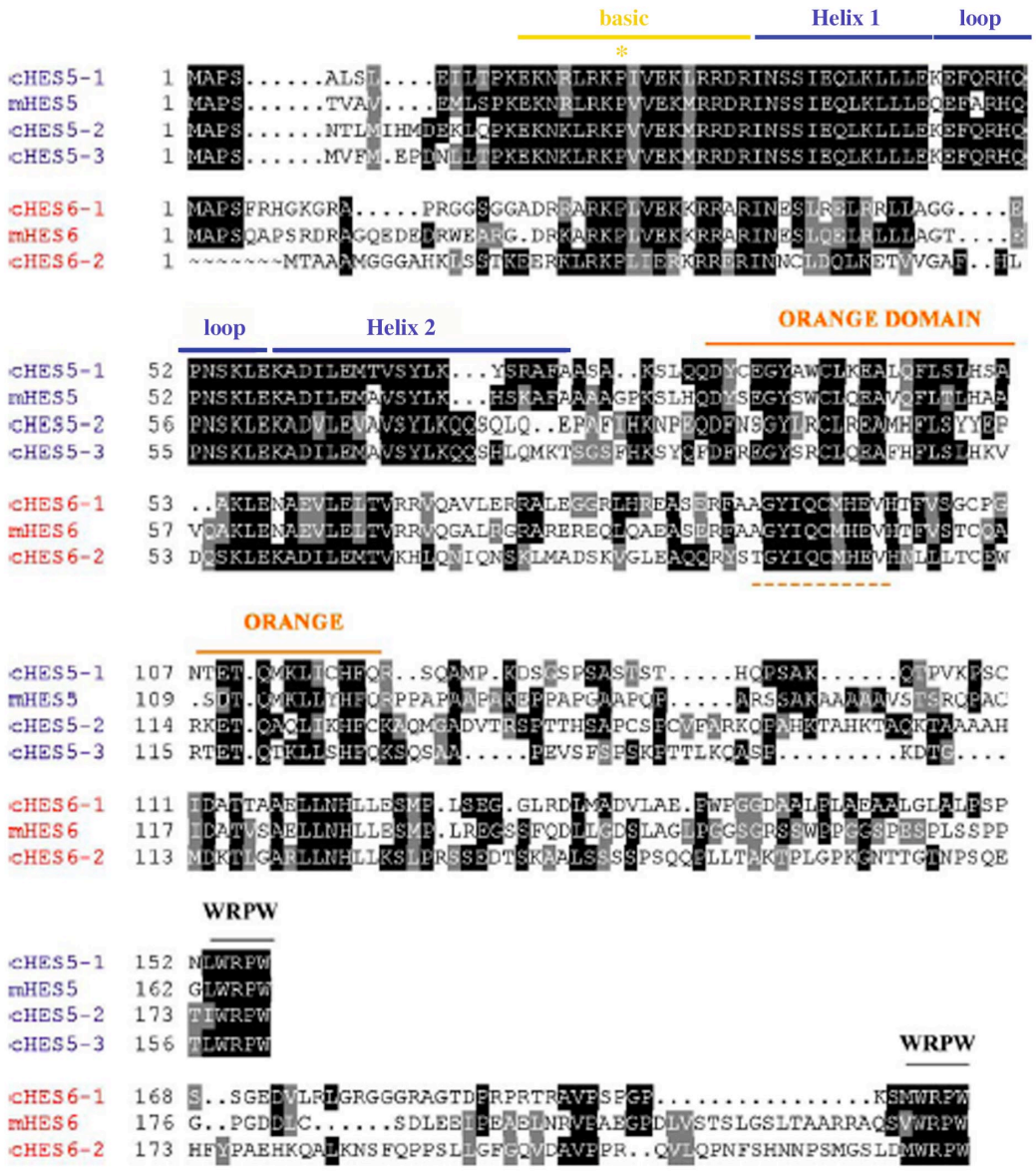


Fig.1 Four domains are evolutionarily conserved among the HES proteins. Sequence alignment of the conserved basic (yellow), Helix-loop-Helix (blue), orange (orange) and WRPW domains of chick HES5-1, HES5-2, HES5-3, HES6-1, HES6-2 and mouse mHES5 and mHES6 proteins. The orange dashed line represents the orange domain conserved motive of the HES6 family (GYIQCHEVH).

Table 1 Identity between the chick HES5 proteins and hHES5. WP-is the whole protein.

% identity bHLH | orange | WP	HES5-1	HES5-2	HES5-3	hHES5
HES5-1	100	80.6|48.5|49.4	83.9|60.3|62.7	83.9|66.7|54.8
HES5-2	80.6|48.5|49.4	100	95|57.6|56.7	82.3 |39.4|45.3
HES5-3	83.9|60.3|62.7	95|57.6|56.7	100	85.5 |60.6|50.2

Analysis of the recently available chick genome reveals that the protein-coding region of each chick *hes5* gene, like the mammalian counterpart, is encoded in 3 exons, whereas the other chick *hes* genes (*hes6, hairy1* and *-2*) contain 4 exons (Fig. 2). The 3 chick *hes5* genes are clustered on Chromosome 21, within a 20 kb region of DNA, adjacent to the *fang1* gene encoding the enzyme pantothenate kinase 4 (Fig. 2). In comparison, both the mouse, rat and human genomes contain only one *hes5* gene, however it is also linked to the *fang1* gene, revealing that this linkage has been conserved throughout evolution.

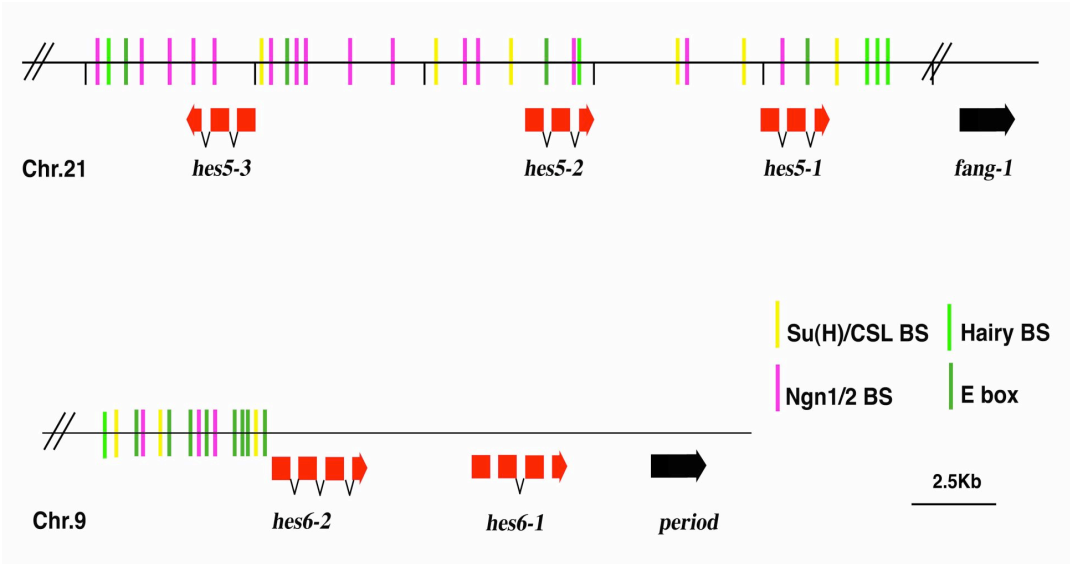


Fig.2 Genome organization of the chick *hes5* and *hes6* genes. The three chick *hes5* genes are clustered on Chr 21, within a 20Kb region of DNA, adjacent to the *fang1* gene. Chick *hes5* genes, like the mammalian counterparts, contain 3 exons, whereas *hes6* genes,like *hairy1* and *–2* contain 4 exons. The chick *hes6* genes (*hes6-1* and *hes6-2*) are also clustered in Chr 9 and in close proximity with the gene *period*. We also analysed the promoter regions for putative binding sites of several transcription factors: we could find several Su(H) binding sites [lower-affinity sequence TGTGTGAA, the net consensus consisted of the five octamers CGTGGGAA, CGTGAGAA, CGTGTGAA, TGTGGGAA, and TGTGAGAA]; several hairy boxes [CACGCG]; Neurogenin1/2 boxes [CAGATG]; E boxes all classes [CANNTG] and most abundant of all were the N boxes [CACNAG] .

The chick *hes6* genes (*hes6-1* and *hes6-2*) are also linked in Chromosome 9 and in close proximity with the gene *period*. In mouse and human only one *hes6* gene exists, however it is also linked to the gene *period* at chromosome 1 and 2, respectively.

III.3.2 The chick *hes5* and *hes6-2* genes are expressed in neural progenitors, but *hes6-2* is also expressed in nascent neurons

In the developing chick CNS, *hes5-1*, *hes5-2* and *hes5-3* transcripts are first detected at HH4-5 (stages according to Hamburger and Hamilton (Hamburger, 1992) in cells at the Caudal Neural Plate (CNP), adjacent and posterior to Hensen's node (Fig. 3A-I).

Previous fate map studies (Henrique *et al.*, 1997; Brown *et al.,* 2000; Mathis *et al.,* 2001) indicate that this region is a stem zone that contains the precursor cells of the caudal part of the CNS (reviewed in Diez Del Corral, 2004). The domain of *hes5* expression in the chick CNP is similar to that of the proneural gene *cash4* (Henrique *et al.,* 1997) and *Delta-1* and these genes continue to be expressed similarly in the caudal stem zone around the regressing Hensen's node, until primary neurulation ends.

It has recently been shown, that FGF signalling is required for expression of *cash-4* in this stem zone, which in turn induces uniform *Delta-1* expression in this proliferative cell group (Akai *et al.*, 2005). Notch activation in this cell population is thought to, together with FGF, maintain the proliferation capacity of this cell group, which constitutes the neural precursor pool that later generates the spinal cord (Akai *et al.*, 2005).

However, as neuronal progenitors leave the CNP to be incorporated in the forming neural tube, they seem to loose transiently *hes5* expression, leading to a gap in *hes5* expression in the transition from the CNP to the neural tube (Fig.3-green arrows).

In contrast to *cash4*, which is only expressed in the CNP (Henrique *et al.,* 1997), the chick *hes5* genes are also expressed in the neural tube, from the onset of neurogenesis. This second wave of *hes5* expression starts at HH6-7 (Fig. 3B,F,J-black arrow), in the neural tube region flanked by the first somite, coinciding with the appearance of the first individual *Delta1*-expressing newborn neurons in the same region (Henrique, 1995), where lateral inhibition starts to operate to maintain a pool of progenitors until the end of development.

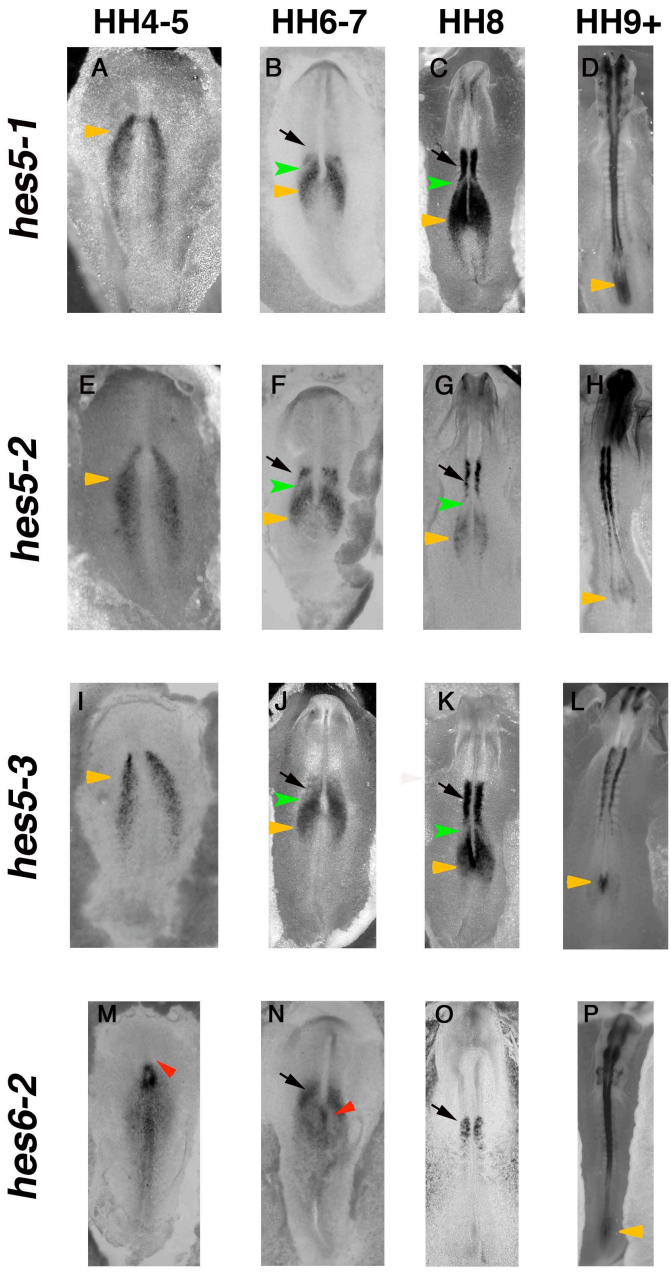


Fig.3 Expression pattern of the chick *hes* genes at early stages of neural development. At HH4-5, the three *hes5* genes show a very similar expression in the Caudal Neural Plate (CNP,yellow arrows) (**A,E,I**), while *hes6-2* starts to be expressed asymmetrically around Hensen's node (**M**, red arrow), showing also weak expression in the primitive streak and adjacent mesoderm (**M**). At HH6-7, all three *hes5* genes are strongly expressed in the CNP (**B,F,J,** yellow arrows) and start to be also expressed in the neural plate region flanking the first somite (black arrow), coinciding with the initial *hes6-2* expression in the same region (**N**, black arrow). However in the transition zone, there is a gap in the expression of the *hes5* genes (**B, F, J, C, G, K**-green arrow). Asymmetric expression of *hes6-2* is still present in the right side of Hensen's node (**N**, red arrow), and very weak expression can be detected also in the ectoderm around it. At HH8-9, the four *hes* genes are expressed throughout the neural tube (**C-P** black arrow) with exception of the hindbrain.

Expression of chick *hes6-2* starts also at HH6-7 in this region, overlapping with the second wave of *hes5* expression. The *hes6-2* gene is not expressed in the CNP stem zone but shows asymmetric expression around Hensen's node at HH5 (Fig.3M), perhaps reflecting the known Notch function during establishment of left-right asymmetry (Raya, 2004). Nevertheless, later, at HH9+ expression of *hes6-2* is also detected at CNP (Fig.3P).

As development proceeds, the expression of the four chick *hes* genes in the forming neural tube correlates well with the described spatio-temporal pattern of neurogenesis (Hollyday, 2001), being detected initially in the ventral spinal cord and later, as neurogenesis proceeds, expanding also dorsally. Transcripts of the four *hes* genes can be detected in the ventricular region where neural progenitors are located and *Notch1* is expressed, being absent from the mantle layer where differentiating neurons are accumulating (Fig. 4A-L). In agreement with their expression in neural progenitors, these *hes* expressing cells are mitotically active, as indicated by their incorporation of BrdU following a brief (30min) exposure (Fig.4M-P).

The chick *hes5-1* and *hes5-3* genes are expressed throughout the ventricular region (Fig.4A,G), spanning the whole dorso-ventral axis (excluding the floor plate), while *hes5-2* shows stronger expression in the ventral half of the neural tube (Fig.4E). Furthermore, double *in situ* hybridization for the *hes5* genes suggest that progenitor cells co-express the three *hes5* genes simultaneously (Fig. 5I).

Comparing the expression pattern of the *hes* genes with the 2 Notch ligands, *Delta1* and *Serrate1*, which have complementary expression domains in the neural tube (Myat *et al.,* 1996), the four chick *hes* genes are transcribed in both domains (Fig.4 /Fig.5), overlapping with *Notch1* across the entire D-V axis of the spinal cord (Fig.4A-L).

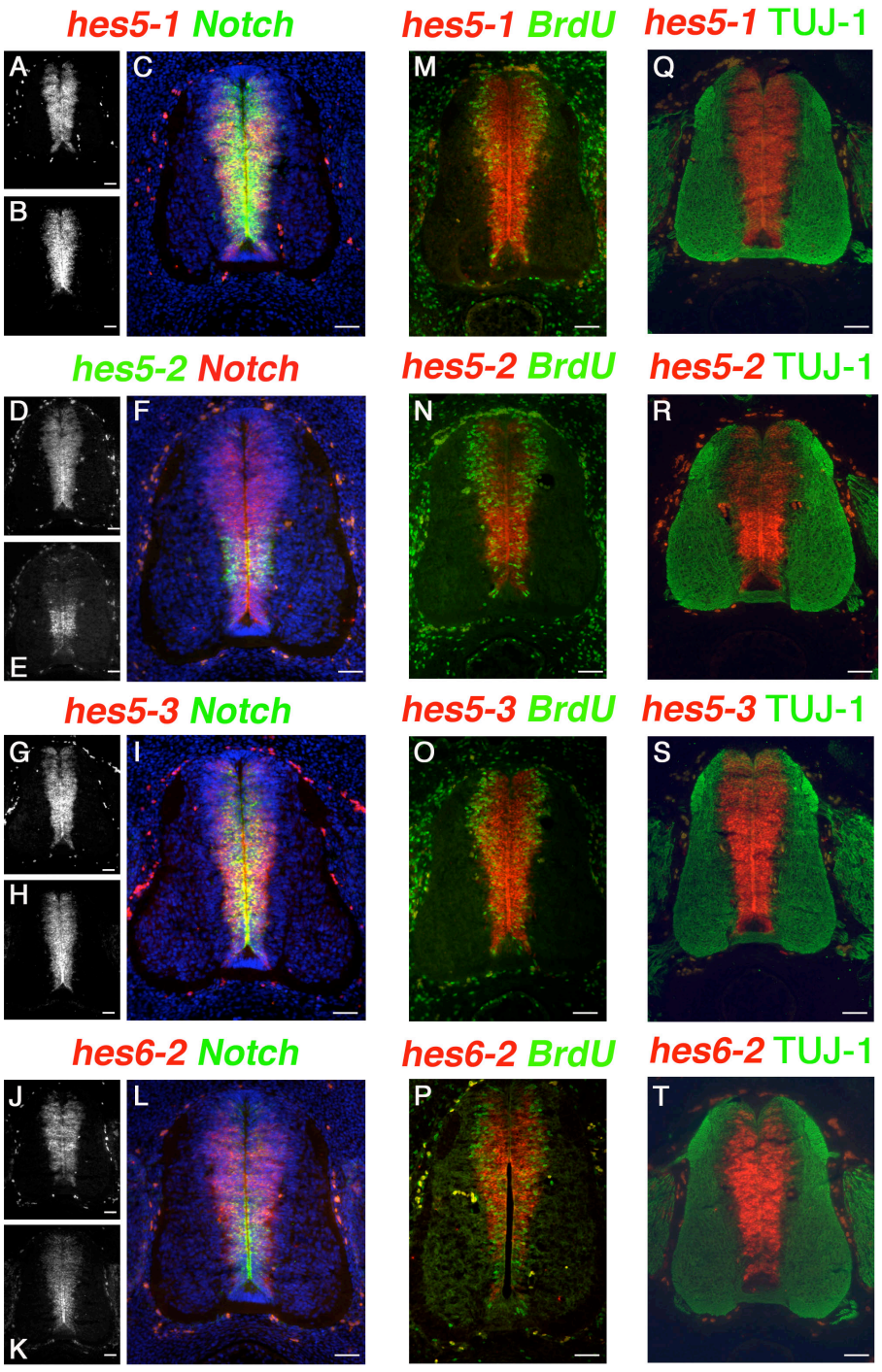


Fig.4 Expression of chick *hes* genes in developing spinal cord. The four *hes* genes are expressed in the ventricular region of the neural tube (**A, E, G, J**) overlapping with *Notch1* expression (**B, D, H, K**). In red are genes where the *in situ* hybridization has been developed with fast red and in green with tyramide-FITC, DNA stained with DAPI in blue. Cells that express the *hes* genes also incorporate BrdU (**M-P**). All the *hes* genes have complementary expression with the neuronal marker TUJ-1 (**Q-T**). Transverse sections of stage HH23 spinal cord. Scale bars 50□m.

Double *in situ* hybridization reveals, however, some differences between the expression of the chick *hes* genes: while all three *hes5* genes are expressed in neuroepithelial cells located apically, with little overlap with *Delta-1* in newborn neurons (Fig.5II-A-C), the cells with stronger *hes6-2* expression are located more basally (Fig.5II-E), the majority of which co-express *Delta-1* (Fig.5II H-J).

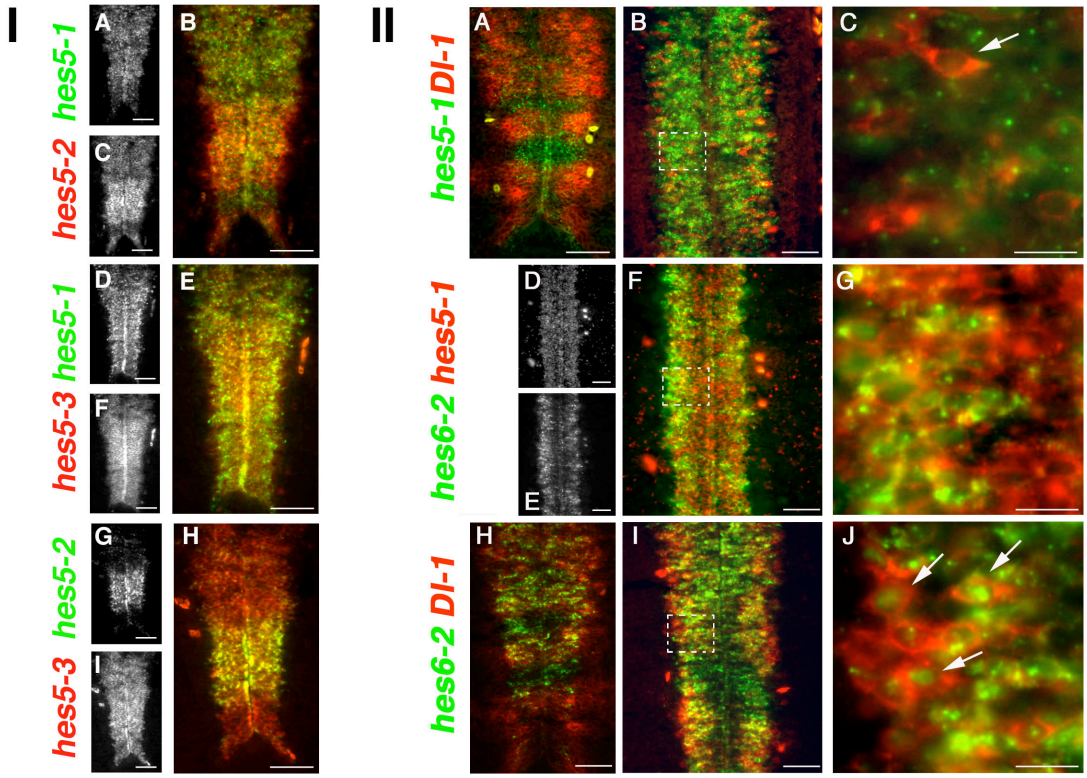


Fig.5 Double *in situ* hybridization unravels some differences between the chick *hes* genes. **I**-Double in situ hybridization between the three *hes5* genes reveals that progenitor cells co-express the 3 *hes5* genes simultaneously (**A-H**). **II-** Double in situ hybridization of the chick *hes5-1*, *hes6-2* and *Delta-1* genes reveals differences in expression between *hes5* and *hes6-2* genes. Double in situ hybridization in transverse (**A,H**) and longitudinal sections (**B –G,I,J**) in developing spinal cord at HH23 [white boxes indicate zoomed regions (**C,G,J**-scale bars 12,5□m)]. *hes5-1* expression is limited to the ventricular zone, with little overlap with *Delta-1* (**A– C**). Nascent neurons with strong *Delta-1* expression (arrow in **C**) do not express *hes5* genes. In contrast, the cells with higher expression of *hes6-2* (arrows in **J**) co-express *Delta-1* (**H– J**) and are more basal (**E**) in comparison to cells expressing *hes5-1* (**D**). In panel (**G**), double in situ with *hes5-1* and *hes6-2* shows that cells with strong *hes6-2* expression are at the edges of the *hes5-1* expression domain. Red and green signals arise from in situ hybridization with DIG and Fluorescein-labeled RNA probes, revealed with Fast-Red and FITC-tyramide, respectively. Scale bars = 50 □m, except in panels (**C**), (**G**) and (**J**) where scale bars=12,5□m.

This indicates that the chick *hes5* genes are expressed only in neural progenitors, while *hes6-2*, although first expressed in progenitors, is highly expressed in cells that are embarking on neuronal differentiation.

In addition, we could also detect *hes* gene expression in the ventral midbrain, but in this region their expression pattern is extremely heterogeneous (Fig.6). The ventral midbrain is characterized by the existence of arcs and inter-arcs, which are neuronal structures that contain functionally and molecularly distinct neurons (Sanders *et al.*, 2002). But this arc-like organization is not just restricted to the mantle layer, the ventricular layer of progenitors cells is also characterized by arc-like patterns in gene expression of the Notch (*Delta-1* and *serrate-1/2*) and WNT signalling pathways (Sanders, 2002). Consistent with this, we also detect a heterogeneous arc-like expression pattern of the *hes* genes, suggesting that the progenitor pool is extremely patterned and that *hes* genes are also operating in this region to control neurogenesis (Fig.6).

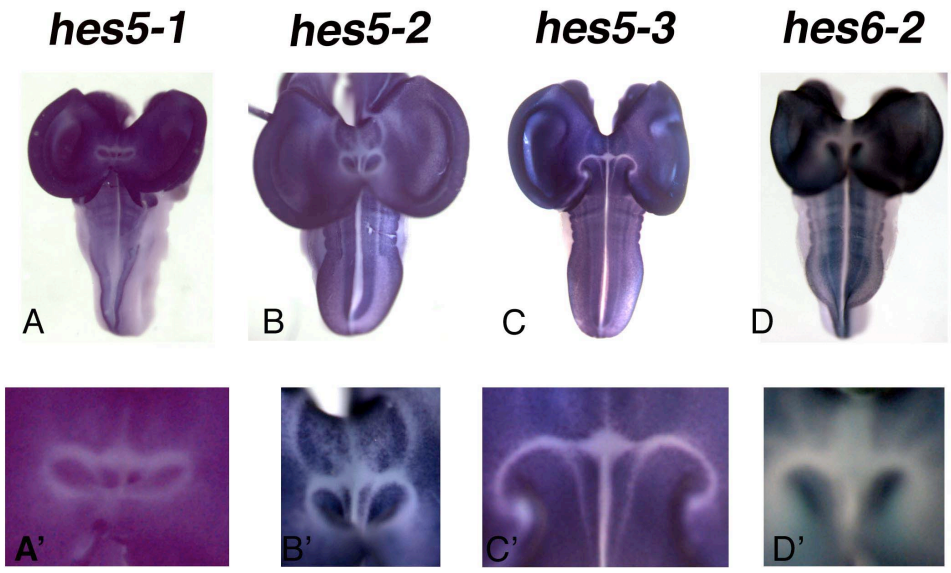


Fig.6 *hes* genes are expressed in the arcs and inter-arcs in the ventral midbrain.

III.3.3 *hes* genes participate in the Notch signalling cascade

In both *Drosophila* and vertebrates, *hes* genes are essential components of the Notch pathway. Their expression is regulated by Notch signalling and some HES proteins

function as downstream effectors of the Notch cascade (Artavanis-Tsakanoas *et al.*, 1999; Kageyama *et al.*, 2005). The similar expression pattern of the chick *hes* and *Notch1* genes suggests that *hes* genes can also implement Notch signals during neurogenesis in the chick embryo. To test this idea, I first assessed how the chick *hes* genes respond to Notch signalling and, second, whether they are able to convey Notch activity during neurogenesis.

In order to know how the *hes* genes respond to Notch signalling, the Notch pathway was activated and the impact on *hes* expression was examined. Then, Notch signalling was blocked and it was evaluated if it had the opposite effect.

To activate the Notch signalling pathway the constitutively active form of the Notch1 receptor (Wakamatsu *et al.,* 2000) was overexpressed in the chick neural tube. The constitutive active form of the Notch receptor comprises the intracellular domain of the Notch receptor (NICD), which is able to translocate into the nucleus and together with the transcriptional factor CSL can activate transcription.

Driving expression of a constitutively active form of the Notch1 receptor (NICD) in the embryonic neural tube leads to the upregulation of the three *hes5* genes and a reduction in *hes6-2* expression (Fig. 7E-H'). Importantly, activation of Notch signalling leads also to the downregulation of the expression of the proneural gene *Ngn-1* and also a decrease in TUJ-1 staining (Fig.7A, C), indicating that neuronal differentiation was inhibited. Moreover, overexpression of NICD also resulted in a downregulation of the expression of the Notch ligand *Delta-1* (Fig.7B, B'), an early neuronal marker (Henrique *et al.*, 1995).

The increase in *hes5* gene expression could be due to a direct induction of *hes5* expression or alternatively, it could be secondary effect of and increase in the number of neural progenitors. To distinguish between these two hypothesis, embryos electroporated with NICD where subjected to a 30min pulse of BrdU to access if the proliferation rate of neuronal progenitors is affected by Notch activation. The results show (Fig.7D, D') that NICD overexpression did not increase the proliferation rate of neural progenitors, instead the number of BrdU positive cells was reduced in the elctroporated side, although not statistical significant (Fig.7D, D'). Therefore, these results strongly suggest that Notch signalling indeed induces *hes5* expression and that Notch signalling inhibits neuronal differentiation, maintaining progenitors in an undifferentiated state but does not induce their proliferation.

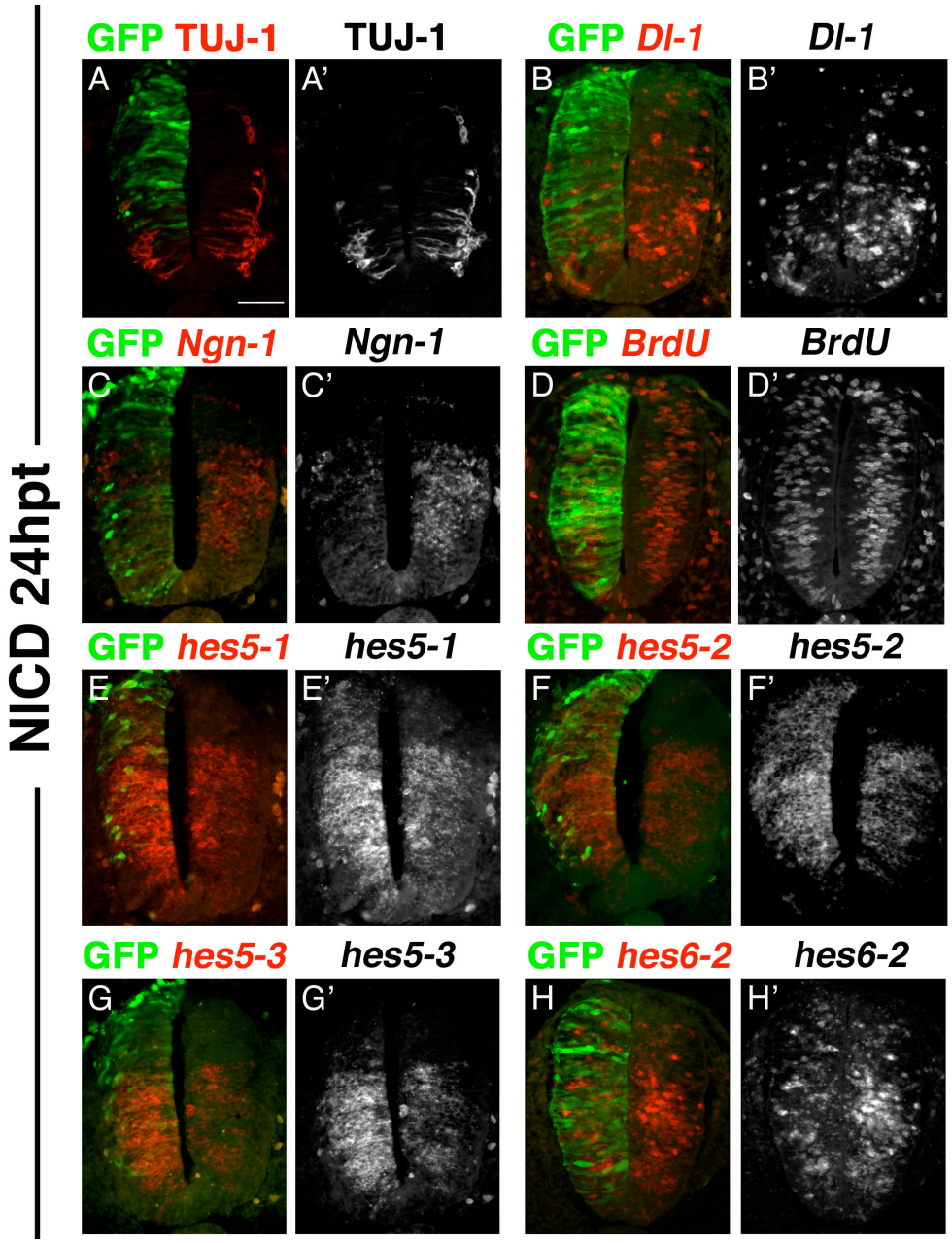


Fig.7 Regulation of the *hes* genes by the Notch pathway. Electroporated cells are shown in green due to the expression of GFP reporter. Overexpression of the activated form of Notch (NICD) led to a decrease in TUJ-1+ cells (**A,A'**), *Delta1* (**B,B'**) and *Ngn1* (**C,C'**) expression, indicating that neurogenesis was inhibited. The chick *hes5* genes, *hes5-1* (**E,E'**), *hes5-2* (**F,F'**) and *hes5-3* (**G,G'**) are up-regulated by NICD. In contrast, *hes6-2* expression is down-regulated by NICD (**H, H'**). However, activation of the Notch pathway did not lead to an increase in BrdU incorporation (**D,D'**), indicating that NICD is not inducing proliferation. Scale bar = 50 □m.

In order to block Notch activity a dominant-negative form of the *Xenopus* homolog of *Drosophila* Suppressor-of-Hairless, (X-Su(H)^{DBM} (Wettstein *et al.,* 1997) was used. This dominant-negative form of Suppressor-of-Hairless is unable to bind DNA but can still bind NICD, forming non-functional complexes with NICD, thus titrating the activated form of Notch available.

In contrast to NICD, forced expression of X-Su(H)^{DBM} leads to an increase in neuronal differentiation, as assessed by the increased number of TUJ-1 positive neurons and the increased expression of *Delta-1* and *Ngn-1* (Fig.8A-C).

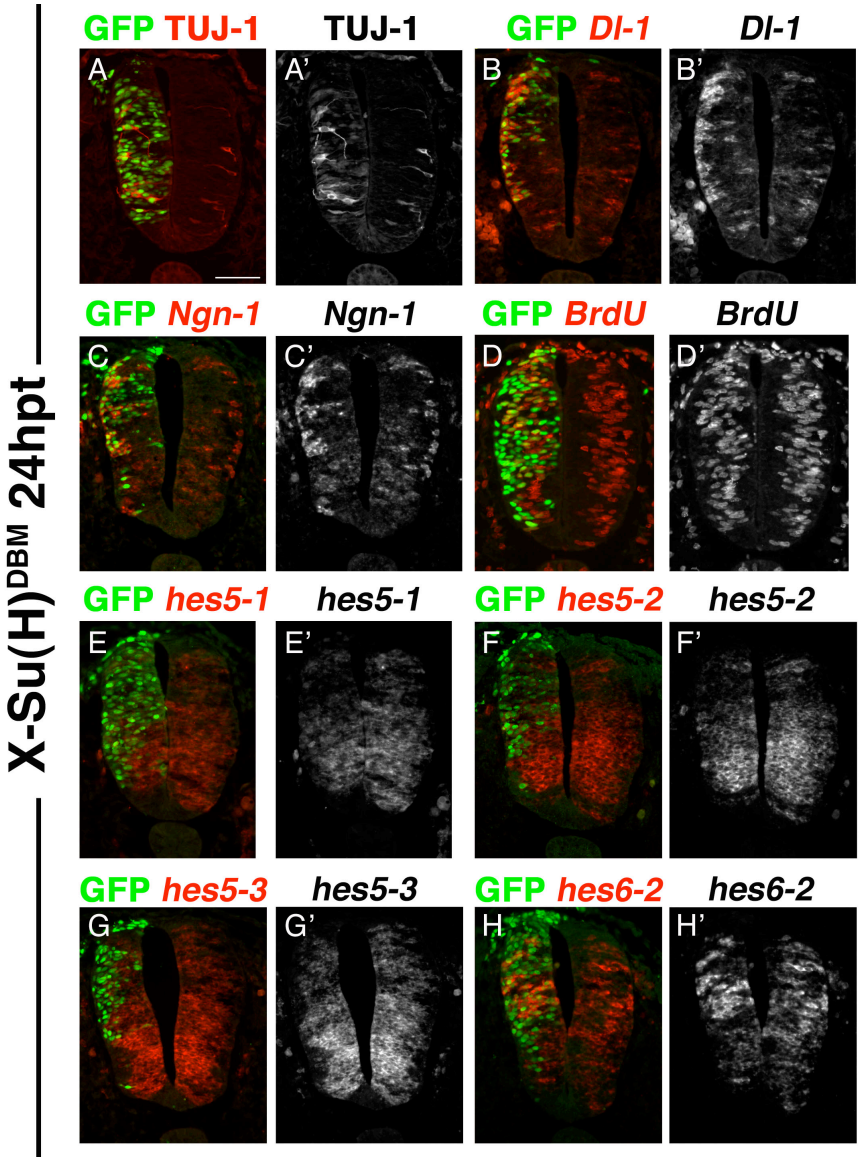


Fig.8 Downregulation of the Notch pathway results in a decrease of *hes5* expression and upregulation of *hes6-2*. Downregulation of the Notch pathway (X-Su(H)^{DBM} overexpression) resulted in an increase in TUJ-1 (**A, A'**) staining *Delta1* (**B, B'**) and *Ngn-1* (**C,C'**) expression. X-Su(H)^{DBM} overexpression resulted in slight increase in *hes6-2* (**H, H'**) expression. In contrast, the expression of the three *hes5* genes was reduced (**E-G'**), scale bar 50□m.

Most importantly, overexpression of X-Su(H)^{DBM} leads to a down-regulation of *hes5* (Fig.8E-G) expression and up-regulation of *hes6-2* (Fig.8H). Together, these results

indicate that all four chick *hes* genes are targets of Notch signalling, although they respond differently to alterations in Notch activity.

The chick *hes5* genes behave as "canonical" Notch targets, since their expression is dependent on Notch activity, and could therefore function as transcriptional effectors of Notch signalling. However, *hes6-2* seems to be repressed by Notch activity and is unlikely to be a direct effector of Notch signalling in the chick neural tube.

To ask whether the chick *hes5* genes are indeed effectors of the Notch signalling pathway during neurogenesis, each of the three *hes5* genes was overexpressed in the embryonic neural tube. In each case, a similar phenotype to that obtained by increased activity of the Notch pathway was detected (Fig.9), namely, a decrease in the number of TUJ-1 positive neurons (Fig.9A, E, I) and a repression of the chick *Ngn1* (Fig.9C, G, K) and *Delta1* genes (Fig.9B,F,J). In addition, *hes6-2* expression is repressed by overexpression of any of the three *hes5* genes (Fig.9D, H, L).

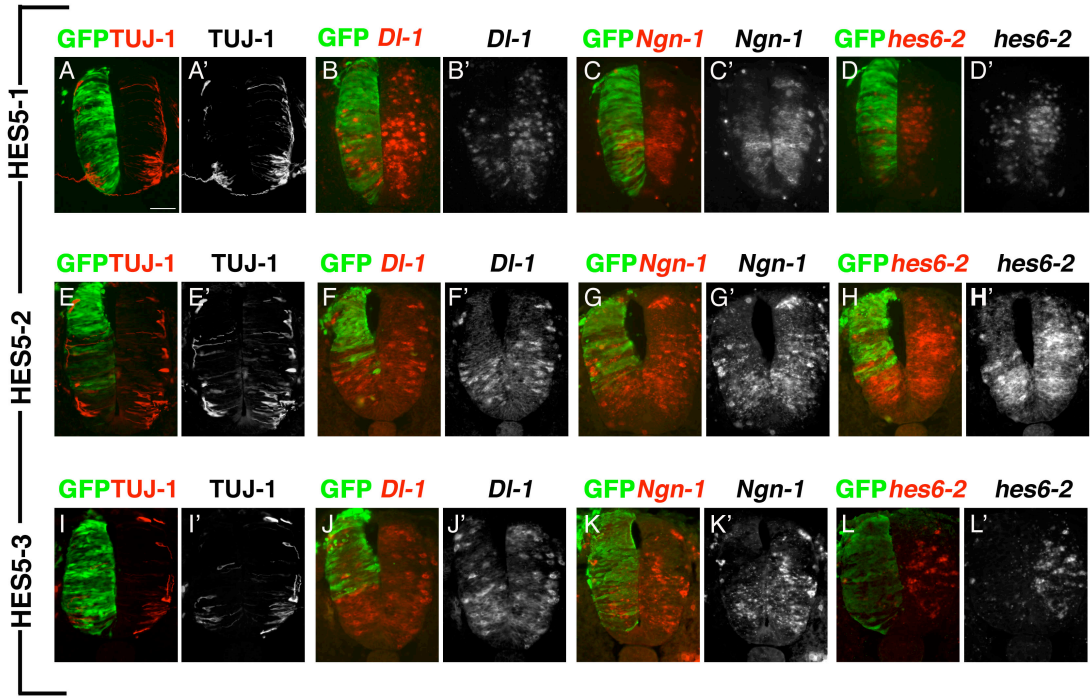


Fig.9 Chick HES5 proteins can mediate Notch activity. Electroporated cells are shown in green due to the expression of GFP reporter. Overexpression of HES5 proteins cause a decrease in TUJ-1+ cells (**A,A', E,E', I,I'**), *Delta-1* (**B,B', F,F', J, J'**) and *Ngn-1* expression (**C,C', G, G', K, K'**), indicating that neurogenesis is inhibited. In addition, both overexpression of HES5 proteins or NICD repress the expression of *hes6-2* (**D,D', H, H', L,L'**). Scale bar = 50□m.

Moreover, 48h after overexpression of NICD or HES5, electroporated cells have a similar behaviour: the cells remain as progenitors and are retained in the ventricular region. In contrast, electroporation of X-Su(H)^{DBM} has the opposite effect– electroporated cells differentiate and migrate, accumulating in the mantle layer (Fig.10).

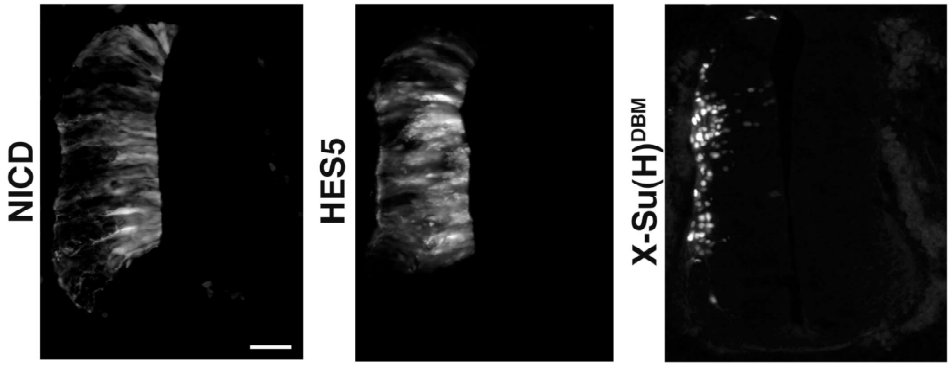


Fig.10 Behaviour of the NICD, HES5 and X-Su(H)^{DBM} electroporated cells 48h post transfection. When NICD or HES5 are overexpressed in the chick neural tube, the electroporated cells have a similar behaviour: the cells are retained in the ventricular region, whereas blocking Notch signalling has the opposite effect–resulting in cell autonomous neuronal differentiation and concomitant migration to the mantle layer.

Overall, these results indicate that the chick *hes5* genes are bonafide Notch effectors during neurogenesis in the embryonic chick neural tube.

III.3.4 Cross-regulation and auto-regulation of the chick *hes5* genes

A remarkable feature of *hes* genes is that they can be negatively regulated by their own products, through direct binding to the respective promoters (Sasai *et al.,* 1992; Takebayashi *et al.*, 1994; Cooper *et al.*, 2000; Hirata *et al.*, 2002; Hirata *et al.*, 2004). This enables the establishment of negative feedback loops in *hes* gene regulation, which might have an important function on the overall architecture of the Notch pathway (Meir *et al.*, 2002). In the chick embryo, the presence of three *hes5* genes raises the possibility of multiple interactions between these genes to modulate Notch signalling.

To check the ability of *hes5* genes to regulate themselves and investigate possible interactions between them, each of the three *hes5* genes was overexpressed in the embryonic neural tube and analysed the effect on the transcriptional output of each

gene [using probes from the 3' untranslated region (UTR), not included in the expression vectors].

The results, summarized in Table 2 (Fig.11), indicate that *hes5-1* and *hes5-2* are indeed able to negatively regulate their own transcription (Fig. 11A,E). In addition, the two genes negatively cross-regulate each other, as shown by the repression of *hes5-2* caused by overexpression of HES5-1, and vice-versa (Fig. 11B,D). By contrast, *hes5-3* is upregulated by overexpression of HES5-1 or HES5-2 (Fig. 11C,F). Furthermore, *hes5-3* is not negatively auto-regulated, as HES5-3 overexpression leads instead to upregulation of the corresponding gene (Fig.11I). HES5-3 overexpression causes also upregulation of both *hes5-1* and *hes5-2* (Fig. 11G,H), raising the question of how can a putative transcriptional repressor lead to simultaneous upregulation of the three *hes5* genes.

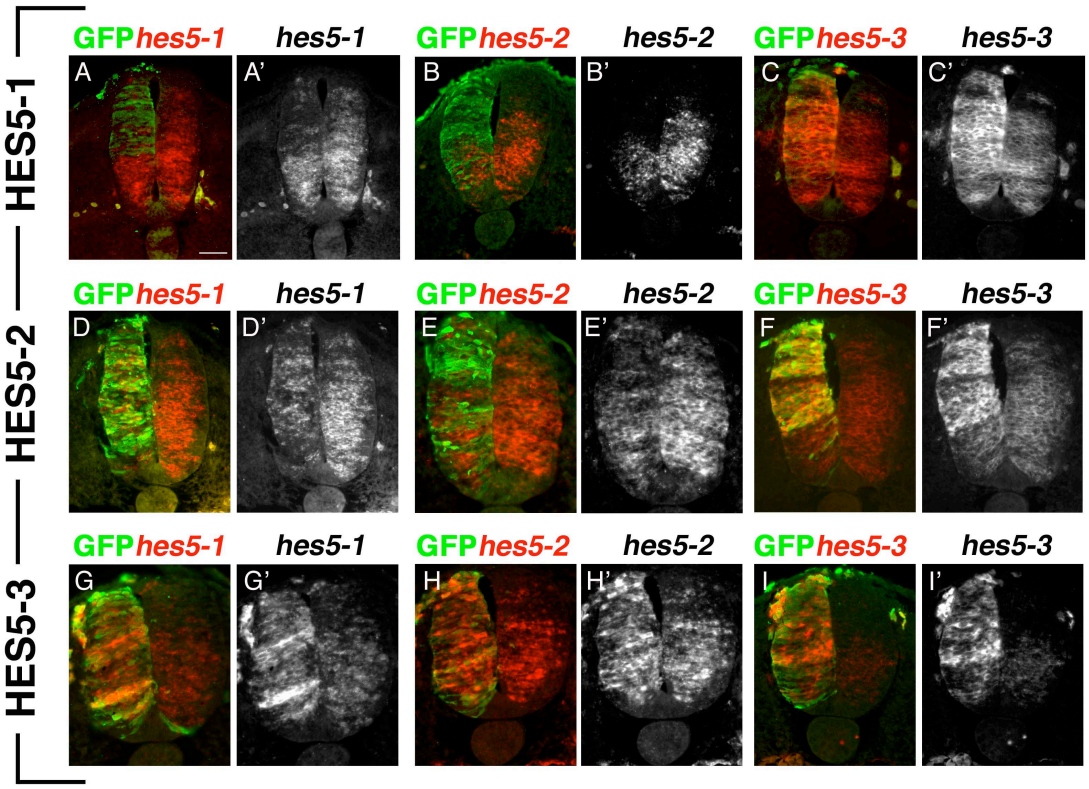


Fig.11 Cross regulations between the chick *hes5* genes. Electroporated cells are shown in green due to the expression of GFP reporter. Embryos were collected 24h after electroporation. Overexpression of HES5-1 results in the down-regulation of the endogenous *hes5-1* (**A,A'**) and *hes5-2* genes (**B,B'**), while it leads to upregulation of *hes5-3* (**C,C'**). Similarly, overexpression of HES5-2 results in the down-regulation of the endogenous *hes5-2* (**E,E'**) and *hes5-1* genes (**D,D'**) and in up-regulation of *hes5-3* (**F,F'**). On the contrary, overexpression of HES5-3 results in the up-regulation of three *hes5* genes (**G– I, G'–I'**). Scale bar = 50□m.

Table 2 Cross-regulations between the chick *hes5* genes.

	hes5-1	*hes5-2*	*hes5-3*
HES5-1	↓	↓	↑
HES5-2	↓	↓	↑
HES5-3	↑	↑	↑

III.3.5 HES6-2 is a common repressor of the three *hes5* genes

The simplest hypothesis to explain how overexpression of HES5-3 results in increased transcription of the three *hes5* genes is to postulate the existence of a common *hes5* repressor, whose activity is repressed by *hes5-3*. In addition, such negative regulator of *hes5* transcription must be also repressed by *hes5-1* and *hes5-2*, since their overexpression results in upregulation of *hes5-3* (but not of themselves, as they are negatively auto-regulated). This common repressor is therefore postulated to play a central role in the concerted regulation of the three *hes5* genes, being able to repress all of them, and being also repressed by any of them.

A good candidate to encode such a common repressor is the *hes6-2* gene, which is actually negatively regulated by all *hes5* genes (Fig. 9D, H, L).

To test this idea and address whether *hes6-2* is indeed able to repress the activity of the three *hes5* genes, 2 different plasmid constructs were electroporated in the embryonic neural tube: one encoding a full-length version of the HES6-2 protein, and another a putative dominant-negative version, in which the C-terminal WRPW domain was replaced by a potent transactivation domain from the viral protein VP16. This fusion protein is expected to bind to the same promoter sites as the normal HES6-2 protein but activate, rather than repress, transcription of target genes (Jimenez *et al.*, 1997).

The results show that HES6-2 overexpression leads to a repression of the three *hes5* genes (Fig.12A-C'), while overexpression of the HES6-2:VP16 fusion has the opposite effect, producing a marked increase on transcription of the three *hes5* genes (Fig. 12D-F'). Together, these results indicate that HES6-2 negatively regulates the transcription of the three *hes5* genes, supporting the model that *hes6-2* functions as a central node on the network of *hes5* regulation (Fig. 13).

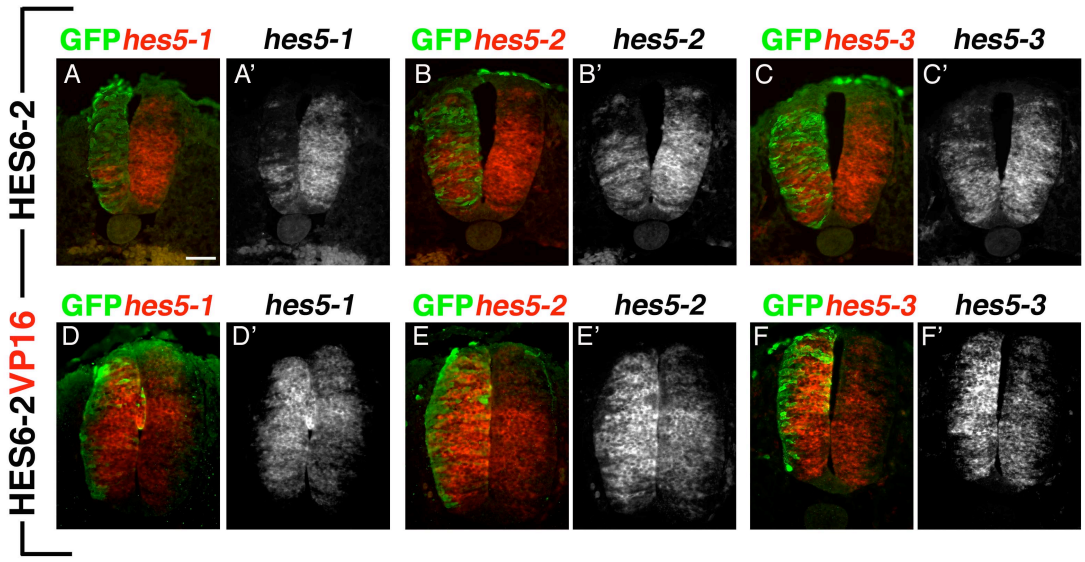


Fig.12 HES6-2 is a common repressor of the *hes5* genes. Overexpression of HES6-2 represses transcription of *hes5-1* (**A,A'**), *hes5-2* (**B,B'**) and *hes5-3* (**C,C'**). In contrast, overexpression of HES6-2:VP16 activates transcription of the three *hes5* genes (**D–F, D'–F'**). Embryos were collected 24 h after electroporation. Electroporated cells are shown in green due to the expression of GFP reporter. Scale bar = 50□m.

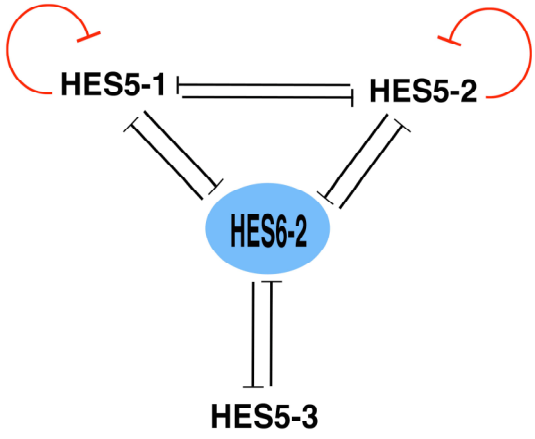


Fig.13 Web of cross-interactions between *hes5* and *hes6-2* genes. HES5-1 and HES5-2 downregulate their own and each other transcription, but upregulate *hes5-3* expression possibly through repressing expression of the common repressor *hes6-2*. HES5-3 upregulates it's own and the others transcription possibly by inhibiting the common repressor HES6-2.

III.3.6 HES6-2 cooperates with the proneural genes to promote neuronal differentiation

The above results suggest that *hes6-2* acts as a negative regulator of Notch signalling by repressing transcription of *hes5* genes. This activity may be important in cells leaving the proliferative zone of the neural epithelium, where *hes6-2* expression

reaches its peak of expression, and may function to reduce Notch signalling and facilitate differentiation into neurons.

To test this idea, we first asked whether the activity of *hes6-2* is sufficient to promote neuronal differentiation.

Our results show that overexpression of *hes6-2*, despite producing a marked decrease in *hes5* expression (Fig.12), does not lead to an increase in neuronal differentiation, instead causes a slight downregulation of TUJ-1, *Delta1* and *Ngn1* expression (Fig.14A-C'). In addition, overexpression of HES6-2 also leads to downregulation of its own expression (Fig.14.D-D').

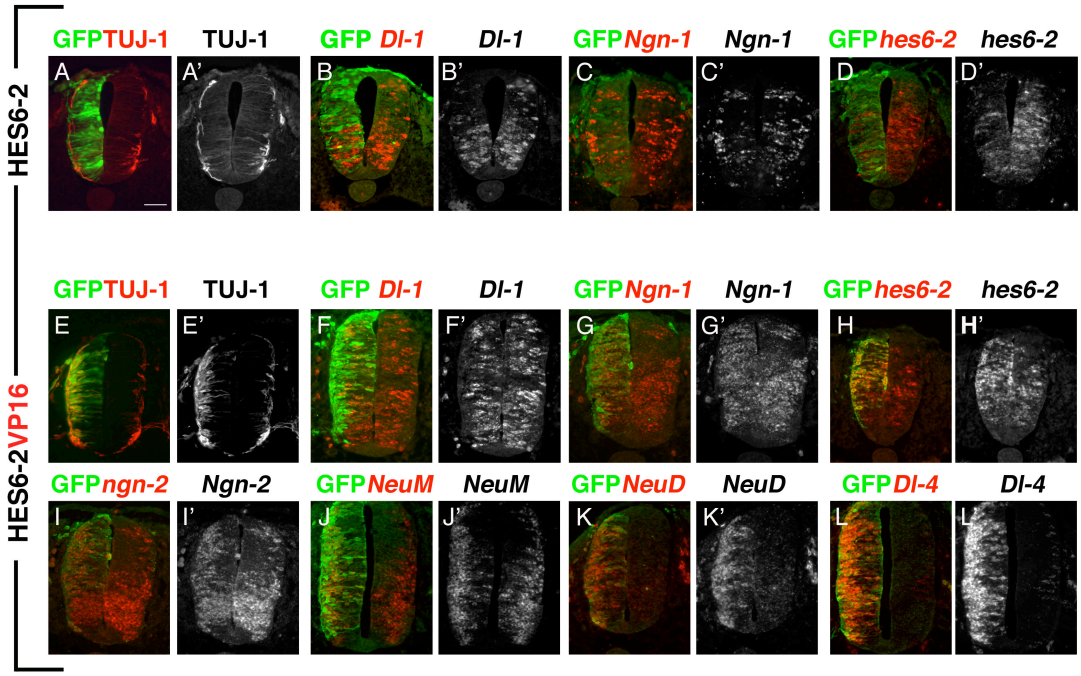


Fig.14 HES6-2 overexpression although represses *hes5* expression, does not lead to an increase in neuronal differentiation. HES6-2 overexpression does not lead to and increase in neuronal production instead seems to inhibit neuronal differentiation. Accordingly, HES6-2:VP16 overexpression leads to an increase in neuronal production, as assessed by the increase in the number of TUJ-1 positive neurons (**E**) and upregulation of the proneural genes *Ngn*-1 (**G**), *Ngn-2* **(I)** and *neuroD* (**K**) expression.

In addition, HES6-2:VP16 overexpression leads to an increase in neuronal production, as assessed by the increase in the number of TUJ-1 positive neurons (Fig.14E-E') and upregulation of the proneural genes *Ngn-1*, *Ngn-2* (Fig.14.G,G',I,I') and *NeuroD* (Fig.14.K,K') expression. Interestingly, the upregulation of the proneural genes and the *Delta* ligands seems selective and at different levels: the proneural genes

Ngn-1 and *Ngn-2* are mildly activated and *NeuroD* is strongly activated, in contrast with the expression of *NeuroM,* which is unchanged. In addition, *Delta-1* expression is unchanged in contrast with *Delta-4*, which like *NeuroD* is strongly induced. Moreover, HES6-2:VP16 overexpression leads to *hes6-2* upregulation, further indicating that HES6-2 is able to negatively regulate its own expression.
Thus, these results show that HES6-2 activity does not promote neuronal differentiation instead HES6-2 seems to have a mild inhibitory effect on neurogenesis.

Nonetheless, *hes6-2* is expressed in two time windows: is expressed in neural progenitors, like *hes5* genes, but also in nascent neurons (Fig. 5). So, it is possible that by overexpressing HES6-2 alone, one is only addressing its first function in neural progenitors and neglecting a later function in cells that are embarking in neuronal differentiation.

Cells that are embarking in neuronal differentiation are characterized not only by their high levels of *Delta-1* expression (Henrique *et al.*, 1995) but also of proneural genes like *Ngn-1* and *Ngn-2* (Bertrand *et al.*, 2002*)*, decisive factors for neuronal commitment.
Indeed, overexpression of proneural proteins (NGN1 or NGN2) in the chick neural tube is enough to drive cells into differentiation, as shown by the increase in *Delta-1* expressing cells and by the subsequent increase in TUJ-1-positive neurons, 24 hours after electroporation (Fig. 15C-D'). The increase in *Delta1* expression is accompanied by up-regulation of *hes6-2* (Fig. 15A, B), indicating that this gene may be a target of proneural genes in nascent neurons. This finding raises the hypothesis that *hes6-2*, although unable to drive neuronal differentiation alone, may function as part of the proneural program, cooperating with the proneural proteins to push cells further into differentiation.
To test this hypothesis, the embryonic neural tube was electroporated simultaneously with expression vectors containing cDNAs encoding HES6-2 and NGN2 (favouring the endogenous conditions of the *hes6-2* expression in nascent neurons). In addition, another group of embryos was electroporated with expression vectors for NGN-2 and HES5-1, and the effects on neurogenesis were compared (Fig.15).

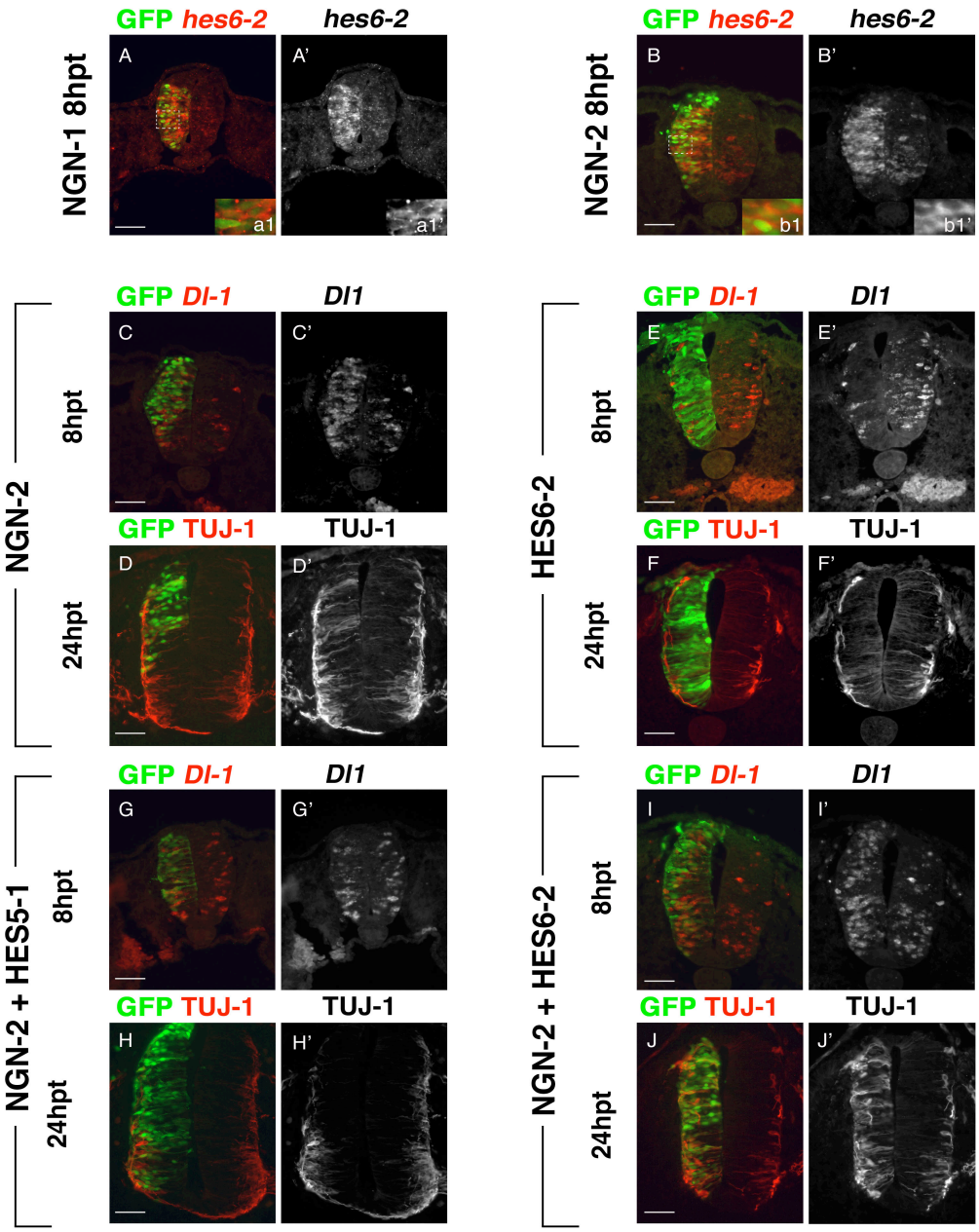


Fig.15 Proneural genes activate *hes6-2* and, together, these genes promote neural differentiation. (**A,B**) Neurogenins activate *hes6-2* transcription, in a cell-autonomous manner, as shown after overexpression of either NGN-1 (**A,A'**) or NGN-2 (**B,B'**). Scale bars 50□m. The white box indicates zoomed region (**a1, b1**). This is accompanied by an increase in *Delta-1* transcription (**C,C'**) and TUJ-1+ cells (**D,D'**), indicating that neurogenesis is promoted by proneural proteins. On its own, overexpression of HES6-2 alone does not promote neuronal differentiation, leading instead to a down-regulation of the neuronal marker TUJ-1 (**F,F'**) and a decrease in the number of *Delta-1* expressing cells (**E,E'**). However, simultaneous expression of NGN-2 with HES6-2 induces the expression of *Delta-1* (**I,I'**) and the appearance of TUJ-1+ cells (**J,J'**) in a synergistic manner, when compared with NGN-2 alone. By contrast, overexpression of NGN-2 with HES5-1 results in an antagonistic effect: 8 h after electroporation, expression of *Delta-1* (**G,G'**) is down-regulated, and 24 h after electroporation, a decrease in the number of TUJ1+ cells is observed (**H,H'**). Electroporated cells are shown in green due to the expression of GFP reporter. Scale bars 50□m.

The results indicate that simultaneous overexpression of NGN-2 and HES6-2 have indeed a synergistic effect on neuronal differentiation, as shown by the higher number of TUJ1-positive neurons (45,4±6% of electroporated cells are also TUJ-1 positive, n=2076)(Fig. 15I-J'), when compared with the overexpression of NGN-2 alone (Fig. 15C-D', 30,6±5% of electroporated cells are TUJ-1 positive, n=1868; P<0,05). By contrast, HES5-1 has an antagonistic effect on NGN-2 activity, as overexpression of NGN-2 and HES5-1 together resulted in a decrease on neuronal production in relation to NGN-2 alone (13,7±6% of electroporated cells are TUJ-1 positive, n=1297; P<0,05) (Fig. 15G-H').

Together these results indicate that *hes6-2* acts in two distinct steps in neurogenesis: First, in neuronal progenitors inhibiting *hes5* expression. Although HES6-2 alone seems to have a mild inhibitory activity on neurogenesis, it is not very efficient comparing with the HES5 proteins. Thus, one possibility is that HES6-2 major function in neuronal progenitors is just to control the switching OFF of HES5 activity. Secondly, in nascent neurons, HES6-2 co-operates with proneural genes, by inhibiting the Notch effectors encoded by the *hes5* genes, and thereby, favouring neuronal commitment.

III.4 DISCUSSION

In this work, I describe four chick *hes* genes that are expressed in the developing nervous system: three *hes5*-like genes (*hes5-1*, *hes5-2* and *hes5-3*) and one *hes6*-like (*hes6-2*). All four genes are expressed in the ventricular zone of the embryonic neuroepithelium, where neural progenitors are located and where the *Notch1* receptor is expressed. Results show that Notch signalling positively regulates the *hes5* genes but reduces expression of *hes6-2*. The four chick *hes* genes appear to be cross-regulated: each *hes5* gene is able to repress *hes6-2*, and all three *hes5* genes seem to be repressed by *hes6-2*. I propose that the function of this *hes5*/*hes6* circuitry is a conserved feature of the Notch pathway, modulating the response of neuroepithelial cells to Notch signals at different phases of their development.

III.4.1 **A simple repertoire of *hes* genes in the chick**

The analysis of the available genome (Release 23.1a.1) and EST databases (Boardman, 2002), indicate that the chick contains 5 genes encoding bHLH-O proteins with homology to the *Drosophila* E(spl) proteins (Fig.1). Three of these genes, named *hes5-1, hes5-2* and *hes5-3*, encode highly related proteins with strong homology to mammalian HES5 proteins (Akazawa *et al.,* 1992;Takebayashi *et al.*, 1995), while the other two genes encode proteins with high homology to mammalian HES6 (Bae *et al.*, 2000; Koyano-Nakagawa *et al.*, 2000; Pissarra *et al.*, 2000), and were named *hes6-1* and *hes6-2.* Completion of the chick genome shall reveal if it contains further members of the *hes* gene family, but analysis of the large collection of chick ESTs now available suggests the existence of only the five *E(spl)*-like genes here reported, plus the two hairy homologues previously described *chairy-1* and *hairy-2* (Palmeirim *et al.*, 1997; Jouve *et al.*, 2000). In comparison, the zebrafish and pufferfish genomes have a much higher number of *hes* genes, at least 19 (Gajewski *et al.*, 2002; Sieger *et al.*, 2004), probably as a result of their genome duplication.

Analysis of the chick genome reveals that the three *hes*5 genes are clustered on a 20 Kb region of DNA in Chromosome 21 (Fig.2), flanking the *fang1* gene. A similar cluster of three *hes5*-like genes is present in the pufferfish genome (Release 23.2c.1), located also in close proximity to the fugu *fang1* gene, suggesting that the *hes5* cluster has been conserved from teleosts (a similar cluster is present in zebrafish) to avians. In mammals, however, a single *hes5* gene flanks the *fang1* gene, implying that the other two genes have evolved differently. Actually, two other *hes* genes – *hes*2 and *hes*3, are present near *hes5* at the tip of Chromosome 4 in mouse (Nishimura *et al.*, 1998), and Chromosome 1 in humans, within a 3 Mb region. This might indicate that the *hes2* and *hes3* genes derive from the ancestral *hes5* cluster but have been dispersed in the chromosome, with their promoter and coding sequences evolving so rapidly that they cannot be ascribed to the *hes5* sub-family anymore. In addition, the mouse *hes2* and *hes3* promoters are unable to respond to Notch activation (Nishimura *et al.*, 1998), suggesting that these genes have lost their capacity to function as effectors of the Notch pathway. This highlights a surprisingly rapid evolution of the Notch pathway circuitry in mammals, which contrasts to the established conservation of its function in various cell-fate decision processes.

III.4.2 The early expression of *hes*5 genes in the Caudal Neural Plate

The analysis of the embryonic expression of the *hes5* and *hes6-2* genes reveal that their transcriptional activity overlap in the developing neural tube but not in the CNP region, where *hes6-2* is not expressed. This difference in the Notch circuitry might reflect the different developmental events taking place in the two regions of the embryo, with the *hes5/hes6-2* circuitry being active only in neural progenitors and nascent neurons within the neural tube, not in their precursors at the CNP.

Recent work (Mathis *et al.,* 2001) have shown that these precursors behave as stem cells, giving rise to similar precursors that remain in the stem zone around Hensen's node, and to cells that, after a transition phase, will end up in the neural tube and constitute the pool of neural progenitors which generate all the cells in the adult spinal cord. The persistence of these stem cells around the regressing Hensen's node and its proliferative state is dependent on FGF signalling, which functions also to prevent the newly formed neural progenitors from starting neurogenesis until they are located in the neural tube region flanking the somites (Diez del Corral *et al.,* 2002). Here, somite-derived signals, mainly retinoic acid, release these progenitors from FGF signalling allowing neurogenesis to begin (Diez del Corral *et al.*, 2003). *hes6-2* expression is initiated in this same region, together with the second wave of *hes5* expression, establishing the *hes5/hes6-2* circuitry.

The expression of *hes5* genes in the CNP is a strong indication that Notch signalling plays a role in cell fate decisions occurring in this region of the embryo, although employing a different genetic circuitry. Actually, recently it has been shown that Notch signalling is downstream of FGF signalling and is required for cell proliferation within the stem zone (Akay *et al.*, 2005). In this region *Delta1* is expressed in a broad and uniform domain, suggesting that is mutual inhibition that is operating instead of lateral inhibition (Akay *et al.*, 2005).

III.4.3 Notch signalling regulates differently the expression of the chick *hes* genes

In *Drosophila*, the *E(spl)* genes are direct targets, and effectors, of Notch signalling in the embryonic nervous system (reviewed in Bray, 1998). The mouse *hes5* gene, an homologue of the *Drosophila E(spl)* genes, is also one of the known targets of Notch signalling in the developing CNS, as shown by the strong reduction of *hes5*

transcription in *Notch1* knock-out mice (de la Pompa *et al.,* 1997; Lutolf *et al.,* 2002). The results presented in this thesis, from experiments involving both gain- and loss-of-function assays for Notch signalling, show that the *hes5* genes are also Notch targets in the developing chick CNS, being positively regulated by activation of the Notch receptor (Fig.7). This regulation is likely to involve direct binding of the CSL-NICD complex to the promoter regions of the *hes*5 genes, which contain various putative CSL-binding sites (Fig.2). This work further indicates that the *hes*5 genes are also effectors of Notch signalling, as their overexpression in the developing neural tube mimics the effects of Notch activation during neurogenesis, i.e., inhibition of neuronal differentiation and repression of the known target genes *Delta1* and *Ngn1* (Fig.9).

In contrast, the *hes6-2* gene is not positively regulated by Notch signalling, being instead repressed when the pathway is activated in the chick neural tube. Conversely, when Notch signalling is reduced, transcription of *hes*6-2 seems to increase. Another vertebrate *hes* gene, *Danio rerio her3*, has also been shown to be repressed, rather than activated, by Notch signalling (Hans *et al.*, 2004). Both the zebrafish *her3* and the chick *hes*6-2 genes contain CSL binding motifs in their promoters, however, they seem to be insufficient to drive transcriptional activation of these genes when Notch signalling occurs. Two hypotheses can be advanced to explain this finding. First, other transcriptional activators, in addition to the CSL-NICD complex, might be needed to effectively promote *hes6-2* activation in the neural tube, the proneural bHLH proteins being good candidates to play this role. Indeed, our results show that NGNs are able to activate *hes*6-2 transcription when ectopically expressed in the neural tube (Fig.15A-B'), supporting a positive role for the proneural bHLH proteins in *hes*6-2 regulation. Similarly, in *Drosophila*, the Ac and Sc proneural proteins were shown to cooperate with the Su(H)/NICD complex to activate transcription of some of the *E(spl)* genes (Kramatschek *et al.*, 1994; Cooper *et al.*, 2000; Nellesen *et al.*, 1999).

A second mechanism to explain why *hes6-2* is not activated by the Notch pathway, despite the presence of CSL binding motifs in its promoter, might involve the counteracting activity of transcriptional repressors that prevent activation by the CSL-NICD complex in the developing CNS. Our finding that each of the 3 *hes*5 genes can repress *hes*6-2 transcription raises the hypot*hes*is that the HES5 proteins might be directly responsible for the *hes*6-2 repression in Notch-responding cells, through

binding to the N- and E-boxes present in the *hes*6-2 promoter. It is even possible that the *hes*6-2 gene is initially induced by Notch signalling, in parallel with the 3 *hes*5 genes, but is quickly down-regulated by the activity of these repressors. This hypothesis could not be assessed in our electroporation assays because at the time when the GFP reporter becomes visible after electroporation, transcription of the NICD transgene and rapid accumulation of the downstream *hes*5 effectors have certainly been underway for sometime already, leading to the detectable repression of *hes*6-2. Thus, the presence of CSL binding motifs in both the *hes*5 and *hes*6-2 promoters opens the possibility that all 4 *hes* genes are equally activated by Notch signalling, with *hes*6-2 being swiftly repressed by the abundant HES5 proteins.

III.4.4 **A cascade of HES activity in neural progenitors**

The analysis of the regulation of the four *hes* genes expressed in the developing neural tube reveals the existence of negative auto-regulatory mechanisms, as well as cross-regulatory interactions between the *hes5* and *hes6-2* genes. These results led to the hypothesis that *hes6-2* functions as a common repressor of the *hes*5 genes, being itself also repressed by these genes. In addition, this circuit of negative feedback regulation between the *hes5* and *hes6-2* genes might play a key role during neurogenesis, modulating Notch activity in both neural progenitors and nascent neurons.

In a simple scenario, when Notch is activated in a neural progenitor, in response to a Delta signal from a neighbouring cell, a fast and massive transcription of the three *hes*5 genes will follow. As Notch effectors, their activity will be essential to implement the decision to stay as a neural progenitor, by repressing the proneural genes (and also *hes*6-2). Later on, negative auto-regulation of *hes5-1* and *hes*5-2 would lead to a downregulation of their own expression, with only *hes5-3* remaining functional. At this point, *hes6-2* might become more active (because their repressors are now less abundant) and would eventually suppress *hes5-3* activity and terminate Notch signalling. Negative auto-regulation of *hes6-2* would finally close a cycle of Notch activity and the cell can again embark on a new process of cell fate decision. This would involve a choice between continuing as a neural progenitor (which requires a new cycle of Notch activity), or commit to neuronal differentiation (which involves a definitive release from Notch signalling). Therefore, neural progenitors would go through cyclic bursts of Notch activity, until they finally commit to differentiation or

instead switch to another fate, like glial progenitor (Gaiano *et al.*, 2002), and I propose that the *hes*5/*hes*6-2 circuitry of negative feedback regulation might play a central role in this mechanism by shutting down the pathway after each Notch activating event.

The existence of cycles of Notch activity in neural progenitors is also supported by the findings of Frade and colleagues (Murciano *et al.*, 2002), who reported that transcription of the *Notch1* gene is switched off when neural progenitors enter S-phase, restarting later to allow the cells to interact with their neighbours and decide their fate. These cycles of Notch activity in neural progenitors might be similar to the cycles described in cells of the presomitic mesoderm (Dale *et al.,* 2003), which also seem to rely on negative feedback of *hes* genes (Lewis, 2003), in the case of the chick, the *hairy1* and *hairy2* genes (Palmeirim *et al.*, 1997; Jouve *et al.*, 2000).

III.4.5 The role of *hes6-2* during neuronal commitment

The analysis of *hes6-2* expression during chick spinal cord development reveals that this gene is expressed at two different phases of neurogenesis: in neural progenitors located in the ventricular zone, close to the apical region of the neural epithelium, and in nascent neurons entering differentiation, located more basally (Fig.5). Expression is higher in the latter, which show also high levels of *Delta1* expression (Henrique, 1995; Myat, 1996). This raises the hypothesis that *hes*6-2, apart from the potential role in neural progenitors discussed above, could also function in cells committing to differentiation, ensuring that these cells are fully released from Notch signalling and can become neurons.

It is known that commitment to neuronal differentiation involves the activity of the proneural bHLH proteins, which trigger a cascade of events leading to cell cycle exit of neural progenitors and full differentiation into neurons (reviewed in Bertrand *et al.*, 2002; Ross *et al.*, 2003). The results presented in this work indicate that *hes*6-2 is a possible target of the proneural bHLH proteins in nascent neurons. The repressor activity of HES6-2 might be crucial to block any HES5-mediated Notch activity in these cells, but this does not seem to be enough to drive neuronal differentiation by itself, as overexpression of HES6-2 in the chick neural tube does not result in increased neurogenesis. However, simultaneous overexpression of NGN2 and HES6-2 lead to a clear increase in neuronal production (Fig.15J-J'), indicating that *hes6-2* cooperates with the proneural genes to promote neurogenesis in the chick spinal cord.

In contrast, HES5 proteins seem to antagonize NGN's proneural activity, as simultaneous overexpression of NGN-2 and HES5-1 results in a decrease in neuronal production (Fig.15G-H). Together, these results indicate that *hes*6-2 functions in nascent neurons to reinforce the decision to enter neuronal differentiation, by supressing the inhibitory activity of the *hes5* genes.

In both mouse and *Xenopus*, *hes*6 homologues are regulated by proneural genes and were shown to promote neurogenesis, but only in regions of the neural plate where the proneural genes are already expressed (Koyano-Nakagawa *et al.*, 2000; Bae *et al.*, 2000), indicating some conservation of *hes6* function in vertebrate neural development. However, in contrast to our findings in the chick, the function of *hes*6 in mouse and *Xenopus* does not seem to involve transcriptional repression of Notch effectors. Instead, *hes6* has been shown to inhibit HES1 activity, through the formation of HES1:HES6 heterodimers that are unable to repress the normal HES1 targets. This correlates to the fact that, contrarily to mHES1, the mHES6 protein cannot bind to N-boxes, due to its shorter loop region in the bHLH domain. Furthermore, HES1:HES6 heterodimers seem more prone to proteolytic degradation, for which phosphorylation of a specific serine residue in mHES6 (Ser183) seems to be crucial (Gratton *et al.*, 2003). In the case of the chick, not only HES6-2 lacks an equivalent serine residue, but also its loop region is 2 aminoacids longer than that of mHES6 [10 in HES6-2, 8 in mHES6, 13 in mHES1, 12HES5], raising the possibility that HES6-2 may have also N-box binding activity. Furthermore, the expression of chick *hairy-2*, which encodes the chick HES1 homologous protein (Jouve *et al.*, 2000), does not correlate with Notch activity in the chick spinal cord, so it is unlikely that HES6-2 functions during neurogenesis by controlling HES1 activity. Instead, our results indicate that HES6-2 has the capacity to directly repress the transcription of the chick *hes*5 genes and might, in this way, modulate Notch activity. In addition, although not addressed in this work, it is also possible that HES6-2 forms inactive heterodimers with the chick HES5 proteins, further hindering their activity as Notch effectors.

In mammals, no interaction between *hes5* and *hes6* were reported yet, but it is possible that *hes6* controls also *hes5* activity during mammalian neural development. The two genes have very similar expression in the developing neural tube (Pissarra *et al.*, 2000; Koyano-Nakagawa *et al.*, 2000; Bae *et al.*, 2000; Takebayashi *et al.*, 1995;

Hatakeyama *et al.*, 2004) and *hes5* is clearly a main Notch effector during mammalian neurogenesis (de la Pompa *et al.*, 1997; Ohtsuka *et al.*, 1999; Lutolf *et al.*, 2002). Also, mouse *hes*6 was shown to promote neuronal differentiation in various assays (Bae *et al.*, 2000; Koyano-Nakagawa *et al.*, 2000;) and it is unlikely that this activity is uniquely mediated by the interaction with *hes1*, whose expression in the developing neural tube is rather restricted (Hatakeyama *et al.*, 2004). Therefore, although the molecular details may vary in different cells, or different animals, *hes6* seems to have a conserved function during vertebrate neurogenesis, as a negative regulator of Notch signalling.

III.5 CONCLUSION

In vertebrates, the Notch pathway has conserved functions in developmental processes as different as neurogenesis and somitogenesis, even if the components and regulatory mechanisms might reveal some variability between different species. This functional flexibility is a consequence of the robustness of the Notch pathway, which leads invariably to a stable, and simple, outcome: making two cells (or two groups of cells) adopting distinct developmental decisions. This robustness was proposed to arise from the existence of several interlaced negative feedback loops, inter- and intra-cellular, that amplify minor differences in the cells' potential and ensures that they stably adopt different decisions (Meir *et al.,* 2002).

One of these negative feedback loops is described in this thesis, involving the circuitry of *hes*5 and *hes*6 activity during neurogenesis. Although the molecular details might be different in chick and mouse, the function of this *hes*5/*hes*6 circuitry may be a conserved feature of Notch signalling in vertebrate neural development. This work shows that, in nascent neurons, HES6-2 represses the Notch effectors encoded by *hes*5 genes, cooperating with the proneural proteins to drive these cells into neuronal differentiation. This work also suggests that the design of the *hes*5/*hes*6 circuitry may support the generation of pulses of Notch activity in neural progenitors, which are responsible for the maintenance of these cells within the neuroepithelium. In this process, the *hes*5 genes act first as effectors of the Notch pathway, to prevent these cells from embarking on neuronal differentiation, after which HES6-2 comes into action to repress *hes*5 activity and terminate Notch signalling. As a result, neural progenitors are driven back into a "neither-ON-nor-OFF state" at the end of each pulse of Notch activity, being able to start afresh a new cell-fate decision process.

Neurogenesis in the vertebrate neural tube can thus be viewed as a reiterative process where cells go through successive events of cell fate decision, mediated by the Notch pathway, until all progenitors are exhausted or move into a different competence state. And the *hes5/hes6* circuitry of negative auto and cross-regulation may contribute to shut down the pathway after each Notch activation event, enabling progenitors to go back to a "neither-ON-nor-OFF state", where they are competent to respond to new cell-fate decision process.

Chapter **IV**

Real-time imaging of Notch activity in the chick neural tube

IV.1 ABSTRACT

Cell-cell signalling mediated by the Notch pathway is vital for many cell fate decisions and patterning events from worms to humans.

By negatively regulating their own expression, Notch signalling targets show an oscillatory behaviour, which in the context of somitogenesis is translated into periodic spatial patterns, the somites. This cyclic *hes* expression is believed to be part of the somite segmentation clock, counting time for the generation of a next somite (Palmeirim *et al.*, 1997; Dubrulle and Pourquie, 2004).

In the context of neurogenesis, it has also been shown that two chick *hes5* genes can negatively regulate their own expression (Fior and Henrique, 2005). In addition, it has also been revealed that a serum chock can elicit *hes1* oscillations in neuroblastoma cell lines (Hirata *et al.*, 2002), opening the possibility that *hes* gene oscillations may occur in other biological systems. However, it is still unclear whether HES proteins generate an oscillatory behaviour or not during neurogenesis, since this might have been unnoticed due to lack of cell synchronization of the neuroepithelium.

A fascinating challenge is to determine if this oscillatory behavior exists in neural development and determine whether oscillations or fluctuations in *hes* gene expression occur in the neuroepithelium and understand its functional relevance.

As a first step towards understanding the complex role of Notch signalling during CNS development, a real-time imaging system to analyse *hes* gene expression with single-cell resolution during neurogenesis was developed.

A reporter construct composed of the *hes5-1* promoter fused to a destabilized Venus protein was generated, which recapitulates the endogenous pattern of *hes5-1* expression and responds to Notch signalling, providing a powerful tool to monitor Notch activity in real time. Initial analysis of time-lapse imaging of the reporter revealed a dynamic gene expression pattern, suggesting that oscillations of *hes5-1* gene expression might indeed occur.

IV.2 INTRODUCTION

The controlled production of neurons relies on a genetic circuitry, conserved from flies to mammals, based on the counteracting activity of two different sets of bHLH proteins: proneural proteins, promoting neural differentiation, and HES proteins, repressing this cell fate decision. The balance between these antagonistic activities and,

consequently, the fate of the cell, is controlled by Notch signalling through Lateral Inhibition. Delta expressing cells activate Notch in neighbouring progenitor cells, leading to the transcriptional up-regulation of genes encoding HES proteins, which repress proneural activity and, thereby, maintain cells undifferentiated. In this way, Delta expressing cells become neurons but at the same time prevent neighbouring cells from following the same fate, maintaining these cells as progenitors.

Cells that remain as progenitors after a Notch activating event will need to downregulate Notch activity to be able to embark again on a new cell-fate decision process. One way to achieve this, might involve the degradation of the NICD protein in the nucleus, triggered by the MAM co-activator (Fryer *et al.*, 2004). In addition, the activity of the downstream HES effectors has also to be restrained.

In the previous chapter, I proposed that the circuitry of negative auto- and cross-regulation of HES repressors could have a crucial role on switching-off Notch activity to enable progenitor cells to go back into a 'naïve' state. Vertebrate neurogenesis could thus be viewed as a reiterative process, in which progenitor cells have to decide again and again whether they are to remain as progenitors or differentiate. This suggests that the Notch signalling cascade is activated transiently in neural progenitors, in a reiterative manner, producing pulses of Notch activity and, therefore, of HES5 expression.

It is well established that the oscillatory expression of several Notch targets plays a crucial role in the molecular mechanism that times the periodic and sequential formation of segments in vertebrates (Palmeirim *et al.*, 1997; Hirata *et al.*, 2002; Bessho *et al.*, 2003; Hirata *et al.*, 2004; reviewed in Guidicelli and Lewis, 2004).

The existence of negative auto-regulatory mechanisms, as well as cross-regulatory interactions between *hes* genes in the developing neural tube (Fior and Henrique, 2005), together with the demonstration of an oscillating expression of *hes1* in neuroblastoma cells (Hirata *et al.*, 2002), raises the attractive possibility that Notch signalling might also have an oscillatory behaviour, although not with a fixed period, during neurogenesis.

Therefore, a fascinating challenge is to determine if this oscillatory behaviour does exist in the neuroepithelium and understand its functional relevance. As a first step to determine if Notch signalling is activated in a reiterative manner and if oscillations of *hes* gene expression occur in the developing neural tube, I developed a real-time

imaging system to analyse dynamic gene expression with single-cell resolution during neurogenesis.

The promoter of the *hes5-1* gene was chosen to build the Notch activity reporter, as this gene is transcribed throughout the entire Notch expression domain, is activated by Notch signalling (Fior and Henrique, 2005) and is the closest homologue of the mouse and human *hes5* genes.

IV.3 RESULTS

IV.3.1 The *hes5-1* gene is the best candidate for a readout of Notch activity in the chick neural tube

The transcriptional activity of the mouse *hes1* gene has been often used as a readout of Notch activity, with its promoter region normally fused to the luciferase reporter in *in vitro* assays (Jarriault *et al.,* 1995). However, *hes1* transcription in the neural tube does not reflect the pattern of Notch activity, as its expression does not significantly overlap with that of *Notch1* and seems unaffected by inactivation of the Notch pathway in the developing embryo (de la Pompa *et al.*, 1997; Lutolf *et al.*, 2002). In contrast, *hes5* transcription is severely reduced in these mutants, suggesting that the *hes5* gene is a bonafide target of Notch activity in the developing nervous system (de la Pompa *et al.*, 1997; Lutolf *et al.*, 2002).

The chick genome contains three *hes-5* homologues, all of which respond to Notch signalling (Fior and Henrique, 2005). Comparison of the protein sequences of the three chick HES5 proteins reveals a high degree of homology between them. However, HES5-1 shows the highest degree of identity and similarity with the mouse and human HES5 protein sequences (Fig.1).

Moreover, the three chick *hes5* genes are clustered on a 20 Kb region of DNA in Chromosome 21, close to the *fang1* gene (Fior and Henrique, 2005). In chick, *hes5-1* is the gene located nearest to *fang1*, further suggesting that it is the closest homologue to the mammalian *hes5* genes.

A

	% Similarity
hHES5/cHES5-1	70,35
hHES5/cHES5-2	62,1
hHES5/cHES5-3	63
mHES5/cHES5-1	85.1
mHES5/cHES5-2	71.2
mHES5/cHES5-3	72.9
cHES5-1/cHES5-2	67.4
cHES5-1/cHES5-3	77.7
cHES5-2/cHES5-3	73.3

B

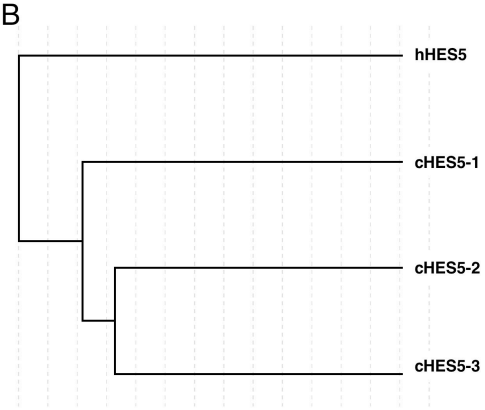


Fig.1 Comparison of the HES5 protein sequences. A. Comparison of the HES5 family protein sequences given by the % of similarity between HES5 proteins (FASTA protein sequence comparison-Pearson, 1998). **B.** HES5 phylogenetic tree. The chick HES5-1 has the higher degree of similarity with human HES5.

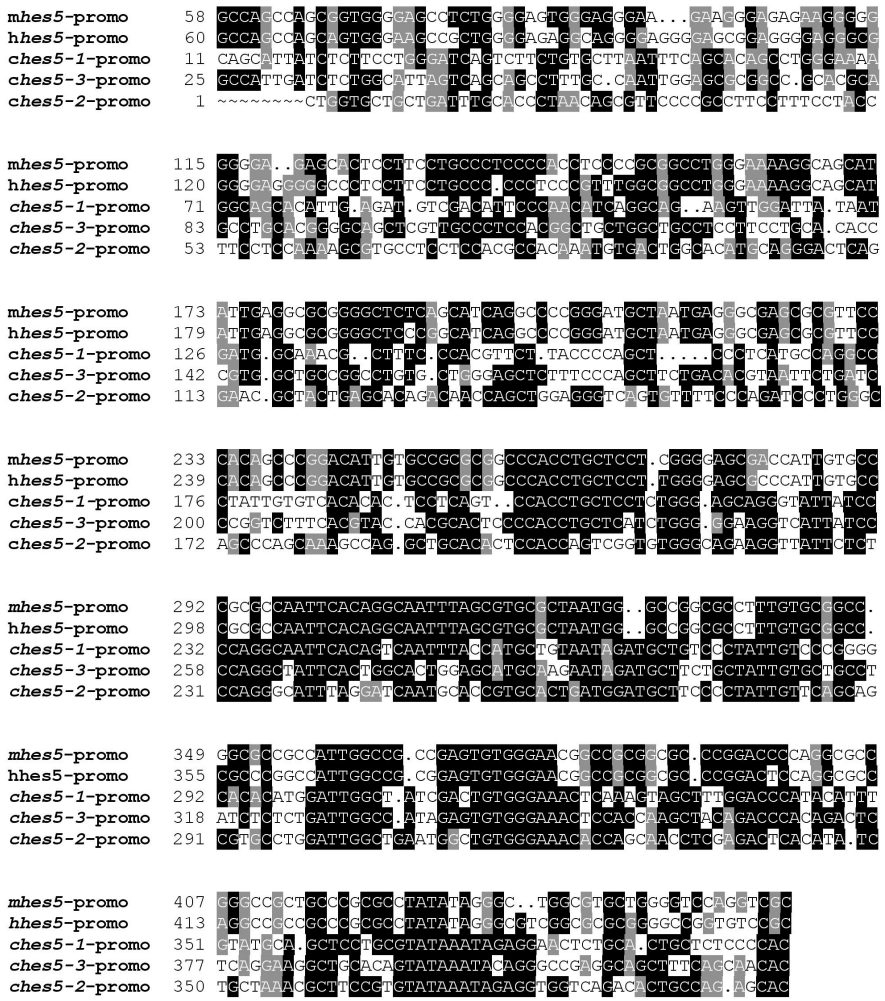


Fig.2 Alignment of the ~600pb promoter region of the chick *hes5* genes with the human and mouse *hes5* promoters.

The *hes5-1* gene shows also the pattern of expression that mostly overlaps with the *Notch1* expression domain in the chick neural tube: like *Notch1*, *hes5-1* expression spans the whole ventricular region of the neural tube (Fior and Henrique, 2005).
In addition, alignment of the promoter regions of the three chick *hes5* genes with the mouse and human *hes5* promoter regions reveals the highest level of homology for the chick *hes5-1* promoter [63,2% *mhes5/hes5-1*; 60.3% *mhes5/hes5-2;* 62% *mhes5/hes5-3*] (Fig.2). Thus, the *hes5-1* promoter can be considered the best candidate to monitor Notch activity in the developing chick neural tube.

IV.3.2 "*In silico*" detailed analysis of the chick *hes5-1* promoter region

hes genes have been shown to be subjected to regulation by CSL transcription factors, proneural proteins and also by auto-regulation by their own products (see Table1).
The promoter region of *hes5-1* was analysed in detail by searching for putative binding sites of these known transcriptional regulators of Notch target genes and also compared with other proximal promoter regions of *hes5*-like genes (Table 1) (Fig.3).

Table 1 Consensus binding sites for the CSL, proneural and HES transcription factors.

CSL binding boxes S BOX	Proneural binding box E BOX-class A CANNTG	HES binding box N BOX
High affinity YGTGRGAA	NGN CATATG CAGATG	CACNAG
Low affinity RTGRGAR	MATH CAGCTG	
REF: Tun *et al.*, 1994: Singson *et al.*, 1994; Jarriault *et al.*, 1995; Bailey and Posakony, 1995; Nellesen *et al.*, 1999;	Kramatschek *et al.*, 1994; Singson *et al.*, 1994; Oellers *et al.*,1994; Cooper *et al.*, 2000; Bertrand *et al.*, 2002; Gazit *et al.*, 2003; Castro *et al.*, 2005; Cave *et al.*, 2005; Lamar and Kintner, 2005	Takebayashi *et al.*, 1993; Davis and Turner, 2001; Hirata *et al.*, 2002

CSL binding sites

Notch activates transcription of *hes* genes by binding to the CSL transcription factor and to the MAM co-activator, forming a ternary activating complex. This ternary complex binds to DNA through the CSL binding sites (S) sites, activating transcription (Mumm and Kopan, 2000). In the absence of Notch signalling, however,

CSL transcriptions factors bind to the same S sites but recruit co-repressor complexes, repressing transcription (reviewed in Bray and Furriols, 2001).
CSL transcription factors bind to optimal consensus high (YGTGRGAA) and low (RTGRGAR) affinity sequences (Tun *et al.*, 1994; Nellesson *et al.*, 1999).
Previous analysis of *hes* promoter regions revealed that most of the elements required for neural expression are located in close proximity to the transcription start site (Nelessen *et al.*, 1999; Davis *et al.*, 2001; Gajewsky and Voolstra, 2002; Castro *et al.*, 2005; Lamar and Kintner, 2005).
I therefore looked in closer detail to the ~600pb upstream of the translation start site, where five putative CSL binding boxes (S boxes) can be found, four of which with an optimal consensus high affinity sequence (Fig.3A).
This region contained a pair of CSL binding sites in a head to head orientation, resembling an SPS (paired S sites S1+S2), spaced by 16 nt. This putative SPS is flanked at 3' end by a TATA and at 5' end by an inverted CAAT box (Fig.3A). This architecture [invCAAT + SPS + TATA] is present in numerous *hes* promoters (Nellesson *et al.*, 1999; Gajewsky and Voolstra, 2002; Cave *et al.*, 2005; Lamar and Kintner, 2005; Ong *et al.*, 2005).

The chick *hes5-1* S1 site has an optimal high affinity consensus sequence that is highly conserved in all the *hes5* genes (Fig.3). Mutation of this site in several *hes* promoter regions (mouse *hes1* and *hes5*, *Xenopus xEsr1* and *xEsr10*) results in abrogation of activation by Notch, indicating that this site is essential and sufficient to elicit a Notch response (Lamar and Kintner 2005, Ong *et al.*, 2005).
In contrast, the S2 site on the chick *hes5-1* proximal promoter does not fit the optimal consensus sequence – it contains two mismatches. The same is true for most of the other *hes5*-like genes in other animals, which also contain two or more mismatches (Lamar and Kintner 2005; Ong *et al.*, 2005).

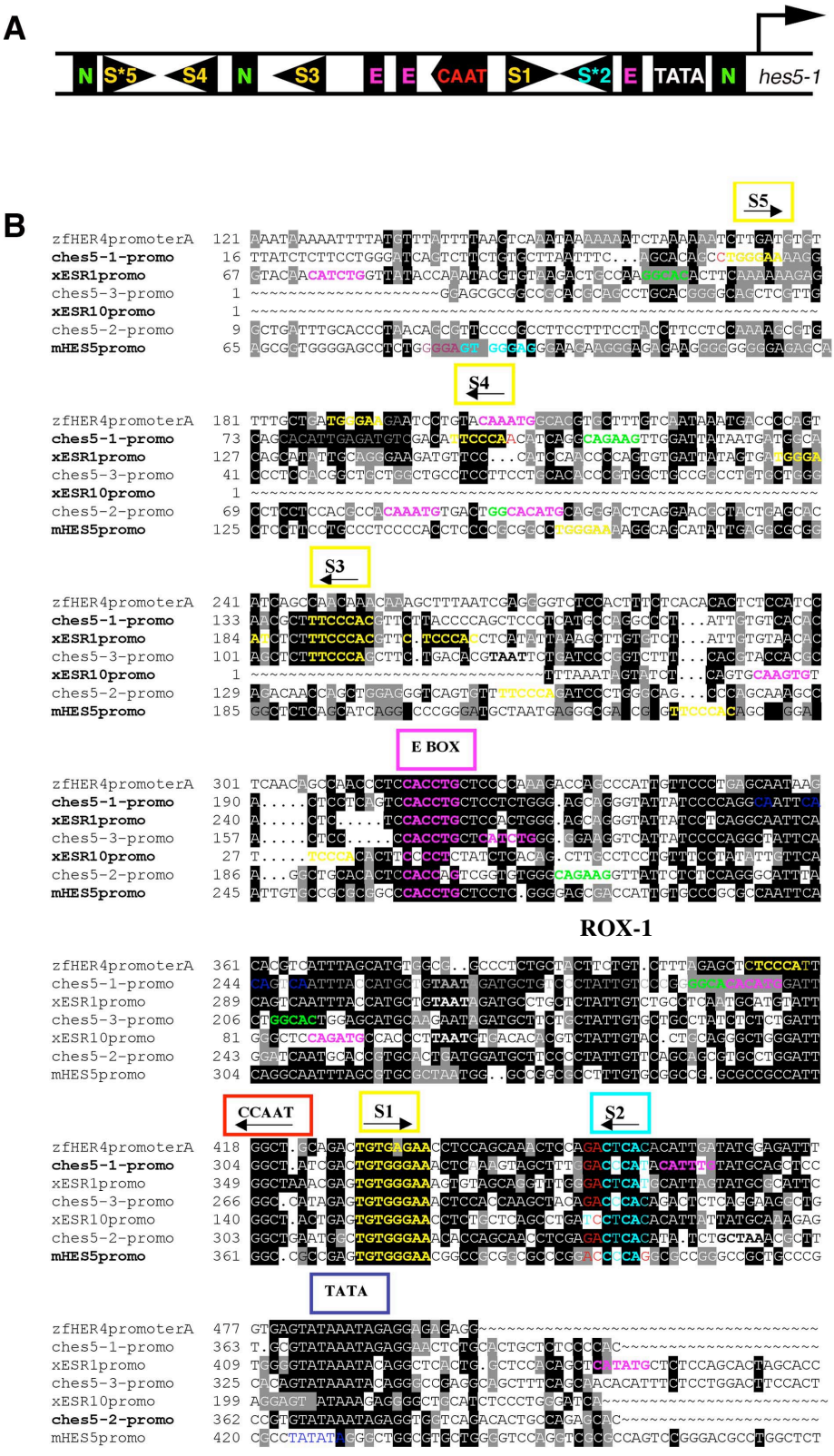


Fig.3 Analysis of the ~600 upstream promoter region of *hes5-1* and comparison with other *hes5* family members A. Summary of the putative binding sites for CSL [S], Proneural [E] and HES [N] transcription factors found in the ~600pb upstream genomic region of *hes5-1*. The S* indicates that the sequences contain one or two mismatches. **B**. Alignment of the proximal~600pb promoter regions of several *hes5*-like genes. High affinity S boxes are in yellow, low affinity S boxes in light blue, E boxes in pink and N boxes in green, mismatches are depicted in red.

In cases where the S2 site fits the optimal consensus sequence [(*E(spl)8*) or S2 sites with one mismatch (*mhes1* and *Esr10*)], mutations on this site also prevent Notch activation. On the contrary, promoters with non-consensus S2 sites (with two or more mismatches) are not affected when further alterations are introduced on this site (*xEsr1* and *mhes5*) (Cave *et al.*, 2005; Lamar and Kintner, 2005; Ong *et al.*, 2005). This implies that S2 sites with more than one mismatch are not functional, suggesting that the SPS motif of *hes5*-like genes (S2 with two or more mismatches) contain only one functional CSL site: the S1 site.

In addition to the putative SPS motif near the inverted CCAAT box, at least more 8 putative CSL binding sites can be detected in the 2kb upstream region of the *hes5-1* promoter (Fig.4), three of which are in the ~600bp proximal region of the transcription start site (S3|S4|S5) (Fig.3A).
The *xEsr1* and the *mhes5* promoter regions also contain several S sites upstream of the S1site (3 in *xEsr1* and 4 in *mhes5*) (Lamar and Kintner, 2005 and Ong *et al.*, 2005). One of these, S3 (Fig.3), is highly conserved in sequence, position and orientation in most *hes5*-like genes (Fig.3B). Mutation of this S3 site in *xEsr1* abrogates enhancer activity in transfected cells and *in vivo* (Lamar and Kintner 2005), indicating that this site is also essential for Notch mediated transcriptional activation.

In conclusion, most of the *hes5* genes have a S2 suboptimal binding site that does not play a significant role on Notch regulation. Instead, a conserved upstream S3 site seems to be necessary for regulation by the NICD-CSL complex, suggesting a unique architecture for the promoter of *hes5*-like genes, which require S sites in a S3-S1 orientation, rather than the classical SPS configuration present in other *hes* genes.

Proneural Input

Expression of several *hes* genes during neurogenesis not only requires direct input from NICD-CSL binding sites, but also contribution from the proneural bHLH proteins through E-box (CANNTG) binding sites (Singson *et al.*, 1994, Cave *et al.*, 2005; Castro *et al.*, 2005; Lamar and Kintner 2005). Proneural bHLH transcription factors have been shown to synergize with NICD-CSL to activate expression of several

hes-like genes from *Drosophila* to vertebrates (Singson *et al.*, 1994, Cave *et al.*, 2005, Castro *et al.*, 2005; Lamar and Kintner 2005).

In agreement with these studies, 15 putative E-boxes were found within the 2kb *hes5-1* promoter region (Fig.4). In particular, a region containing a cluster of 8 E-boxes can be found at ~750bp upstream of the translation start site, including 3 E-boxes that have been characterized as preferential binding sites for the MATH1 bHLH proneural protein (Bertrand *et al.* 2002; Castro *et al.*, 2006) (Fig.4). A similar cluster of MATH1-E boxes binding sites was also found in both the human and mouse *hes5* promoter regions (Krizhanovsky *et al.*, 2006).

All *hes5*-like genes contain several E-boxes in their promoters, one of which seems highly conserved in position and orientation (cCACCTGc, highlighted in Fig. 3), with exception of *xEsr10* (Lamar and Kintner, 2005) (Fig.3). However, when this E-box was mutated in *xEsr1,* no alterations in *xEsr1* expression was detected, suggesting that its contribution to the regulation of *hes* expression might be compensated by other E-boxes.

Fig.4 Summary of the putative binding sites for CSL, proneural and HES transcription factors found in the 2kb upstream genomic region of *hes5-1*. The -2kb region of the *hes5-1* promoter region contains 10 putative CSL binding sites (S), 6 high affinity sites which are depict in yellow, and the other four low affinity sites are in light blue. The S* indicates that the sequences contain one or two mismatches. 15 Proneural putative binding boxes-Eboxes (E, in pink), were found, three of which are putative MATH-1 sites (M-in pink) and one is a NGN putative binding box (Ng in pink). Four putative HES binding sequences-Nboxes (N-in green) were also found. The figure is not to scale, the first ~600pb proximal to the transcription start site are separated from the other 1400pb by two vertical bars.

HES auto and cross regulation

In the previous chapter, it was shown that chick *hes5-1* is able, not only to negatively regulate its own expression but is also subjected to cross regulation by other HES proteins, namely HES5-2 and HES6-2 (Fior and Henrique, 2005).

HES proteins have been shown to repress transcription by directly binding to E boxes or to N-boxes (Sasai *et al.*, 1992; Takebayashi *et al.*, 1994; Van Doren *et al.*, 1994;

Jennings *et al.*, 1999; Hirata *et al.*, 2000). Although no $E_{B/C}$ boxes are present at the *hes5-1* promoter region, it contains four N-boxes, three of which are in the ~600bp proximal to the translation starting site (Fig.3 and Fig.4).

A similar architecture of N-boxes is present in the mouse *hes1* promoter region, containing also four N-boxes, three of which have been shown to be necessary for negative auto-regulation (Takebayashi *et al.*, 1993).

In addition, it is noteworthy to mention that a highly conserved sequence (CTATTGT) is present in all the proximal promoter regions of the chick *hes5* genes, being however absent from the mouse and human *hes5* promoters (Fig.3). This sequence seems to be a putative binding site for the oxygen sensing transcription factor ROX-1 (Kwast *et al.*, 2002), which may participate in the regulation of chick *hes5* expression in the vascular system (Fior and Henrique, 2005). Consistent with the absence of this ROX-1 binding box, the mouse *hes5* gene is not expressed in the developing vascular system.

IV.3.3 **Generation of a live gene reporter system for Notch signalling activation during chick neurogenesis**

To test the hypothesis that Notch signalling is activated transiently and in a reiterative manner in neuroepithelial cells a real time imaging system was developed to visualize these putative pulses of Notch activity.

The reporter system must be designed to mimic as much as possible the transient nature of Notch activity. Such reporter has to be expressed in the right cells at the right time (dependent on the promoter regulation), and present only during the transient period of Notch activity (dependent on the protein and mRNA stabilization/degradation signals).

As discussed above, the promoter region of the *hes5-1* gene is highly conserved relatively to the mouse and human *hes5* promoters and was therefore chosen as a sensor to detect Notch activity *in vivo*, in the chick neural tube. As reporter, the VNP (Venus-NLS-PEST) protein was chosen, due to the fast maturation time and strong fluorescence intensity of the VENUS protein, a derivative of the Yellow Fluorescent Protein (YFP) (Nagai *et al.*, 2002). The nuclear localization signal (NLS) at the C-end of VENUS helps to localize the fluorescent signal within the cell nucleus, allowing

better fluorescence signal quantification, while the PEST sequence (Li *et al.*, 1998) aims to shorten the half-life of the whole protein. Furthermore, to adjust the half-life of the VNP mRNA to that of the endogenous *hes5-1* mRNA, the 3'UTR of this gene was also introduced in the final construct (Fig. 5).

Fig.5 The *hes5-1* reporter. Vector with VNP reporter under the control of the *hes5-1* 2kb promoter region. The VNP was fused to the *hes5-1* 3'UTR to impose on the VNPmRNA the *hes5-1*mRNA half-life. The three fragments where inserted in the modified backbone of the pCAGs vector (lacking the β-actin promoter and the CMV enhancer sequences).

IV.3.4. The Phes5-1VNP reporter mimics *hes5-1* endogenous expression

To determine if the isolated 2kb fragment of the *hes5-1* promoter contained all regulatory sequences necessary for the proper expression of the *hes5-1* gene in the chick neural tube, two groups of embryos were electroporated: one with the reporter (Phes5-1VNP vector) and another with a control vector in which the VNP expression was under the control of the constitutively active β-actin promoter (β-actinVNP).
48h after transfection, the expression of the VNPmRNA was analyzed by *in situ* hybridization on sections and compared with the pattern of *hes5-1* expression. This is confined to the ventricular zone of the neural tube (Fior and Henrique, 2005) (Fig.6A), where neural progenitors are located.

Analysis of electroporated embryos show that VNPmRNA expression under the control of the *hes5-1* promoter (P*hes5-1*VNP) was restricted to the Ventricular zone (Vz), indistinguishable from the *hes5-1* endogenous expression pattern (Fig.6- compare A and B). In contrast, the *VNP* mRNA in control embryos, driven by the constitutive promoter (β-actinVNP), spanned the whole width of the neural tube, preferentially accumulating in the mantle layer of the neural tube (Fig.6C).

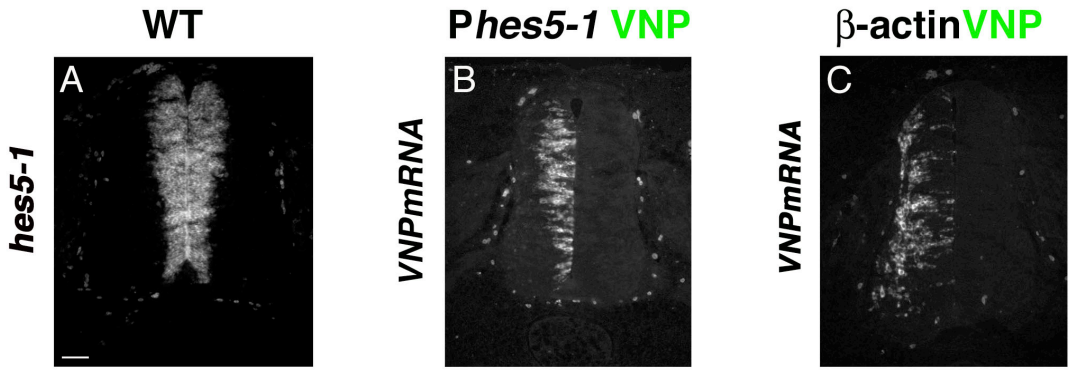


Fig.6 Expression of the P*hes5-1*VNP reporter mimics *hes5-1* endogenous expression. A. *In situ* hybridization for *hes5-1* in a wild-type embryo. **B**. *In situ* hybridization for VNP in embryos electroporated with P*hes5-1*VNP. **C**. *In situ* hybridization for VNP in embryos electroporated with VNP under the -actin promoter. Scale bar=50m

To further confirm that reporter activity occurs in cells that express the endogenous *hes5-1* gene, double *in situ* hybridization for VNP and the *hes5-1* coding region was performed. Since the Phes5-1VNP vector includes the *hes5-1* 3'UTR, a probe excluding the *hes5-1* 3'UTR was generated.

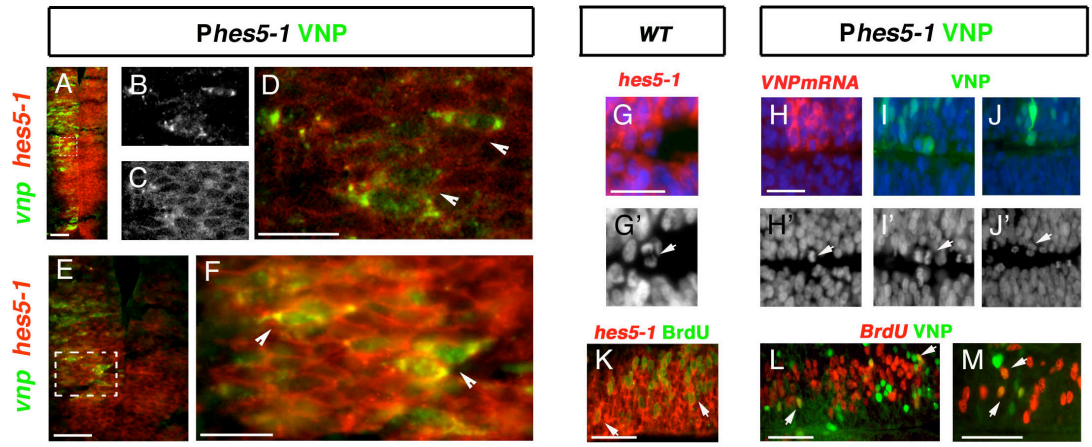


Fig.7 The reporter is expressed in cells that express *hes5-1* and are mitoticaly active. A-F. Double in *in situ* hybridization for VNP and *hes5-1* coding region in Phes5-1VNP electroporated embryos. **B-D**. Confocal microscope image of A-48hpt embryo section. **F.** Zoom of E**,** image acquired in a fluorescent microscope-24hpt embryo section. **G** *hes5* mRNA expression (red) in wt embryos is found in cells undergoing mitosis and S phase-**K**-BrdU incorporation in green. VNPmRNA (red) and protein (green) under *hes5-1* promoter (Phes5-1VNP) are also found in cells undergoing mitosis (**H-J'**) and also incorporate BrdU (**L,M**)**.** Red and green signals arise from *in situ* hybridization with DIG and Fluorescein-labeled RNA probes, revealed with Fast-Red and FITC-tyramide, respectively. DNA is stained with DAPI in blue (G', H;', I',J'). A, E, K, L, M - Scale bar=50m. D, F-Scale bar=12.5m. G-J'-Scale bar=25m.

Confocal analysis confirmed co-localization of the two mRNAs, within the same cells, confirming that the reporter is being expressed in *hes5-1* expressing cells (Fig. 7 A-D). Furthermore, we could detect VNP protein and mRNA in cells that are mitotically active (Fig. 7 H-J') and that incorporate BrdU (in S phase) (Fig. 7L,M), like the endogenous *hes5-1* expressing cells (Fig. 7 G, G',M).

These results strongly suggest that the 2kb genomic fragment of *hes5-1* contains the essential signals to elicit expression of the reporter in the right cells at the right time in the chick neural tube: the VNPmRNA expression pattern mimics the pattern of endogenous *hes5-1* mRNA in the chick neural tube.

IV.3.5 **Distribution of Phes5-1VNP reporter activity in the neural tube**

Next, we examined the distribution of the reporter activity by electroporating the chick neural tube with the Phes5-1VNP vector and comparing VNP protein distribution to that driven by the control vector (β-actinVNP). After 48h, the fates of the VNP positive cells were determined by comparing the expression of the VNP protein with the expression of HU neuronal marker (Fig.8A and A').

The majority of VNP expressing cells under the β-actin promoter control were found out of the Vz, in the HU domain (Fig.8A', B'). By contrast, the majority of VNP expressing cells under *hes5-1* promoter were found inside the Vz and very few cells were found in the HU domain (Fig.8A, B).

In order to determine and quantify the distribution of the reporter activity within the neuroepithelium, the two groups of embryos electroporated with Phes5-1VNP and β-actinVNP were subjected to a 30'pulse of BrdU to label cells in S phase, delimiting the frontier between the Vz and the Mantle Layer (ML). Cells that lay inside the Vz and VNP positive were counted and compared to the number of VNP cells that were located in the mantle layer.

In control embryos, 48h after transfection, the majority of the VNP-positive cells (under the control of the β-actin promoter) migrated out of the Vz to the mantle layer (73,8%± 7.1sd in the mantle layer and 26, 2% in the ventricular region, n=1420 cells, 8 sections, 4 embryos) (Fig.8B' and E).

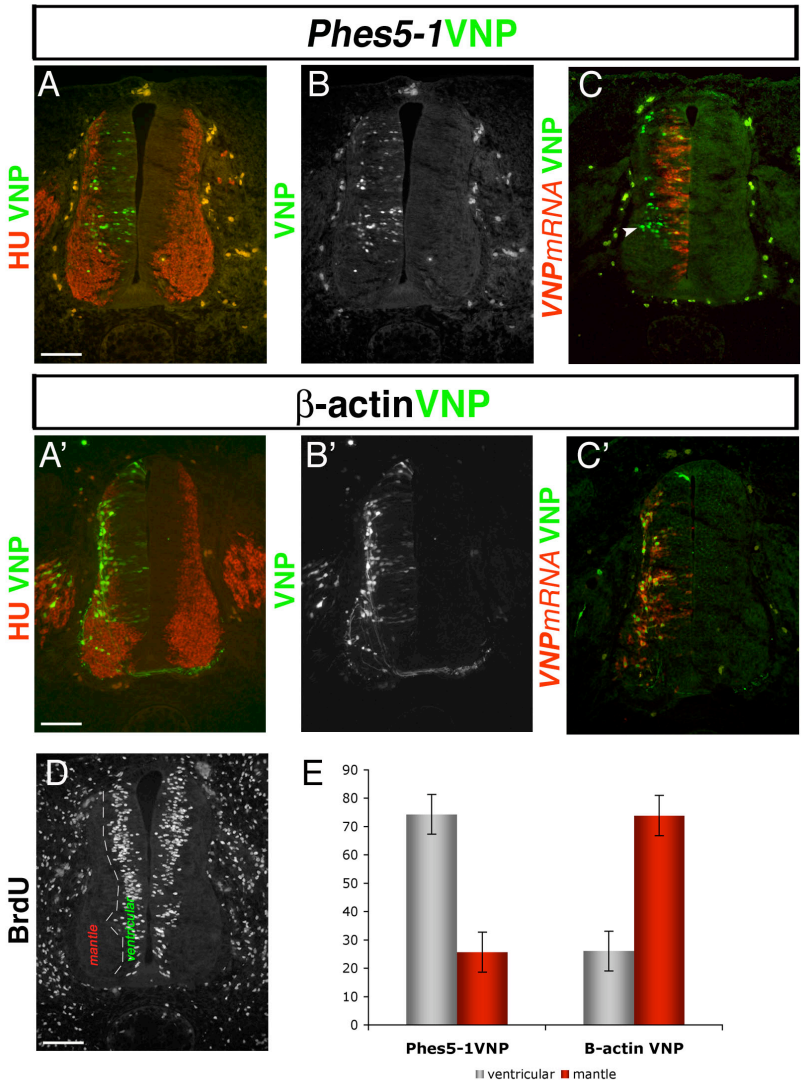


Fig.8 Distribution of Phes5-1VNP reporter activity in the chick neural tube. A-C'. 48hpt with the vectors Phes5-1VNP and β-actin-VNP, electroporated embryos were analysed for the relative distribution of VNPprotein (**B, B'**) with the HU neuronal marker (**A, A'**) and VNP mRNA (**C, C'**) was analysed. **A'-C'. D**. Transverse section of a wt embryo subjected to a 30'pulse of BrdU. **E.** Chart illustrating the distribution of the VNP positive cells under the control of the *hes5-1* promoter vs VNP under the control of the β-actin promoter in the Vz (light grey) and in the mantle layer (red). Scale bars = 50μm.

By contrast, when Phes5-1VNP was transfected the distribution of the VNP expressing cells had a strikingly opposite distribution: 74,3% ± 7sd in the Vz (n=2842 cells, 10 sections, 4 embryos) and 25,6% in the mantle layer (Fig.8B and E).

These results show that the promoter is restricting VNP expression to the ventricular zone, which was expected since *hes5-1* is expressed in the Vz. However, not 100% of the cells with reporter activity are in the ventricular region: 25,6% of the electroporated cells were found out of this layer. This could be due to either promoter leakness or to VNP stability. If the promoter is leaky, VNP mRNA should be found in

these cells located out of the Vz. On the other hand, if it is a problem of VNP stability the VNP mRNA should be only in the Vz and the cells out of the Vz would only express the protein and not the mRNA.

To test these two possibilities, the VNP protein was visualized using an anti-GFP antibody, after *in situ* hybridization for VNPmRNA (Fig.8C and C').

Although not all the VNP cells are visualized due to epitope destruction after the *in situ* procedure, we could detect VNP-positive cells that do not express the VNP mRNA in the Phes5-1VNP electroporated embryos (Fig.8C arrow). This implies that the VNP protein is still present in cells that no longer express the VNP mRNA. Moreover, these cells that no longer express the VNPmRNA are cells that are located out of the Vz, suggesting that these might be cells that have activated Notch – *hes5-1* expression, but subsequently undergone the neuronal pathway and migrated out of the Vz.

IV.3.6 **Notch signalling induces Phes5-1VNP reporter expression**

The results presented above show that the Phes5-1VNP reporter could drive the expression of VNP mRNA in the same expression domain as the endogenous *hes5-1* gene. *hes5-1* expression is regulated by Notch signalling, since it is induced when NICD is ectopically expressed and is downregulated when Notch signalling is reduced (Fior and Henrique, 2005).

To examine whether the *hes5-1* reporter responds to Notch signalling, two groups of embryos were electroporated: one group (control) in which the Phes5-1VNP reporter was co-electroporated with a control of electroporation efficiency vector- β-actin-nRFP [nuclear Red Fluorescent Protein driven by the constitutively active β-actin promoter], and another group where the same vectors were co-electroporated with a constitutively active form of the Notch receptor-NICD, driven also by the β-actin promoter (β-actin -NICD).

First, the kinetics of the fluorescent signal increase was followed *in vivo* over time, taking pictures with the same exposure (2.5 seconds) every hour. When NICD was co-electroporated with the Phes5-1VNP reporter, VNP expression was detected as soon as 4hpt and its intensity strongly increased with time (Fig. 9A-D). By contrast, in the absence of constitutive Notch activation, similar levels of reporter activity could only be detected 24h after electroporation (Fig. 9H-I). Signal was strikingly stronger in

presence of NICD, strongly suggesting that the reporter responds to Notch signalling (Fig. 9 compare C/D with H/I, n=10).

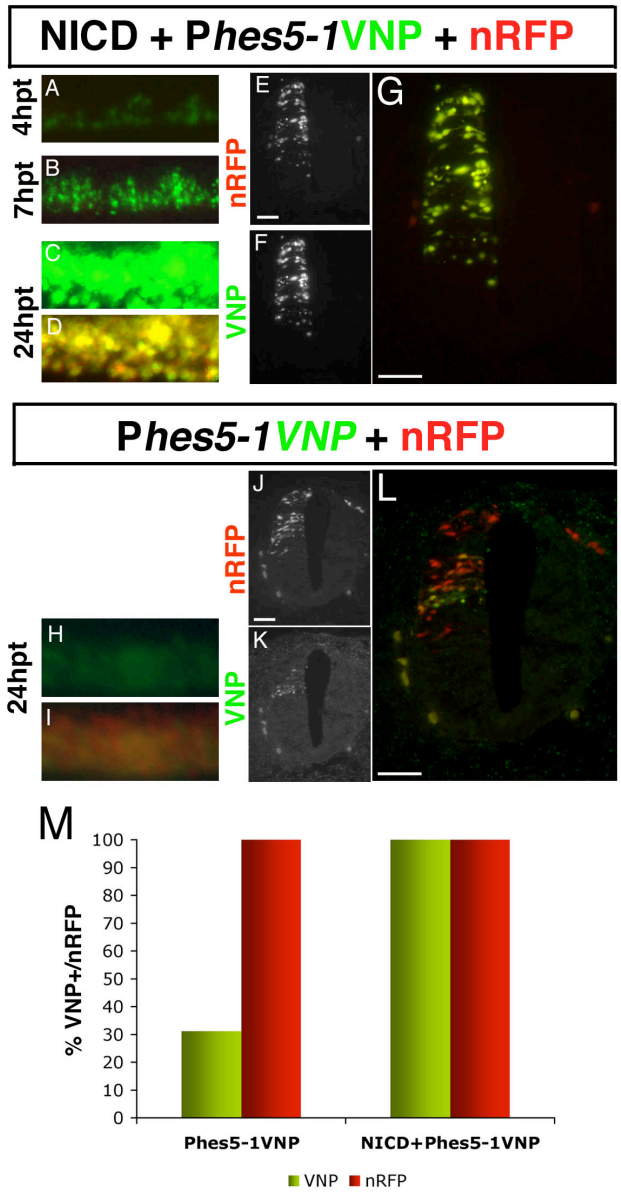


Fig.9 The Phes5-1VNP reporter responds to Notch signalling. **A-D and H-I**.*In vivo* time course of VNP intensity in embryos electroporated with [A-D-β-actin-NICD+Phes5-1VNP+β-actin-nRFP] and [H-I-Phes5-1VNP+β-actin-nRFP] and
D and I are merged images of the green (VNP) and red (nRFP) signals **E-G and J-L.** Transverse sections of the embryos electroporated with [β-actin-NICD+Phes5-1VNP+β-actin-nRFP] and [H-I-Phes5-1VNP+β-actin-nRFP] 24hpt. **M**. Chart illustrating the % of electroporated cells that express VNP under the *hes5-1* promoter in the absence (31,7%) or presence of NICD (>100%). Scale bars = 50μm

Next, the number of VNP and nRFP cells was quantified 24h post electroporation. In the control group the number of nRFP positive cells was higher than VNP positive cells: only 31,7% ± 8.1sd of the transfected cells (RED) (n=806 cells, 3 embryos, 19 sections) express the reporter (VNP) (Fig. 9M). This was expected since the *hes5-1* promoter is not constitutively active.

By contrast, when β-actin-NICD is co-transfected with Phes5-1VNP reporter and β-actin-nRFP, 101.4% ± 2,36 of the transfected cells (RED) are VNP positive (n=1125

cells, 3 embryos, 17 sections) (Fig. 9M). The percentage is higher than 100% probably because the Venus protein is brighter and faster maturating, enabling a better detection than the nRFP fluorescent protein.

These results indicate that the Phes5-1VNP reporter is being activated by NICD.

IV.3.7 **Time-lapse imaging of the reporter Phes5-1VNP system**

The results above show, that the VNP expression driven by the Phes5-1VNP reporter recapitulates the pattern of endogenous *hes5-1* expression and that the reporter responds to Notch signalling.

Although the VNP protein is too stable to be an ideal reporter for *hes5-1* mRNA expression, the vector can still be used as a reporter of Notch activity; it is activated by Notch and is expressed in the right cells at the right time.

To monitor the behaviour of the Phes5-1VNP reporter *in vivo*, this reporter was electroporated into the chick neural tube and embryos were collected 4h later. Neural tube slices were quickly prepared and visualized under confocal microscopy for periods up to 12h. Fluorescence images were recorded in a confocal Zeiss microscope spanning 42 z stacks with 3 m step size at 10.4 min intervals up to 12h.

The individual fluorescent cells were identified manually, and the stacks with higher fluorescent signal were followed manually throughout the time-lapse series to create the movies (supplementary material).

Initial analysis of the fluorescent profiles of single nuclei revealed a dynamic expression of the *hes5-1* reporter, which can be grouped in four categories (Fig.10):

I- Maximum expression sustained for 2-3.4h followed by downregulation of expression to background values (less than 50 Fluo int.)(n=4).

II- Maximum expression sustained for 2-3.4h followed by downregulation of expression to 100-150 Fluo int.(n=3).

III- Maximum expression sustained for 40min-1h followed by downregulation of expression until 100-150 Fluo int., followed again by upregulation until maximum intensity for another hour, to be downregulated to background values (less than 50 Fluo int.) (n=4) [movie in supplementary material].

IV- Oscillating levels of expression between 50-150 Fluo int. with a period of +/- 40 min (n=3).

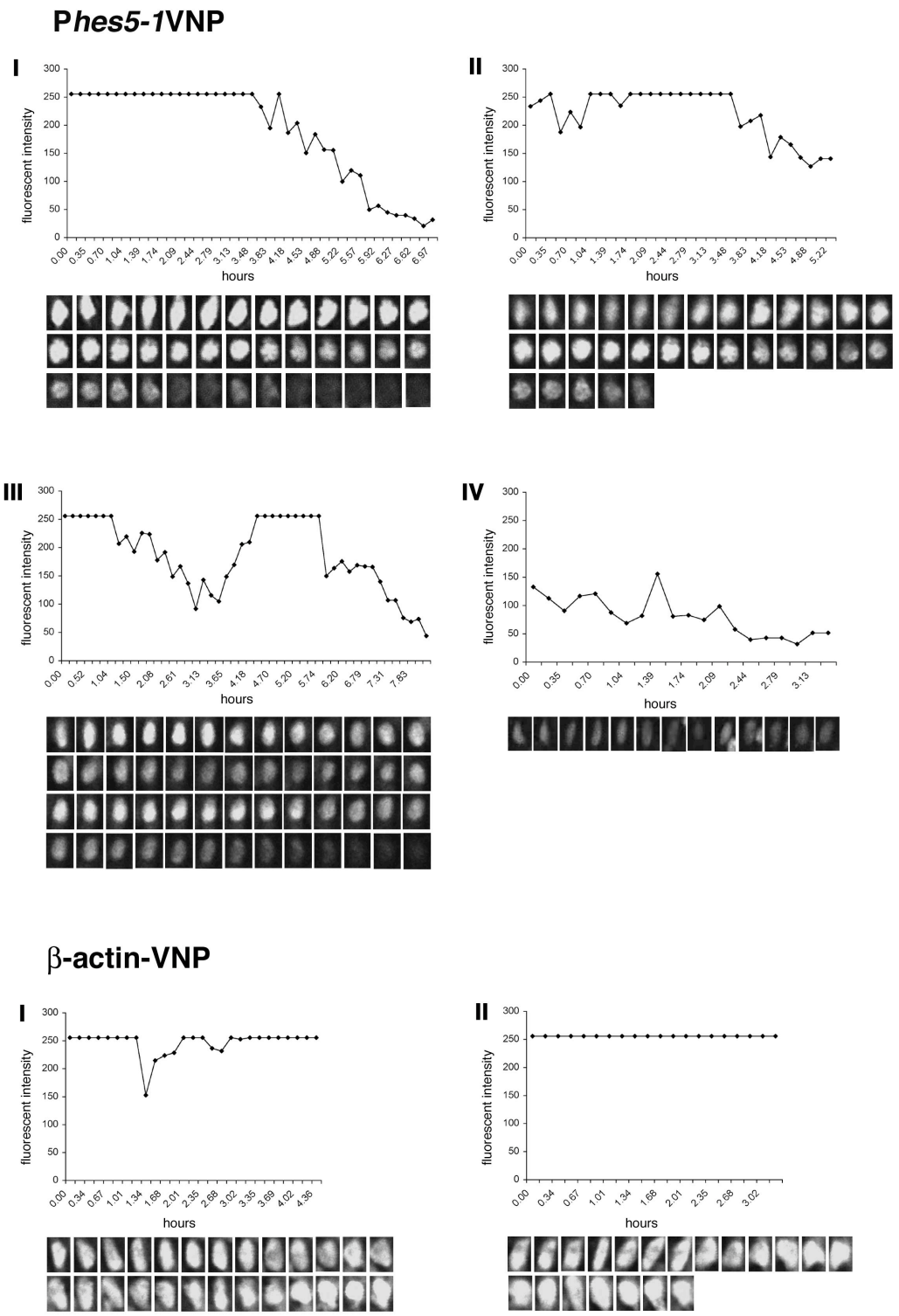


Fig.10 Real time imaging of *hes5-1* reporter at the single cell level. Single cell profiles of fluorescent intensity of cells expressing VNP under the control of *hes5-1* promoter can be classified in four categories I-IV (see text for details). Analysis of the fluorescent profiles of single nuclei in which the VNP is under the control of the constitutively active β-actin promoter, showed constant maximum expression with no apparent oscillatory behaviour. Movies are supplied in a CD in attachment.

By contrast, analysis of the fluorescent profiles of single nuclei in which the VNP is under the control of the constitutively active □-actin promoter, showed constant maximum expression (Fig. 10) and no apparent oscillatory behaviour.

These initial results reveal that *hes5-1* shows a dynamic expression pattern and that the *hes5-1* reporter system provides a useful tool to monitor Notch activity. In the future with a systematic and statistical analysis it will be possible to determine whether oscillation of *hes5* expression indeed occur.

IV.4. DISCUSSION

In this study, I have generated a real-time imaging system to analyse *hes5-1* expression with single-cell resolution during chick neurogenesis.

A 2Kb fragment of the *hes5-1* genomic sequence was used to drive expression of a destabilized form of the Venus fluorescent reporter. This reporter faithfully recapitulates the endogenous expression pattern of *hes5-1* and responds to Notch signalling, providing a useful tool to monitor Notch activity in real-time with a high spatial and temporal resolution.

Preliminary analysis of time-lapse imaging of the reporter revealed that *hes5-1* has a dynamic gene expression pattern. The dynamic single cell profiles also suggest that indeed Notch signaling could be activated in a reiterative manner.

IV.4.1 The chick *hes5-1* promoter comprises a S1-S3 architecture

The chick *hes5-1* promoter was considered the best candidate to be a Notch reporter in the chick neural tube because it is expressed throughout the *Notch* expression domain, is activated by NICD (Fior and Henrique, 2005) and its promoter is closely related to the mouse and human *hes5* genes. Only *hes5* has been shown to be directly regulated by the Notch pathway *in vivo* during neurogenesis: its expression is downregulated in *Notch1* and *CBF-1* mutant mice, whereas expression of *hes1* (which is widely used to monitor Notch activation) is hardly unaffected during CNS development (de la Pompa *et al.*, 1997, Lutolf *et al.*, 2002).

The detailed analysis of the chick *hes5-1* promoter and comparison with other HES promoters revealed that *hes5*-like genes have a distinct promoter configuration than

hes1-like genes. They contain a S3-S1 architecture instead of the classical SPS configuration. In addition, they present a E box conserved in position and orientation (Fig.11).

Fig.11 Two different types of S architectures can be found in *hes* genes. The SPS is found in *hes1*-like genes and the S3IS1 architecture is found in many *hes5*-like genes.

The S3-Ebox-S1-CAAT architecture is present in all *hes5*-like genes promoter regions with exception of *Xenopus xEsr10*.

Strikingly, the *xEsr1* promoter region, which has S3-S1 configuration, is activated by NICD 100-fold over reporter alone, levels 10 times greater than those seen in comparable essays of *xEsr10* and *mhes1*, which have the classical SPS configuration (Lamar and Kintner 2005).

Besides *hes5*-like genes, there are many other Notch target genes that do not have SPS binding sites, as for example *Vg* (Kim *et al.* 1996), *CyclinD1* (Ronchini and Capobianco, 2001), *p21* (Rangarajan *et al.*, 2001; Talora *et al.* 2001), *Nrarp* (Pirot *et al.*, 2001; van Grunsven *et al.* 2004), *Hey* (Maier and Gessler 2000), *Nodal* (Raya *et al.* 2003), *E(spl)mδ* (Nelesson *et al.*, 1999; Castro *et al.*, 2005) and *E(spl)mβ* (Nelesson *et al.*, 1999), suggesting that other S architectures are important for a proper Notch activation.

IV.4.2 The reporter recapitulates *hes5-1* endogenous expression

I have generated a fluorescent reporter system to follow in real-time the expression of the Notch target gene *hes5-1*. The reporter must be short lived to account for transient Notch activation and to visualize possible *hes5-1* oscillations/fluctuations in gene expression. In order to destabilize the reporter, a PEST sequence and the 3'UTR of *hes5*-1 was fused to the Venus fluorescent protein. The Venus reporter contains a nuclear localization signal (NLS) to increase the fluorescent intensity within the cells in order to facilitate fluorescent quantification.

To drive expression of the reporter, I used a 2Kb *hes5-1* regulatory sequence that contains putative sites both for Notch and proneural induction (10 CSL binding sites + 15 E boxes) as well as for HES negative feedback (4 N boxes).

The results show that VNPmRNA expression under the control of the *hes5-1* promoter (Phes5-1VNP) was restricted to the ventricular zone (Vz), indistinguishable from the *hes5-1* endogenous expression pattern (Fig.6 compare A and B).

Double *in situ* hybridization for VNP and the *hes5-1* coding region, followed by confocal analysis, confirmed that the reporter was being expressed in cells that express the endogenous *hes5-1* gene. Furthermore, the reporter is detected in mitotically active progenitor cells, like the endogenous *hes5-1* expressing cells (Fig. 7).

Therefore the results show that the 2kb genomic fragment of *hes5-1* contains the essential regulatory information to elicit expression of the reporter in neural progenitors that express *hes5-1*.

IV.4.3 Spatial and temporal distribution of the VNP reporter

During development of the vertebrate CNS, neurons are generated from the progenitor cells that lie within the ventricular zone of the neural tube, and after terminal mitosis they migrate outside this region and accumulate in the mantle zone. The endogenous *hes5-1* expression is restricted to the ventricular zone in neural progenitors (Fior and Henrique, 2005) and VNPmRNA under the *hes5-1* promoter is also restricted to the same region.

The HES5-1 protein distribution is unknown since no antibodies are available. However, since the related mouse HES1 protein is short lived (Hirata, 2002) it is expectable that HES5-1 protein will also have a quick turnover, and that the spatial distribution of the protein will be similar to the mRNA, being restricted to the ventricular zone.

We found that the majority (73,4%) of VNP expressing cells driven by the *hes5-1* promoter are located in the Vz. However, not 100% of the cells are in the ventricular region, 25,6% were found out of this layer. This seems to be due to VNP protein stability since these cells expressed the VNP protein but no longer expressed the VNPmRNA and were located out of the Vz.

To overcome these problems, the half-life of the endogenous *hes5-1* mRNA and protein must be determined and several fluorescent reporters with shorter half-lives

must be engineered, in order to select the one with the most similar temporal dynamics to the endogenous HES5-1 protein. To decrease VNP protein half-life more protein destabilizing elements such as one or two copies of Ubiquitin can be added (Luker *et al.*, 2003, Voon *et al.*, 2005).

IV.4.4 Phes5-1VNP reporter is induced by Notch signalling

hes5-1 is a bonafide target of the Notch pathway as its expression is induced when the Notch receptor is activated and downregulated when Notch signalling is impaired (Fior and Henrique, 2005). Our results show that the Phes5-1VNP reporter is induced by Notch signalling implying that this reporter can be used to detect Notch signalling *in vivo*, in the chick neural tube. Nevertheless, further confirmation of Notch regulation requires mutation of the crucial S boxes, presumably S1 and S3, and demonstration that mutation of these boxes abrogates promoter expression and no longer responds to NICD.

IV.4.5 Dynamic behaviour of *hes5-1* expression

Preliminary time-lapse analysis of single nuclei revealed a dynamic expression of the *hes5-1* reporter, however with different behaviours, which were grouped in four categories. The results suggest that a cell is able to respond at least twice to Notch signalling (Fig.10-III, movie supplementary material), since levels of *hes5-1* expression reach maximum levels twice. This may suggest that progenitor cells are able to respond to Notch signalling in a reiterative manner until they finally differentiate. These preliminary results are in agreement with the data from Mizutani and Saito (2005) where this authors show that progenitors that had been temporarily subjected to Notch activation at an early stage can generate neurons at later stages.

It is noteworthy to say that these cells that activated Notch signalling twice did not divide, suggesting that a progenitor cell after receiving a Delta signal and activating Notch does not necessarily enter in S phase and mitosis. This is in agreement with our previous studies where overexpression of NICD or HES5 did not lead to an increase in cell proliferation (Fior and Henrique, 2005), instead cells remained within the ventricular zone even 48hpt, without differentiating but also without accelerating the cell cycle (ChapterIII, Fig7, Fig10).

However, a more systematic and statistical analysis of the profiles of single nuclei is mandatory to interpret the dynamic behaviour of *hes5-1*. Moreover, by combining this reporter with a proneural or *Delta* reporter it will be possible to follow the fates of the progenitor cells and therefore correlate Notch activity profile with the behaviour of the neuroepithelial cells.

Chapter V

General Discussion

General Discussion

The building of the vertebrate nervous system involves the production of a large number and variety of neurons and glial cells. The differentiation of neural progenitors into the various types of neurons and glia occurs over a long period of time. This prolonged process allows progenitor cells to change not only their intrinsic competence but also to 'listen' to the extrinsic cues that change during development, permitting the diversification of cells fates (Edlund and Jessell, 1999). Therefore, mechanisms must exist to restrain differentiation and temporarily maintain progenitor cells in an undifferentiated state so that they can respond to later differentiation signals.

Notch signalling through Lateral Inhibition is one of the major mechanisms to restrain neural differentiation and maintain a pool of progenitors throughout neural development (Lewis, 1998). The present study focus on the molecular events that occur downstream of Notch activation during neurogenesis, aiming to understand the logic that underlies the controlled production of neurons throughout development.

In Chapter III, I described the expression, function and regulation of four novel *hes* genes that are expressed in the chick developing nervous system: three *hes5*-like genes (*hes5-1*, *hes5-2* and *hes5-3*) and one *hes6*-like (*hes6-2*). All four *hes* genes are expressed in the ventricular zone of the embryonic neuroepithelium, where neural progenitors are located and where the *Notch1* receptor is expressed. I showed that Notch signalling positively regulates the *hes5* genes but reduces expression of *hes6-2*. Moreover, this work showed that *hes5* genes are downstream effectors of Notch signalling, inhibiting neuronal differentiation. By contrast, *hes6-2* acts as a negative regulator of Notch signalling, cooperating with proneural proteins to induce neuronal differentiation.

Importantly, this work also revealed a new circuitry of feedback regulation between the *hes* genes during neurogenesis: each HES5 protein is able to repress *hes6-2*, and all

three *hes5* genes are repressed by HES6-2. In addition, two of the *hes5* genes (*hes5-1* and *hes5-2*) are also able to negatively regulate their own expression. I propose that this HES5/HES6 circuitry of negative feedback regulation functions to restrict the duration of Notch signalling in neural progenitors, ensuring that the pathway is shut down after each event of Notch activation. Progenitors could then go back to a state where they are again competent to respond to environmental cues and decide their fate. Vertebrate neurogenesis can thus be viewed as a reiterative process in which progenitors activate the Notch signalling cascade transiently, time and again, until they finally commit to differentiation.

In Chapter IV, I describe experiments developed to test this model and determine if pulses of Notch activity indeed occur during neurogenesis. A real-time imaging system to monitor Notch activity *in vivo* was developed, using a reporter construct composed of the *hes5-1* promoter fused to a destabilized fluorescent protein. This reporter recapitulates the endogenous pattern of *hes5-1* expression and responds to Notch signalling, thus providing a valuable tool to follow individual cells experiencing Notch activity in real time, and study their behaviour throughout neurogenesis. The first results obtained with this system revealed a dynamic pattern of Notch activity, suggesting that Notch signalling may indeed be activated reiteratively in neural progenitors and that fluctuations of *hes5* expression occur in these cells.

Here, I will further discuss several points previously examined in Chapters III and IV and discuss and explore the model that has been developed during the course of this work.

V.1 On the genomic organization of the vertebrate *hes* genes

In 'silico' analysis of the chick genome reveals that the three *hes5* genes are clustered on a 20 Kb region of DNA in Chromosome 21, next to the *fang1* gene (ChapterIII-Fig. 2). A similar cluster of three *hes5*-like genes is present in the pufferfish genome (Release 23.2c.1), located also next to the *fang1* gene, suggesting that the *hes5* cluster has been conserved from teleosts (a similar cluster is present in zebrafish) to avians. The chick *hes6* genes (*hes6-1* and *hes6-2*) are also linked in Chromosome 9, in close proximity with the gene *period*. In mouse and human, only one *hes6* gene exists and is also linked to the gene *period* at Chromosome 1 and 2, respectively.

In zebrafish, several pairs of *her* genes (*hes*-related genes) seem to be linked as well (see Table 1). Similarly to the chick, where the three *hes5* genes operate during neurogenesis, pairs of genes in zebrafish have been shown to operate in the same developmental processes: *her1* and *her7* are part of the molecular clock that controls somitogenesis (Henry *et al.*, 2002; Oats *et al.*, 2002), whereas *her5* and *her11* regulate the formation of the midbrain-hindbrain boundary (Geling *et al.*, 2004; Ninkovick *et al.*, 2005) (Table 1).

In *Drosophila melanogaster*, the seven genes which encode E(spl)-like bHLH-O proteins are all present in the E(spl) complex and the architecture of this complex has been conserved throughout the 60 million years that separate *D.melanogaster* and *D.hydei* (Maier *et al.*, 1993). Given that the function of individual *E(spl)* genes seem to be redundant during *Drosophila* development (Davis and Turner, 2001), it is intriguing why this cluster architecture of redundant genes has been maintained. One possibility is that the clustering of redundant genes (or genes that can compensate for the loss of each other) permits the sharing of regulatory modules. Shared modules can be used to direct expression of genes which cooperate in the same developmental program, in the same expression domains (Gibert and Simpson, 2003). In addition, sharing of enhancer modules presumably could facilitate auto- and cross-regulation and the establishment of regulatory feedback-loops.

Table 1 The genomic organization of some vertebrate *hes* genes in different animal models

zebrafish		chick		mouse	
gene	Chr	gene	Chr	gene	Chr
her1 /her7	*5*	*hes5-1/hes5-2/hes5-3*	*21*	*hes1*	*16*
her6	*6*	*hairy1b*	*21*	*hes2*	*4*
her5/her11	*14*	*hairy2*	*9*	*hes3*	*4*
her4/her9	*23*	*hes6-1/hes6-2*	*9*	*hes5*	*4*
her8	*?*			*hes6*	*1*
her12	*?*			*hes7*	*11*
her8.2/her13.2?	*?*				
her15					

As already mentioned, a cluster-like organization has been also conserved in zebrafish and chick, where *hes* genes operating in the same developmental process seem to be organized in clusters. Mammalian *hes* genes, however, are not organized in clusters (Table 1). Although *hes2*, *hes3* and *hes5* are present in the same Chromosome in

mammals, they are spread within a 3 Mb region, with *hes5* located close to *fang1*. In the chick genome, *hes5-1* is also close to *fang1*, suggesting that mammalian *hes2* and *hes3* derive from an ancestral *hes5* cluster but have been dispersed in the Chromosome.

The rule of conservative changes states that 'only those changes that change essentially nothing can be tolerated' (reviewed in Ghysen, 2003). It is possible, therefore, that evolutionary pressure was exerted on the regulatory modules to guarantee that the overall regulation, including auto- and cross-regulations, is maintained, rather than in the preservation of several redundant genes organized in genomic clusters.
Accordingly, although mouse *hes1* and *hes5* are located in different chromosomes, cross regulation between these *hes* genes also seems to occur, since mouse mutants for *hes1* and *hes5* reveal upregulation of *hes5* and *hes1* transcripts, respectively (Cau *et al.*, 2000; Hatakeyama *et al.*, 2004).

Moreover, the promoter regions of *hes* genes have maintained several independent transcriptional modules throughout evolution, from *Drosophila* to vertebrates, including mouse and human. These enhancer modules are characterized by having CSL binding sites (S boxes) together with E boxes (Nellesen, 1999; Gajewski *et al.*, 2002; Lamar and Kintner, 2003; Ong *et al.*, 2005; Cave *et al.*, 2005; this work) and can occur in at least two different types of configuration (Fig.1A):

- The SPS (pair of S sites in a head to head orientation) + E box configuration (SPS+E), present in some of the *hes* genes as mouse *hes1* and *Drosophila E(spl)m8 and m*□ (Nellesen *et al.*, 1999; Gajewski *et al.*, 2002; Cave *et al.*, 2005). These promoters also contain E boxes next to the TATA box, however this is not true for all the genes within this group.
- The S3-E-S1 configuration, present in some of the *hes5* homologues as chick *hes5-1*, mouse *hes5,* human *hes5* and *Xenopus Esr1* (Lamar and Kintner, 2003; Ong *et al.*, 2005; this work). In this configuration the E box is conserved in sequence, position and orientation.

In addition to the SPS+E and S1-E-S3 conserved modules, *hes1* and *hes5-1* proximal promoter regions contain more S and E boxes, as well as several N boxes (Fig. 1B), which might underlie the negative auto and cross-regulation between *hes* genes.

In summary, although the mammalian Notch-responsive *hes* genes present a different genomic architecture and are not organized in clusters, they have maintained similar transcriptional regulatory modules (S and E boxes) and are able to cross regulate each other.

V.2 The neurogenesis 'syntagm' is printed in the promoter regions of the network players

Besides the conservation of the binding sites for CSL/NICD and proneural proteins in the promoter regions of *hes* genes, the promoter regions of proneural genes have also regulatory modules highly conserved throughout evolution, from Cnidarians to vertebrates. These include binding boxes for the HES-like repressors (N boxes), suggesting that the relationship between proneural and *hes* genes has been maintained for at least 600-700 million years (Rebeiz *et al.*, 2005). In addition, similarly to the *Drosophila Delta* promoter region (Kunisch *et al.*, 1994), it has been recently shown that the mouse, chick and zebrafish *Delta1* promoter region also contains binding sites for proneural proteins (Castro *et al.*, 2006).

Overall, this suggests that evolutionary pressure contributed to maintain the Proneural/Notch/HES 'syntagm' (Fig.1C). By maintaining regulatory modules in the promoter regions of the Notch-responsive *hes* genes, proneural and *Delta* genes, the cross-interaction network is guaranteed, regardless of whether the genes are organized in cluster or not, and dispersal was allowed since it did not change the overall coherence/function of the circuit (Ghysen, 2003).

Thus, the ancient relationship between proneural, *Notch*, *Delta* and *hes* genes – the "syntagm", seems to be 'printed' in the promoter regions of the network players (Fig.1.C). While the E boxes in the proneural promoters allow positive autoregulation, the N boxes permit negative regulation by the HES repressors. *hes* promoters also seem to contain conserved N and E boxes, which permit negative autoregulation and positive regulation by proneural proteins, respectively. Finally, E boxes in the *Delta* promoter provide the inter-cellular link between proneural and *Notch*/*hes* genes (Fig.1C): activation of *Delta* transcription by proneural proteins will lead to activation

of Notch in neighbouring cells and, thereby, to transcriptional up-regulation of the *hes* genes (binding of NICD-CSL to the conserved S boxes in *hes* promoters).

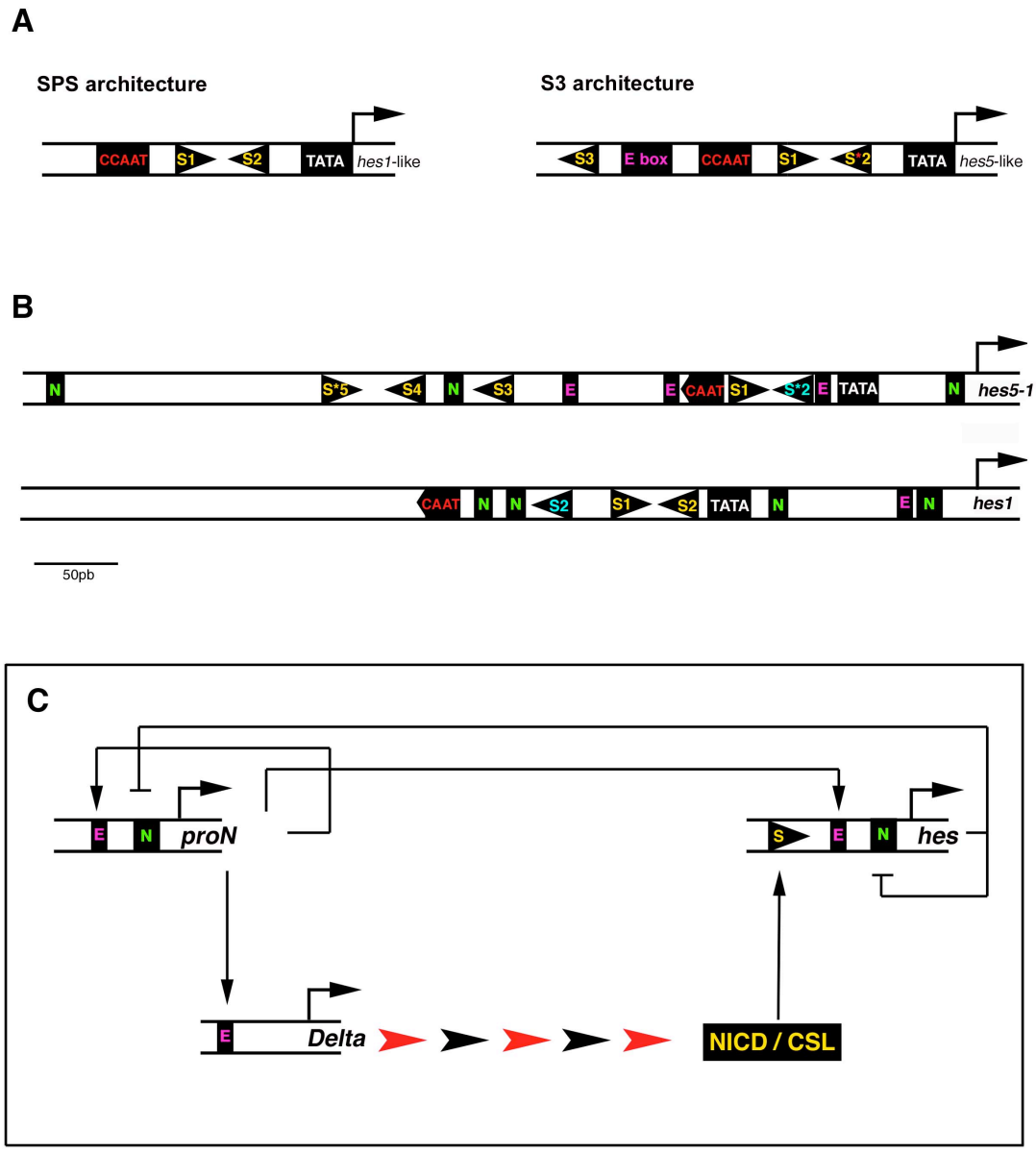


Fig.1 Notch responsive promoters architecture and the neurogenesis 'syntagm'. A.Two different types of conserved configurations of *hes* proximal promoter regions. *hes1-like* promoter regions have been shown to contain a pair of S sites in a head to head orientation (SPS), flanked by a TATA box at 3' and an inverted CAAT box at 5' (Ong *et al.*, 2005, Nellesson *et al.*, 1999). In addition, these promoters also contain an E box near the TATA box. In contrast, *hes5-like* proximal promoter regions contain a suboptimal SPS, also flanked by a TATA and an inverted CAAT box, in which the S2 presents two or more mismatches (*). However, *hes5-like* proximal promoter regions also contain another S box and E box upstream, both highly conserved in sequence, position and orientation (S3) (Lamar and Kintner, 2005; this thesis-ChapterIV). **B**.Detailed description of the several S, E and N boxes present in the chick *hes5-1* and mouse *hes1* proximal promoter regions. **C**-The Neurogenesis 'SYNTAGM'- the conserved web of genetic cross-interactions is "printed" in the promoters of the players. High affinity S boxes are in yellow, low affinity S boxes in light blue, E boxes in pink and N boxes in green.

V.3 Neurogenesis as a reiterative process

Although there has been great progress in understanding the principles that govern embryonic development (Wolpert, 1996, 2006) how the timing of developmental events is controlled is still poorly understood and unexplored. Actually, during normal development, it is important not only to control the timing of events that lead to the activation of signalling cascades but also to limit the activity in time. Thus, mechanisms to terminate signalling are crucial to control the duration of the signal and allow responding cells to progress in their developmental path. Failure in temporal control may cause disease, like T cell acute lymphoblastic leukemia (T-ALL), which involves unrestrained Notch activity in T cells (Elissen *et al.*, 1991; reviewed in Lai, 2004).

This work, by taking into account the kinetics of Notch signalling during neurogenesis provides a novel perspective of the logic that underlies the controlled production of neurons. Moreover, a mechanism to control the duration of Notch signalling is also advanced, based on the new circuitry of *hes* gene regulation involving the *hes5* and *hes6* genes.

During neurogenesis progenitor cells have to decide again and again whether they are to remain as progenitors or differentiate. Since this decision is based on Notch-mediated Lateral Inhibition, this implies that the Notch signaling cascade is activated transiently in neural progenitors, in a reiterative manner, producing pulses of Notch activity and, therefore, of *hes* expression. This also means that, after each pulse of Notch activity, the signaling cascade has to be downregulated in order to allow progenitors to become competent again to respond to environmental cues and start a new cell-fate decision process.

One way to achieve this down-regulation involves the rapid degradation of the nuclear form of the Notch receptor (NICD) triggered by the co-activator MAM (Fryer *et al.*, 2004). However, the activity of the downstream effectors, namely the *hes* genes, has also to be restrained. In this work, I propose that the circuitry of negative auto- and cross-regulation of HES activity is also included in the neurogenesis 'syntagm' and plays an active role to promote the complete desensitization of the Notch pathway in neural progenitor cells in order to sustain the cycles of Notch activity.

In the mouse the scenario seems simplified: instead of the cross regulation between three *hes5* genes, probably negative auto and cross-regulation of *mhes5* and *mhes1* (Hatakayama *et al.*, 2004) will be sufficient to shut down the pathway after each Notch activation event, and mHES6 would play the equivalent role to HES6-2 in nascent neurons, inhibiting any residual Notch activity in these newly committed cells (Koyano-Nakagawa *et al.*, 2000; Bae *et al.*, 2000).

V.4 Real-time imaging of *hes5-1* expression with single cell resolution

To test this model and visualize Notch activity during chick neurogenesis, a real-time imaging system with single-cell resolution was developed, based on the transcriptional output of the Notch target gene *hes5-1*. A 2Kb fragment of the *hes5-1* promoter region was used to drive expression of a destabilized form of the Venus fluorescent protein. Results show that this reporter faithfully recapitulates the endogenous expression pattern of *hes5-1* and responds to Notch signalling.

Initial analysis of this reporter system to follow *hes5-1* transcription in neural progenitors revealed a highly dynamic transcriptional regulation of this gene. Moreover, single cell analysis suggests that Notch signalling in individual progenitors could indeed be activated in a reiterative manner, supporting the hypothesis that progenitor cells do not maintain sustained levels of Notch activity for long periods, but instead go through bursts of signalling, each time a cell fate decision process occurs.

This is consistent with previous findings in *Drosophila*, where it has been shown that neurectodermal cells which expressed *E(spl)* genes as result of Notch activation can subsequently re-enter the neural pathway (Jennings *et al.*, 1994). Thus, although Notch-E(spl) activity does inhibit neuroblast segregation, inhibited cells are competent to respond to a subsequent signal and become neuroblasts.

Similar findings in the mouse embryo are also consistent with the above hypothesis: as shown by Mizutani and Saito, neural progenitors which have been temporarily subjected to Notch activation at an early stage might generate neurons at later stages, skipping the early neural fate (Mizutani and Saito, 2005). These experiments show that Notch activity does not cause a permanent block on the progenitors' competence to differentiate, which are still able to respond to the appropriate cues.

V.5 Neurogenesis could be controlled by two interacting loops

During vertebrate somitogenesis, Notch signalling reveals a cyclic activation pattern that coincides with the period of somite formation (reviewed in Giudicelli & Lewis, 2004). Several genes from the Notch pathway and from other signalling pathways (Dequeant *et al.* 2006) have been shown to transcriptionally oscillate in cells of the presomitic mesoderm, as part of a molecular machinery that regulates the periodicity of somite formation. It has been proposed that the generation of such cyclic gene oscillations relies on two interacting loops: an intracellular negative feedback-loop established by HES transcription factors on the promoter of their own genes, and an inter-cellular loop involving Notch activation by Delta in adjacent cells (Lewis, 2003). The first loop should drive a cell-autonomous oscillation of *hes* gene expression based on a time-delayed feedback mechanism, while the second should account for the rhythmic activation of Notch to maintain synchrony between adjacent cells (Lewis, 2003).

One might also consider the neurogenesis 'syntagm' might involve two similar interacting loops, which may also generate an oscillatory behaviour of *hes* gene expression (Fig. 2):

- An inter-cellular loop mediating Lateral Inhibition (Fig.2A): high levels of proneural proteins up-regulate *Delta* expression in the nascent neuron (cell A), which will activate Notch in a neighbouring progenitor (cell B). Notch activation leads to a burst of *hes* transcription in cell B and HES repressors will then inhibit proneural gene transcription and activity, thus impairing neuronal commitment in cell B. Next, negative auto-and cross-regulation of HES repressors on their own genes would downregulate *hes* expression in the neural progenitor. Then, if this progenitor contacts with a Delta-expressing cell, it would activate Notch again and up-regulate *hes* expression once more.

This intercellular loop would therefore result in an oscillatory behaviour of *hes* expression with high amplitude. However, in contrast with what occurs in the PSM, cells in the asynchronous neuroepithelium do not signal at the same time, so these oscillations would have a variable period, dependent on the frequency in which this cell contacts with a Delta-expressing cell.

- An intra-cellular loop established by two negative feedback loops:

A first loop involving the activity of proneural proteins activating expression of their own repressors – the *hes* genes (Kramatschek *et al.*, 1994; Singson *et al.*, 1994; Oellers *et al.*, 1994; Cooper *et al.*, 2000; Castro *et al.*, 2005; Cave *et al.*, 2005; Gazit *et al.*, 2004; Lamar and Kintner, 2005). This first loop was proposed to generate intermediate levels of proneural expression (Meir *et al.*, 2002).

A second loop established by the negative feedback of HES transcription factors on the promoter of their coding genes. Mathematical modelling showed that feedback inhibition with transcriptional delay may account for oscillatory gene expression (Lewis, 2003; Monk, 2003). Thus, it is possible to envision that this intracellular loop (negative auto and cross-regulation between *hes* genes) could generate cell-autonomous oscillations of *hes5* expression, with low amplitude [since these oscillations would occur in the absence of Notch activity]. These *hes5* oscillations would have a fixed period, or not, depending on the time-delays of proteins and mRNA synthesis (Lewis, 2003; Monk, 2003).

Therefore, the intermediate levels of proneural proteins (Meir *et al.*, 2002), together with fluctuations of *hes5* expression could characterize the "neither-ON-nor-OFF" steady state, in which progenitors are ready and competent to receive information to differentiate or not. Thus, the competent state would lie within this 'noisy' low amplitude range of *hes* expression.

It is noteworthy, however, that the negative feedback on proneural expression and their positive autoregulation (Fig.2B) could possibly generate an oscillating behaviour of proneural expression. In this case, the "neither-ON-nor-OFF" could have oscillating antiphasic expression of *hes* and proneural genes. Nevertheless, in order to simplify the rational, I will consider that the "neither-ON-nor-OFF" state is characterized by intermediate levels of proneural expression together with oscillating *hes* expression (Fig.2B).

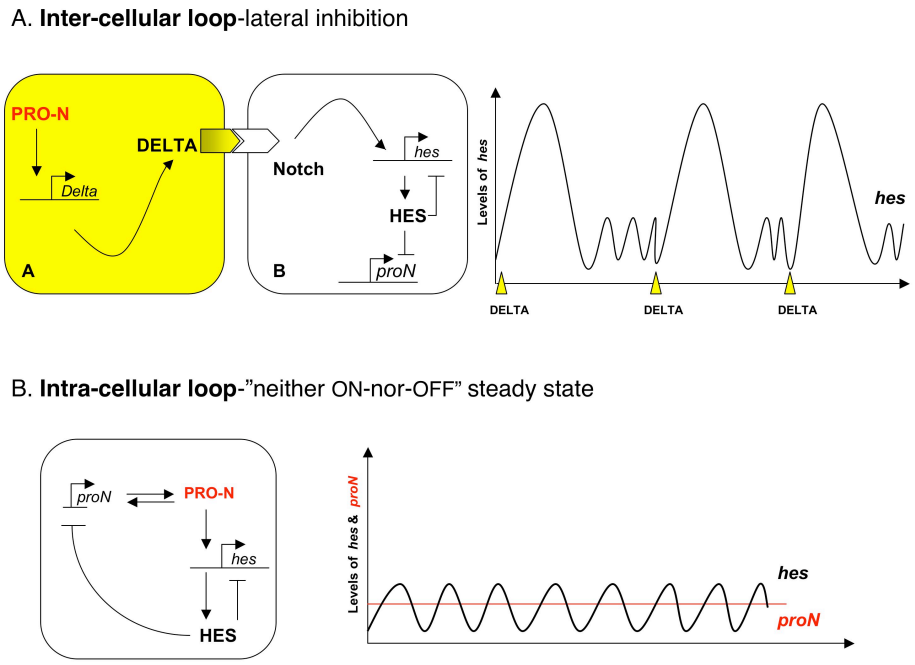


Fig.2 Neurogenesis could be controlled by two interacting loops. A. An intracellular loop based on HES negative feedback with a time delay could possibly generate low amplitude oscillations of *hes5* expression. **B**. An inter-cellular loop based on Delta-Notch signalling would activate HES expression to high levels, inhibiting neurogenesis, thus instructing the cell to remain as progenitors.

As already mentioned, in contrast with the PSM, cells in the asynchronous neuroepithelium do not signal at the same time, so the high amplitude oscillations that result from the inter-cellular loop would have a variable period, dependent on the encounter with a Delta-expressing cell.

Interestingly, this asynchronous activation of Notch in the neuroepithelium could also generate a "side-effect" of Lateral Inhibition – leading to the appearance of a heterogeneous population of progenitors with different levels of *hes* expression (Fig.3). For instance, in time1 (Fig.3.t1), when the first nascent neuron expresses Delta, it will activate Notch in the neighbouring cells. Notch activation then leads to a burst of HES expression (Fig.3 t2), which after a time delay will start decreasing due to auto, cross-regulation and mRNA and protein decay (Fig.3.t3). If in t2 a second Delta expressing nascent neuron is "born", it will activate Notch in the neighbouring cells, leading again to a burst of HES expression in t3. Finally if in t3 a third Delta

expressing neuron is "born", activating Notch in the neighbouring cells, this would generate several states of *hes* expression within the neuroepithelium.

Thus, in contrast to what occurs during somitogenesis, where Notch seems to 'tune' cells to the same behaviour (Jiang et al, 2000; Lewis, 2003), the 'de-phased' and asynchronous Notch activation in the neuroepithelium leads to an heterogeneous progenitor population, with variable and varying levels of *hes* expression (Fig.3).

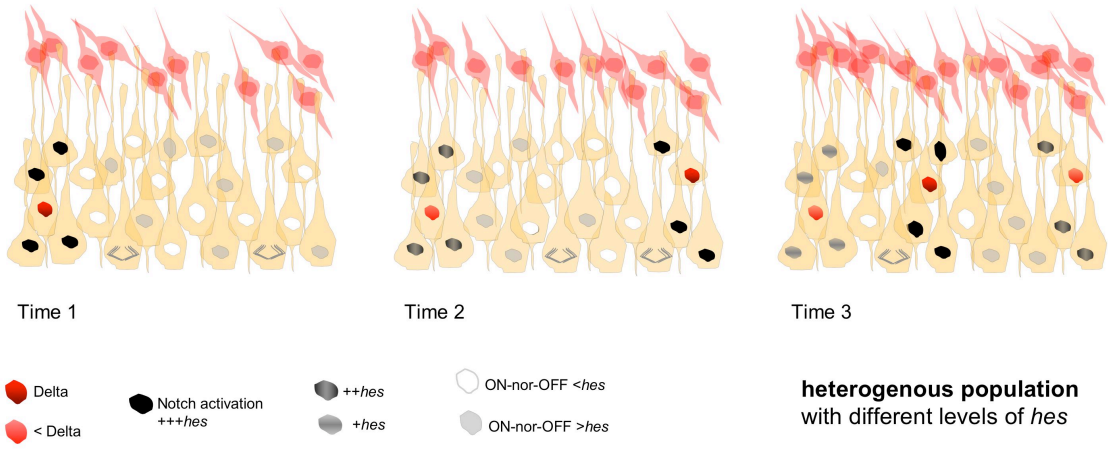


Fig.3 Lateral Inhibition generates progenitors with different levels of *hes* expression. At t3 there will be progenitors at several states of *hes* expression, some with high levels (activated at t3-black), others with intermediate levels (that were activated by Notch at t2-dark grey) and others already with low levels of *hes* (that were activated by Notch at t1-light grey).

V.6 Feedback-loops, fluctuations of *hes5* expression and physiological relevance

Small differences in protein abundance [cellular noise] may confer advantages or disadvantages to development (Raser and O'Shea, 2005). A positive example comes from the nervous system where stochastic activation of an odorant receptor (OR) gene followed by negative feedback may generate the diversity of olfactory neurons (ON), each expressing only one type of the ~1500 OR (Raser and O'Shea, 2005; Serizawa *et al.*, 2004). Since ONs expressing a particular OR gene project their axons to a specific set of glomeri in the olfactory bulb (OB), the odorant stimuli is converted into a topographic map of activated glomeri on the OB. In addition, it has been proposed that intrinsic noise can generate fluctuations in the relative levels of two alleles of the same gene, which may potentially result in cells expressing no allele, one or both alleles. If the two alleles are functionally different, the population of cells may acquire

heterogeneity, which may contribute, for instance, to the phenomenon of hybrid vigour (Raser and O'Shea, 2005).
In this way, a pool of genetically identical cells may exhibit significant diversity even when they have identical histories of environmental exposure (Raser and O'Shea, 2005). Similarly, cells in an equivalent group may have similar potential but not identical.

According to this view, it is possible that the postulated 'side efect' of LI and the "neither-ON-nor-OFF" steady state with the oscillatory 'noisy' expression of *hes* genes could have a positive impact on neurogenesis. Since HES repressors inhibit proneural expression/function, it is possible to envision that the different levels of *hes* expression within progenitors could provide different states of receptiveness to differentiating signals. This implies that even cells within the same equivalence group would respond differently to these signals, thereby increasing the heterogeneity of the progenitor population.
For instance, if a progenitor cell receives a differentiating cue (e.g. RA, in the spinal cord) when it has just experienced a Notch activation event, it will not respond since it has very high levels of HES proteins to counteract the proneural proteins. However, if a progenitor cell has downregulated Notch activity and lies within the "neither-ON-nor-OFF" competent state, it can respond to the differentiation cues in two ways: if it is at the higher peak of HES expression (Fig.4A), it could be less responsive to these cues. In contrast, if the progenitor cell receives a differentiation signal when the levels of HES repressors are low (Fig.4.B), this cell would be more prompt to upregulate the proneural genes and therefore embark on differentiation.
In this way, the possible oscillatory 'noisy' *hes* expression could result into different states of receptiveness to differentiating cues. These different states of competence might provide yet another mechanism, besides LI, to delay neuronal differentiation, providing TIME to allow changes in the competence state of neural progenitors and in the extracellular cues, thus permitting the formation of the correct numbers and various neural cell types.

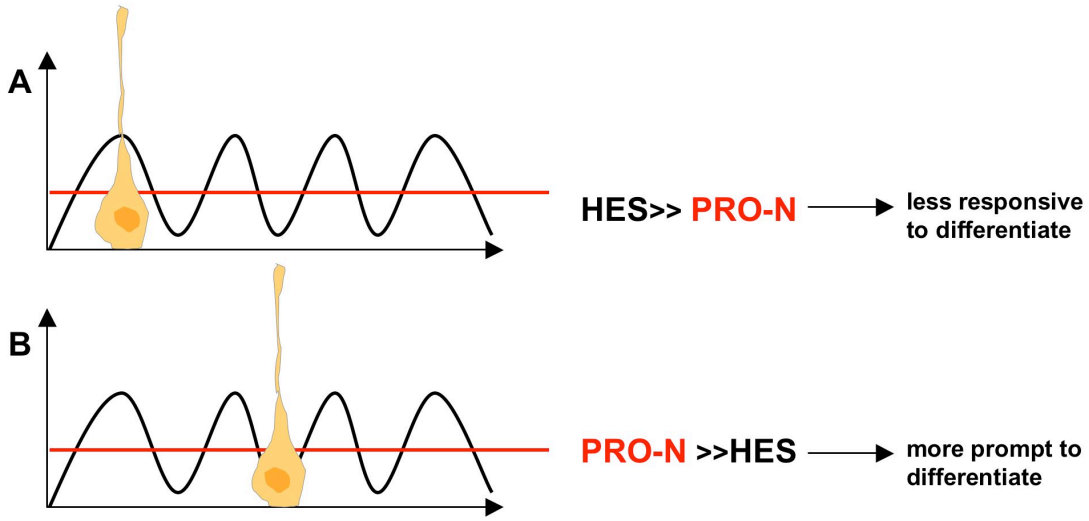


Fig.4 Different levels of HES expression may provide different states of receptiveness to the differentiating signals. A. High relative levels of HES repressors may delay the response to differentiating cues. **B.** High relative levels of proneural proteins may render cells to respond more rapidly to the differentiating cues.

Thus, although instability/noise may counteract the robustness of developmental programs (Martinez-Arias and Hayward, 2006), in the nervous system, which is the organ with more cellular diversity, instability and stochasticity may give a hand generating all its astonishing complexity.

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Appendix

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Genomes & Developmental Control

A novel *hes5*/*hes6* circuitry of negative regulation controls Notch activity during neurogenesis

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Abstract

HES transcriptional repressors are important components of the Notch pathway that regulates neurogenesis from *Drosophila* to vertebrates. These proteins are normally induced by Notch activity and inhibit neural commitment by antagonizing the activity of proneural genes. We describe here four chick *hes* genes that are expressed during neurogenesis: three *hes5*-like genes (*hes5-1*, *hes5-2* and *hes5-3*) and one *hes6*-like (*hes6-2*). We show that *hes6-2* represses transcription of the *hes5* genes, thus functioning as a negative regulator of Notch signaling. Conversely, *hes6-2* may be repressed by *hes5* activity. In cells committing to differentiation, we find that *hes6-2* is up-regulated by proneural genes and contributes to the proneural program of neuronal commitment by preventing Notch activity in these cells. In neural progenitors, Notch signaling produces an initial burst of *hes5* activity, which represses *hes6-2*. However, as *hes5* transcription declines due to negative auto-regulation, *hes6-2* may become active and inhibit the remaining *hes5* activity to end Notch signaling. These cells can then enter a new cycle of fate decisions and will be kept as progenitors if a new pulse of Notch activity occurs. Maintenance of progenitors during vertebrate neurogenesis therefore requires that these cells go through successive cycles of Notch activity. We propose that the *hes5*/*hes6* circuitry of negative cross-regulations is a conserved feature of the Notch pathway that underlies these cycles in neural progenitors.

Keywords: Notch signaling; *hes* genes; Neurogenesis

Introduction

A conserved feature of the genetic circuitry regulating neurogenesis, in animals as different as flies and mammals, is the antagonism between two different sets of basic helix–loop–helix (bHLH) proteins. Proneural proteins of the Achaete–Scute and Atonal/Neurogenin families play a positive role in promoting the commitment of a cell to a neural fate, while bHLH-Orange (bHLH-O) proteins from the Hairy and Enhancer of Split (HES) family repress this cell fate decision. The balance between the activity of these two sets of bHLH proteins and, therefore, the final fate of the cell, is dictated by a cell–cell communication system known as lateral inhibition, mediated by the Notch receptors and their ligands Delta/Serrate (reviewed by Campos-Ortega, 1994). In *Drosophila*, where this system was first studied, proneural proteins are expressed in groups of ectodermal cells, called proneural clusters, which thereby acquire the potential to follow a neural fate. Although each cell in a group has an equivalent potential, only one of them becomes neural and inhibits its neighbors from adopting a similar fate by activating the Notch pathway in the latter cells. Notch activation leads to transcriptional up-regulation of genes encoding HES proteins, which suppress the activity of the proneural genes and, thereby, keep these cells uncommitted. In this way, one cell in the equivalence group realizes its neural potential and ensures that other cells are prevented from doing so.

This basic mechanism has been well conserved during animal evolution and controls the development of a great variety of cell types, not only of neural cells (reviewed by Artavanis-Tsakonas et al., 1999; Lewis, 1998). In recent

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years, the molecular details of the Notch signaling pathway have been the focus of intense research, and regulation at different levels of the pathway has been shown to have important contributions to its final outcome (reviewed by Lai, 2004; Schweisguth, 2004). Despite its complexity, a unique feature of the Notch cell–cell communication system is that it mediates a simple binary decision, ensuring that cells acquire one of two alternative fates, the nature of which depends on the embryonic context and developmental time. This unique feature is based on the robust design of the Notch pathway, at the core of which an inter-cellular feedback loop functions to amplify small differences in the potential of the cells, leading invariably to a distinct outcome in each of them.

In *Drosophila*, the bHLH-O proteins that mediate Notch activity in responding cells are encoded by the *Enhancer of split* Complex [*E(spl)*-C], which contains seven genes clustered in a single 60 kb complex and seems to have mostly overlapping functions (Bray, 1997; Knust et al., 1992). *Drosophila* contains other genes encoding bHLH-O proteins, like the *hairy* and *deadpan* genes, which do not mediate Notch activity, although they also regulate neural development.

The HES family of bHLH-O proteins is characterized by the presence of a conserved proline at the basic region and a WRPW tetrapeptide at the carboxy-terminus, which was shown to interact with the co-repressor encoded by the *groucho* gene (Paroush et al., 1994). Another domain, located after the bHLH region, was also found to be conserved among HES proteins and named Orange domain (Dawson et al., 1995), being important for the specificity of protein–protein interaction between various HES proteins. These are known to be DNA-binding transcriptional repressors and the recognition sequences to which they bind have been characterized (N-boxes and ESE-boxes), being different from the E-boxes recognized by proneural proteins (Davis and Turner, 2001). The main mechanism of transcriptional repression by HES proteins is based on the WRPW-mediated recruitment of Groucho, which interacts with and inhibits the transcriptional machinery. HES proteins might also block the activity of the proneural bHLH proteins by direct protein–protein interaction, forming heterodimers that are unable to promote neural commitment. Therefore, different mechanisms can be used by the HES proteins to counteract the activity of the proneural proteins during neurogenesis.

The first *hes* genes described in vertebrates were a homologue of *hairy* and a homologue of the *E(spl)* genes, which were given the names of *hes1* and *hes3*, respectively (Sasai et al., 1992). Several other vertebrate genes of the *hes* family have since then been described, some of which were shown to participate in Notch signaling during neurogenesis (reviewed in Davis and Turner, 2001). However, the general regulation of *hes* gene function during neural development is still poorly understood. In the mouse, for instance, four *hes* genes are expressed in the developing neural tube (*hes1*, *hes3*, *hes5* and *hes6*), with distinct, but partially overlapping patterns. Only *hes5* has been shown to be directly regulated by the Notch pathway in vivo (de la Pompa et al., 1997; Lutolf et al., 2002), but its deletion does not phenocopy Notch inactivation during neurogenesis (Ohtsuka et al., 1999). In addition to *hes5*, two other *hes* genes (*hes1*, *hes3*) have to be inactivated to cause complete elimination of the neural progenitor pool and premature neuronal differentiation (Hatakeyama et al., 2004), as expected for a total absence of Notch signaling (Henrique et al., 1997a). Since neither *hes1* nor *hes3* is direct target of Notch signaling in the neural tube, this apparent redundancy in *hes* function raises the question of whether *hes1* and *hes3* normally function as Notch effectors in the neural tube and how they interact with *hes5* to control neurogenesis. The *hes6* gene might participate also in this network of *hes* genes, as it was shown to act as a negative regulator of *hes1* (Bae et al., 2000; Gratton et al., 2003; Koyano-Nakagawa et al., 2000). Whether it interacts also with *hes3* and *hes5* and how these interactions contribute to the Notch pathway's function during neurogenesis remains to be known.

In this paper, we address the regulation and function of *hes* genes in the developing spinal cord of the chick and how they participate in the cascade of events in the Notch pathway that regulate neuronal production. We show that a series of interactions between the *hes5* and *hes6* genes, and of those with the proneural genes, are important to control different steps along neural development. In particular, we show that the Notch effectors *hes5* genes are transcriptionally repressed by the product of the *hes6-2* gene, which may function as a negative regulator of Notch activity, both in neural progenitors and nascent neurons. We propose that this *hes5*/*hes6* circuitry of negative regulation is a key mechanism to ensure a proper modulation of Notch activity throughout neurogenesis.

Materials and methods

cDNA cloning

Initial PCR cloning was performed with cDNA prepared from HH12 chick embryos and degenerated primers targeted at the bHLH and WRPW regions conserved in the HES gene family. The PCR fragments were then used to screen a HH17 spinal cord cDNA library and full-length cDNAs were obtained for 4 different *E(spl)*-like genes.

Digoxigenin and Fluorescein-labeled RNA probes for the 3 *hes5*-like genes, *hes6-2*, *Delta-1*, *ngn-1* and *Notch1* were synthesized by T3 or T7 RNA polymerase, from plasmid templates containing the full-length cDNAs (partial cDNA for *Notch1*). Antisense RNA probes complementary only to the 3′UTR of the four *hes* genes were also synthesized.

Embryo electroporation

Super-coiled plasmid DNA was injected into the neural tube of HH11–12 chicken embryos at a concentration of 2 μg/μl in PBS, with exception of the *hes6-2* construct, which was used at 1 μg/μl. Platinum electrodes (Nepagene CUY613G), distanced 4 mm between anode and cathode, were placed parallel to the neural tube, and embryos were pulsed 4 times (30 V/50 ms), using a Electro Square Porator™ ECM830 (BTX). Embryos were incubated for 8 h or 24 h and then harvested.

Plasmid constructs to express the chick HES proteins were generated in pCAGsIRES-GFP, which is derived from the pCAGs vector (Niwa et al., 1991) and contains an IRES-GFP cassette downstream of a polylinker followed by a stop codon in each frame (Bekman, E. and Henrique, D., unpublished). cDNAs for the 4 chick *hes* genes were cloned in the polylinker, after removal of the 5′ and 3′ UTR, and all contain the same consensus Kozak sequence surrounding the initial ATG. The vector encoding a HES6-2:VP16 fusion was made by fusing a *hes6-2* cDNA lacking the WRPW domain (prepared by PCR with the primers 5′-CGGAATTCGCCACAATGACGGCCGCAG3′/5′CGGGATCC-GGGCTGCAGGACCT-3′) and the VP16 sequence (amino acids 412–490). All vectors were checked by sequencing.

For construction of cNicd@pCAGsIRES-GFP, a *Not*I 2.5kb DNA fragment from cNicd-pYDF30 (Wakamatsu et al., 2000) was subcloned in the pCAGsIRES-GFP vector. The vectors pCIG and X-Su(H)^{DBM}@pCIG were kindly provided by Andy McMahon (Megason and McMahon, 2002). The cDNAs encoding the full-length rat proteins NGN-1 and NGN-2 (Mizuguchi et al., 2001) were subcloned in pCIG.

For each construct, a minimum of 6 electroporated embryos were analyzed by in situ hybridization and immunofluorescence, with at least 10 sections from each embryo scored for phenotypes. Images presented in figures are representative of each experiment. Controls were done by electroporating the pCAGsIRES-GFP vector alone and no alterations in gene expression were observed, with any of the probes here described. Statistical analysis of results presented in Fig. 10 ("NGN-2" vs. "NGN2+ Hes5-1" and "NGN-2" vs. "NGN2+ HES6-2") was done using *t* test.

In situ hybridization and immunofluorescence

Chicken embryos were collected and fixed in 4% paraformaldehyde/PBS at 4°C. Whole-mount in situ hybridizations were done as described (Henrique et al., 1995), with modifications. For hybridization on cryostat sections, fixed embryos were cryoprotected in 15% sucrose in PBS, embedded in 7.5% gelatin/15% sucrose/PBS and cryosectioned (12 μm). Hybridization on cryostat sections was done as previously described (Myat et al., 1996), with modifications. Double in situ hybridization on cryostat sections was done with DIG- and fluorescein-labeled RNA probes. The fluorescein-labeled probe was first detected with AP-conjugated anti-Fluo antibody (Roche) and developed with Fast-Red substract (Roche). After washing in PBS, sections were blocked and incubated with HRP-conjugated anti-DIG antibody (Roche), followed by FITC-Tyramide amplification, as recommended by the manufacturer (Perkin-Elmer). Electroporated cells were visualized after in situ hybridization using a polyclonal antibody against GFP (AbCam). Detailed protocols are available upon request. The Tuj1 antibody (Lee et al., 1990) was kindly provided by A. Frankfurter (Univ. Virginia).

Results

The chick genome contains three hes5 and two hes6 homologues

Using degenerate PCR and cDNA library screening, we have cloned 4 new members of the *hes* gene family in the chick. Three of these genes encode highly related proteins with strong homology to the mammalian HES5 protein and were named *hes5-1*, *hes5-2* and *hes5-3*. The other gene encodes a protein with homology to mammalian HES6 and was named *hes6-2*, since there is another gene in the chick genome encoding a bHLH protein with even higher homology to mammalian HES6 (*hes6-1*; Fig. 1). The chick *hes6-1* gene is not expressed during neurogenesis in the neural tube and was not studied further.

Full-length cDNAs encoding the three chick *hes5* genes (*hes5-1*, *hes5-2* and *hes5-3*) and the two *hes6* genes (*hes6-1* and *hes6-2*) predict proteins of 157, 178, 154, 206 and 228 amino acid residues, respectively, with all the structural features of the E(spl) subfamily of bHLH-O transcriptional repressors (Davis and Turner, 2001). The three chick HES5 proteins show a high degree of homology between each other in the bHLH region (around 95% identity) and to human HES5 (80 to 83% identity) but show more divergence in the Orange domain (47 to 75% identity between them and to hHES5). As the Orange domain confers specificity for protein–protein interaction (Dawson et al., 1995), the three chick HES5 proteins may have slightly different properties.

The chick HES6-1 and HES6-2 proteins display only 56 and 52% identity with human HES6 in the bHLH domain, respectively, but have more than 60% identity at the Orange domain, in which we could identify a signature sequence for the HES6 subfamily (Fig. 1). The bHLH domain of the chick HES6 proteins, like those of mouse and human HES6, contains a shorter loop region when compared to the other HES proteins.

Analysis of the recently available chick genome reveals that the protein-coding region of each chick *hes5* gene, like the mammalian counterpart, is encoded in 3 exons, whereas the other chick *hes* genes (*hes6, hairy1* and *-2*) contain 4

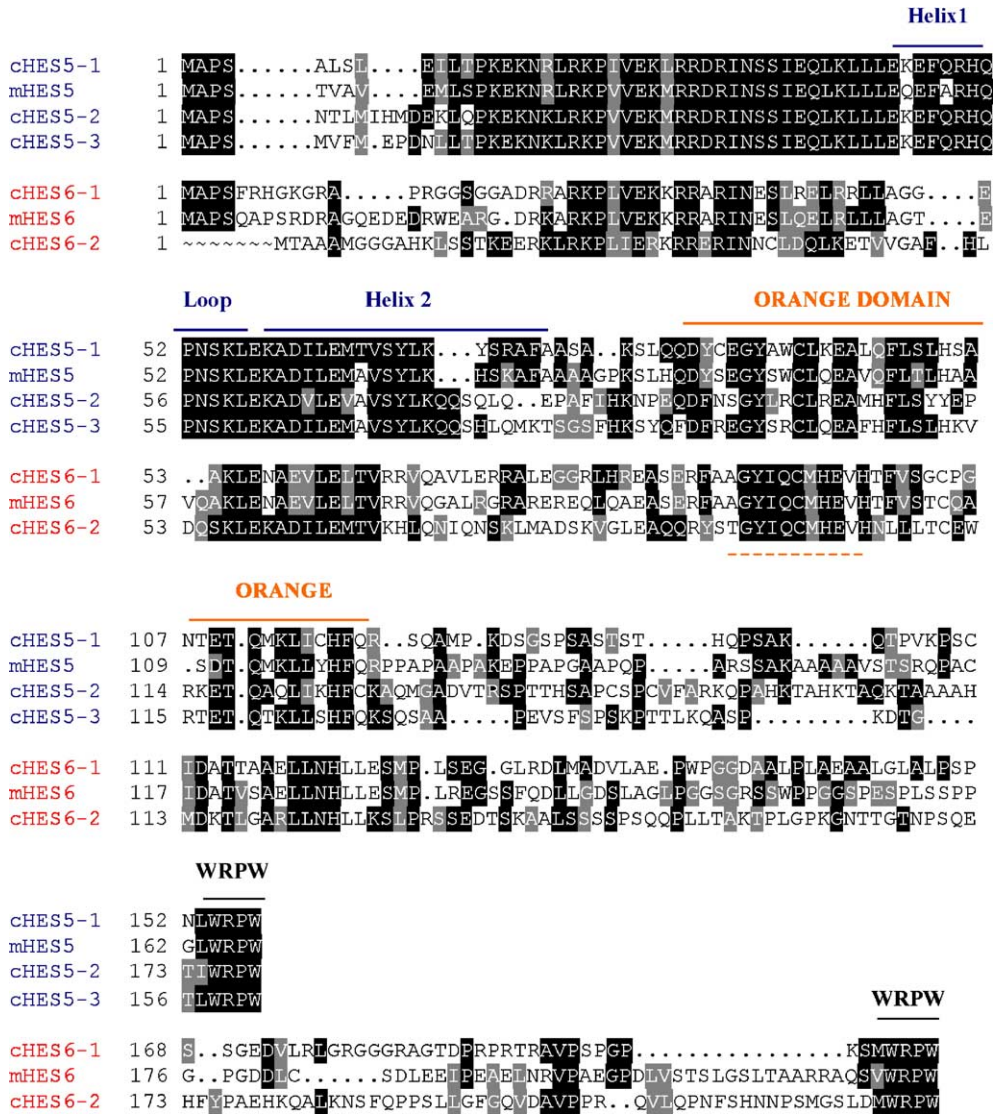

Fig. 1. Sequence alignment of the chick HES proteins with the mouse HES5 and HES6 proteins. The three HES5 proteins show clear homology to mHES5, with HES5-1 being the closest homologue. The chick HES6-1 protein was predicted from various ESTs (Boardman et al., 2002) (sequence identifier 332379.4) and shows higher homology to mHES6 than HES6-2. A HES6-specific motif at the Orange domain can be identified in all HES6 proteins (orange dashed line).

exons (Sup. Fig. 1). The 3 chick *hes5* genes are clustered on Chromosome 21, within a 20 kb region of DNA, adjacent to the *fang1* gene encoding the enzyme pantothenate kinase 4 (Supplementary Fig. 1). A similar cluster of three *hes5*-like genes exists in the Fugu genome, also near the *fang1* gene (data not shown). In comparison, both the mouse, rat and human genomes contain only one *hes5* gene, also linked to the *fang1* gene, revealing that this linkage has been conserved throughout evolution.

The chick hes5 and hes6-2 genes are expressed in neural progenitors, but hes6-2 is also expressed in nascent neurons

In the developing chick CNS, *hes5-1*, *hes5-2* and *hes5-3* transcripts are first detected at HH4-5 (stages according to Hamburger and Hamilton (1951)) in cells at the Caudal Neural Plate (CNP), adjacent and posterior to Hensen's node (Figs. 2A–I). Previous fate map studies (Brown and Storey, 2000; Henrique et al., 1997b; Mathis et al., 2001) indicate that this region is a stem zone that contains the precursor cells of the caudal part of the CNS (reviewed by Del Corral and Storey, 2004). The domain of *hes5* expression in the chick CNP overlaps with that of the proneural gene *cash4* (Henrique et al., 1997a), and both genes continue to be expressed similarly in the caudal stem zone around the regressing Hensen's node, until primary neurulation ends. This expression pattern of the *hes5* and *cash4* genes in the stem zone suggests that Notch signaling has a role in regulating cell fate decisions in this region.

In contrast to *cash4*, however, the chick *hes5* genes are also expressed in the neural tube, from the onset of neurogenesis. This second wave of *hes5* expression starts at HH6–7 (Figs. 2B,E,H), in the neural tube region flanking the first somite, and coincides with the appearance of the

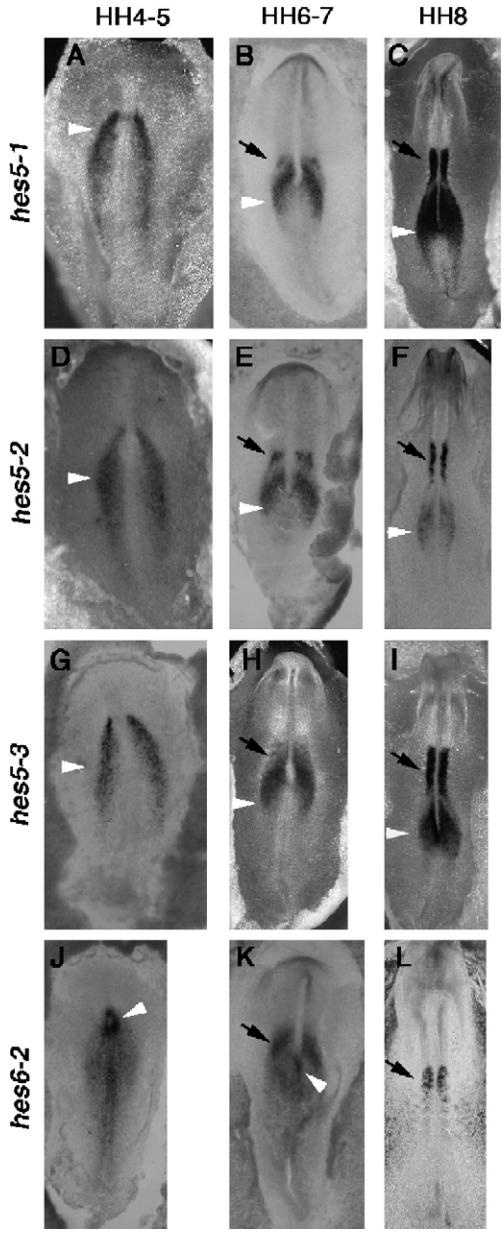

Fig. 2. Expression pattern of the chick *hes* genes at early stages of neural development. At HH4-5, the three *hes5* genes show a very similar expression in the Caudal Neural Plate (CNP, white arrows) (A,D,G), while *hes6-2* starts to be expressed asymmetrically around Hensen's node (J, white arrow), showing also weak expression in the primitive streak and adjacent mesoderm (J). At HH6-7, all three *hes5* genes are strongly expressed in the CNP (B,E,H, white arrows) and start to be also expressed in the neural plate region flanking the first somite (black arrow), coinciding with the initial *hes6-2* expression in the same region (K, black arrow). Asymmetric expression of *hes6-2* is still present in the right side of Hensen's node (K, white arrow), and very weak expression can be detected also in the ectoderm around it. At HH8-9, the four *hes* genes are expressed throughout the neural tube (C,F,I,L, black arrow) with exception of the hindbrain.

first *Delta1*-expressing newborn neurons in the same region (Henrique et al., 1995). Expression of chick *hes6-2* starts also at HH6–7 in this region, overlapping with the second wave of *hes5* expression. The *hes6-2* gene is not expressed in the CNP stem zone but shows asymmetric expression around Hensen's node at HH5 (Fig. 2J), perhaps reflecting the known Notch function during establishment of left–right asymmetry (Raya et al., 2004).

As development proceeds, the expression of the four chick *hes* genes in the forming neural tube correlates

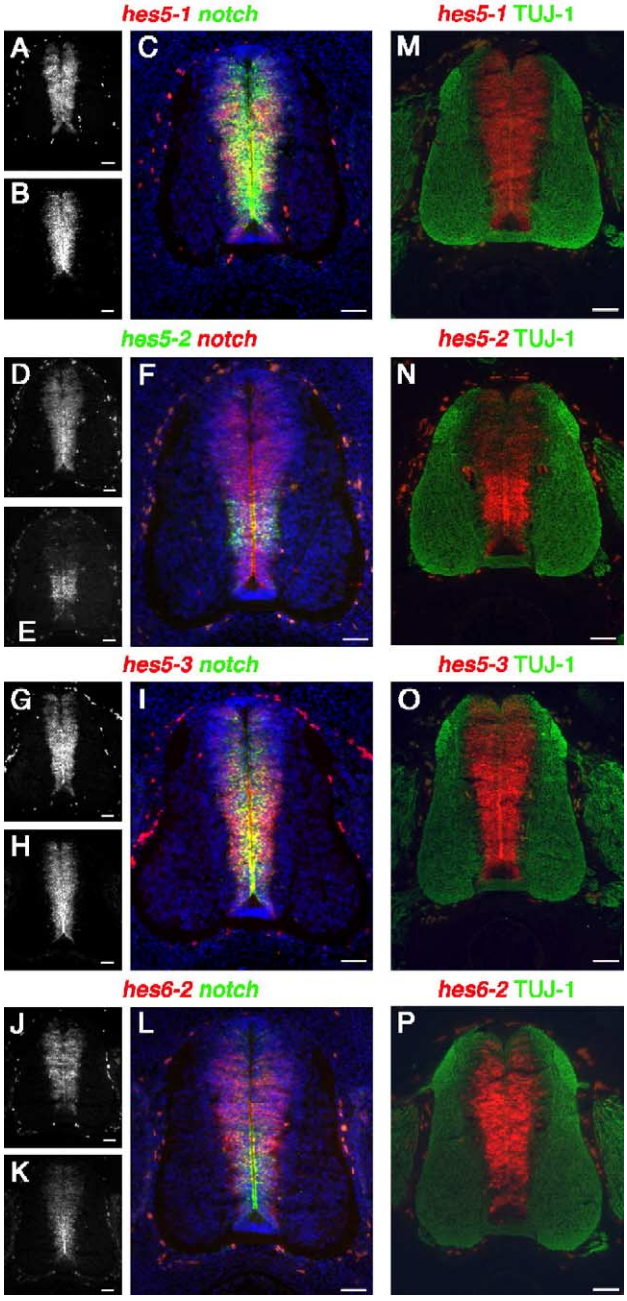

Fig. 3. Expression of chick *hes* genes in the developing spinal cord at HH23 in transverse sections. The four *hes* genes are expressed in the ventricular region of the neural tube (A,E,G,J) where *Notch-1* is expressed (B,D,H,K). Merged images (C,F,I,L) show overlap in expression (DAPI in blue. Red and green signals arise from in situ hybridization with DIG and Fluorescein-labeled RNA probes, revealed with Fast-Red and FITC-tyramide, respectively). Expression of *hes* genes is absent from differentiated neurons, as shown by the complementary labeling with the neuronal marker TUJ-1 (M–P). Scale bars = 50 μm.

well with the described spatio-temporal pattern of neurogenesis (Hollyday, 2001), being detected initially in the ventral spinal cord and later, expanding also dorsally (data not shown). Transcripts of the four *hes* genes can be detected in the ventricular region where neural progenitors are located and *Notch1* is expressed, being absent from the mantle zone where differentiating neurons are accumulating (Figs. 3A–P). The chick *hes5-1* and *hes5-3* genes present a more homogeneous expression in the ventricular region (Fig. 3A), spanning the whole dorso-ventral axis (excluding the floor plate), while *hes5-2* shows stronger expression in the ventral half of the neural tube (Fig. 3E). Comparing with the 2 Notch ligands, *Delta1* and *Serrate1*, which have complementary expression domains in the neural tube (Myat et al., 1996), the four chick *hes* genes are transcribed in both domains, overlapping with *Notch1* across the entire D–V axis of the spinal cord (Figs. 3A–L). Double in situ hybridization reveals, however, some differences between the expression of the chick *hes* genes: while all three *hes5* genes are expressed in neuroepithelial cells located apically, with little overlap with *Delta-1* in newborn neurons (Figs. 4A–C), the cells with stronger *hes6-2* expression are located more basally (Fig. 4E), the majority of which co-express *Delta-1* (Figs. 4H–J). This indicates that the chick *hes5* genes are expressed only in neural progenitors, while *hes6-2*, although first expressed in progenitors, is most highly expressed in cells that are embarking on neuronal differentiation.

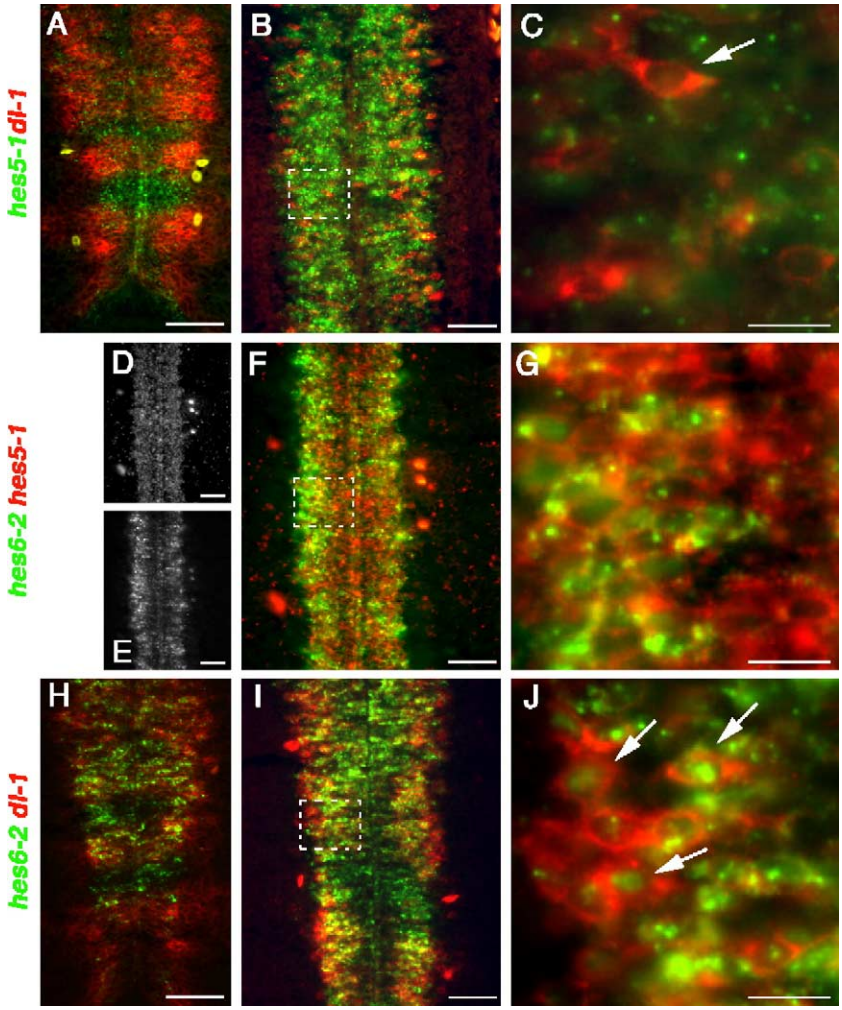

Fig. 4. Double in situ hybridization of the chick *hes5-1*, *hes6-2* and *Delta-1* genes reveals differences in expression between the *hes5* and *hes6-2* genes (expression of *hes5-2* and *hes5-3* is identical to *hes5-1*). Double in situ hybridization in transverse (A,H) and longitudinal sections (B–G,I,J) in developing spinal cord at HH23 [white boxes indicate zoomed regions (C,G,J-scale bars 12,5μm)]. *hes5-1* expression is limited to the ventricular zone, with little overlap with *cDelta-1* (A–C). Nascent neurons with strong *cDelta-1* expression (arrow in C) do not express *hes5* genes. In contrast, the cells with higher expression of *hes6-2* (arrows in J) co-express *cDelta-1* (H–J) and are more basal (E) in comparison to cells expressing *hes5-1* (D). In panel (G), double in situ with *hes5-1* and *hes6-2* shows that cells with strong *hes6-2* expression are at the edges of the *hes5-1* expression domain. Red and green signals arise from in situ hybridization with DIG and Fluorescein-labeled RNA probes, revealed with Fast-Red and FITC-tyramide, respectively. Scale bars = 50 μm, except in panels (C), (G) and (J).

The hes genes participate in the Notch signaling cascade

In both *Drosophila* and vertebrates, *hes* genes are essential components of the Notch pathway. Their expression is regulated by Notch signaling and some HES proteins function as downstream effectors of the Notch cascade. The similar expression pattern of the chick *hes* and *Notch1* genes suggests that the *hes* genes also implement Notch signals during neurogenesis in the chick embryo. To test this idea, we first assessed how the chick *hes* genes respond to Notch signaling and, second, whether they are able to convey Notch activity during neurogenesis.

Driving expression of a constitutively active form of the Notch1 receptor (Wakamatsu et al., 2000) in the embryonic neural tube leads to up-regulation of the three *hes5* genes and a reduction in *hes6-2* expression (Figs. 5C–F′). By contrast, blocking Notch activity by overexpressing a dominant-negative form of the *Xenopus* homolog of *Drosophila* Suppressor-of-Hairless, (X-Su(H)^{DBM} (Wettstein et al., 1997), leads to a down-regulation of *hes5* expression and up-regulation of *hes6-2* (Figs. 5I–L′). Together, these results indicate that all four chick *hes* genes are targets of Notch signaling, although they respond differently to alterations in Notch activity. The chick *hes5*

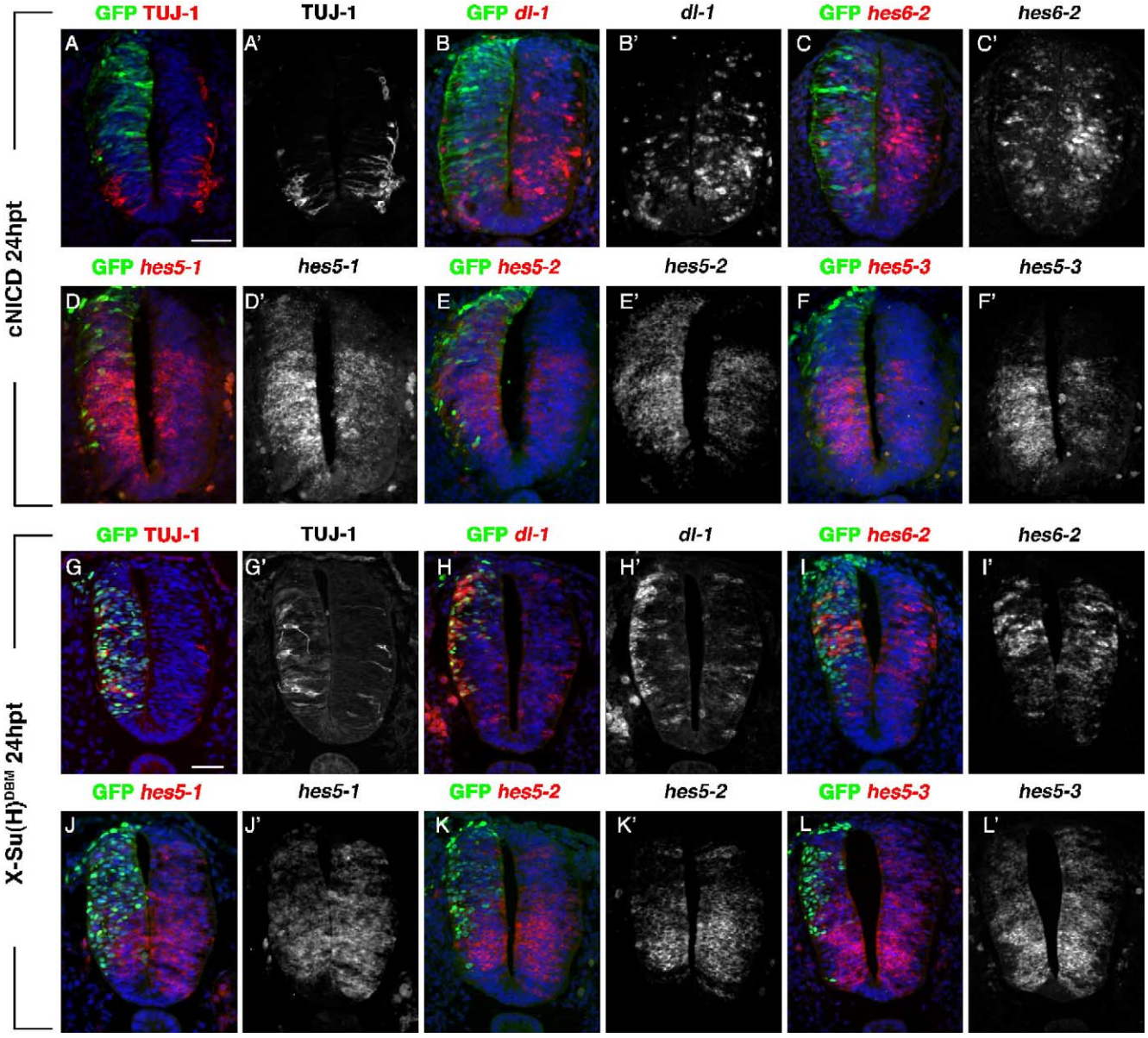

Fig. 5. Regulation of the *hes* genes by the Notch pathway. Electroporated cells are shown in green due to the expression of GFP reporter. The activated form of Notch (NICD) led to a decrease in TUJ-1+ cells (A,A′) and *cDelta-1* expression (B,B′), indicating that neurogenesis was inhibited. The chick *hes5* genes, *hes5-1* (D,D′), *hes5-2* (E,E′) and *hes5-3* (F,F′) are up-regulated by NICD. In contrast, *hes6-2* is down-regulated by NICD (C,C′). Down-regulation of the Notch pathway (due to X-Su(H)^{DBM} overexpression) leads to an increase in TUJ-1+ cells (G,G′) and *cDelta-1* expression (H,H′). X-Su(H)^{DBM} overexpression results also in a slight increase in *hes6-2* expression (I,I′). In contrast, the expression of the three *hes5* genes is reduced (J–L, J′–L′). Scale bars = 50 μm.

genes behave as "canonical" *hes* genes, since their expression is dependent on Notch activity and could therefore function as transcriptional effectors of Notch signaling. However, *hes6-2* seems to be repressed by Notch activity and is unlikely to be a direct effector of Notch signaling in the chick neural tube.

To ask whether the chick *hes5* genes are indeed effectors of the Notch signaling pathway during neurogenesis, each of the three *hes5* genes were overexpressed in the embryonic neural tube. In each case, we detect a similar phenotype to that obtained by increased activity of the Notch pathway (Fig. 5), namely, a decrease in the number of Tuj-1 positive neurons and a repression of the chick *ngn1* and *Delta1* genes (Figs. 6A–C and data not shown). In addition, *hes6-2* expression is repressed by overexpression of each of the three *hes5* genes (Figs. 6D,D′ and data not shown), as well as by overexpression of an activated form of the Notch1 receptor (Figs. 5C,C′). These results indicate that the chick *hes5* genes are bonafide Notch effectors during neurogenesis in the embryonic chick neural tube.

Cross-regulation and auto-regulation of the chick hes5 genes

A remarkable feature of *hes* genes is that they can be negatively regulated by their own products through direct binding to the respective promoters (Cooper et al., 2000; Hirata et al., 2002, 2004; Sasai et al., 1992; Takebayashi et al., 1994). This enables the establishment of negative feedback loops in *hes* gene regulation, which might have an important function on overall architecture of the Notch pathway (Meir et al., 2002). In the chick embryo, the presence of three *hes5* genes raises the possibility of multiple interactions between these genes to modulate Notch signaling. To check the ability of *hes5* genes to regulate themselves and investigate possible interactions between them, we overexpressed each of the three *hes5* genes in the embryonic neural tube and analyzed the effect on the transcriptional output of each gene (using probes from the 3′ untranslated region, not included in the expression vectors). Our findings, summarized in Table 1, indicate that *hes5-1* and *hes5-2* are indeed able to negatively regulate their own transcription (Figs. 7A,E). In addition, the two genes negatively cross-regulate each other, as shown by the repression of *hes5-2* transcription caused by overexpression of HES5-1, and vice-versa (Figs. 7B,D). By contrast, *hes5-3* is up-regulated by overexpression of HES5-1 or HES5-2 (Figs. 7C,F). Furthermore, *hes5-3* is not negatively auto-regulated, as HES5-3 overexpression leads instead to up-regulation of the corresponding gene (Fig. 7I). HES5-3 overexpression causes also up-regulation of both *hes5-1* and *hes5-2* (Figs. 7G,H), raising the question of how can a putative transcriptional repressor lead to simultaneous up-regulation of the three *hes5* genes.

hes6-2 is a repressor of the three hes5 genes

The simplest way to explain how overexpression of HES5-3 results in increased transcription of the three *hes5* genes is to postulate the existence of a common *hes5* repressor, whose activity is itself repressed by *hes5-3*. In addition, such negative regulator of *hes5* transcription must be also repressed by *hes5-1* and *hes5-2*, since their overexpression results in up-regulation of *hes5-3* (but not

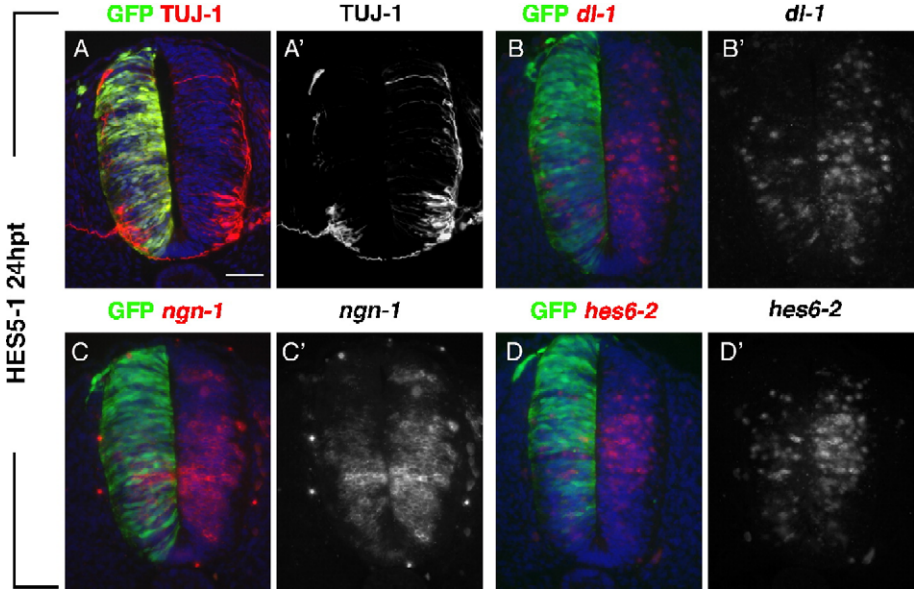

Fig. 6. *hes5-1* can mediate Notch activity. Electroporated cells are shown in green due to the expression of GFP reporter. Overexpression of HES5-1 causes a decrease in TUJ-1+ cells (A,A′), *Delta-1* (B,B′) and *ngn-1* expression (C,C′), indicating that neurogenesis is inhibited. In addition, both overexpression of HES5-1 and NICD repress the expression of *hes6-2* (D,D′). Overexpression of HES5-2 and HES5-3 also inhibits neuronal differentiation and *hes6-2* expression (data not shown). Scale bars = 50 μm.

Table 1
Summary of the cross-regulations between the chick *hes5* genes

	ches5-1	ches5-2	ches5-3
HES5-1	↓	↓	↑
HES5-2	↓	↓	↑
HES5-3	↑	↑	↑

While *hes5-1* and *hes5-2* are able to negatively auto-regulate their expression, as well as repress each other, they both lead to an increase in *hes5-3* transcription. Strikingly, HES5-3 overexpression leads to an increase in its own expression, as well as that of *hes5-1* and *hes5-2*.

of themselves, as they are negatively auto-regulated). This common repressor is therefore postulated to play a central role in the concerted regulation of the three *hes5* genes, being able to repress all of them and being also repressed by any of them (Fig. 8).

A good candidate to encode such a common repressor is the *hes6-2* gene, which is actually negatively regulated by the *hes5* genes (Fig. 6D and data not shown). To test this idea and address whether *hes6-2* is indeed able to repress the activity of the three *hes5* genes, 2 different plasmid constructs were electroporated in the embryonic neural tube: one encoding a full-length version of the HES6-2 protein and another a putative dominant-negative version, in which

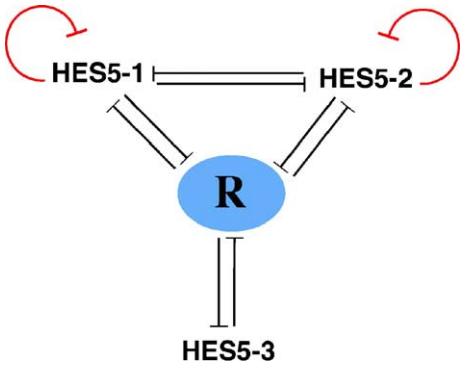

Fig. 8. Interactions between the three *hes5* genes indicate the existence of a common repressor (R) that itself might also be repressed by each of the *hes5* genes. Since *hes5-3* is unable to negatively auto-regulate its own expression, the repressing activity of this gene on the putative repressor leads to up-regulation (de-repression) of all the *hes5* genes, as shown in Table 1.

the C-terminal WRPW domain is replaced by a potent transactivation domain from the viral protein VP16. This fusion protein is expected to bind to the same promoter sites as the normal HES6-2 protein but activate, rather than repress, transcription of target genes (Jimenez and Ish-Horowicz, 1997). Our results show that HES6-2 over-

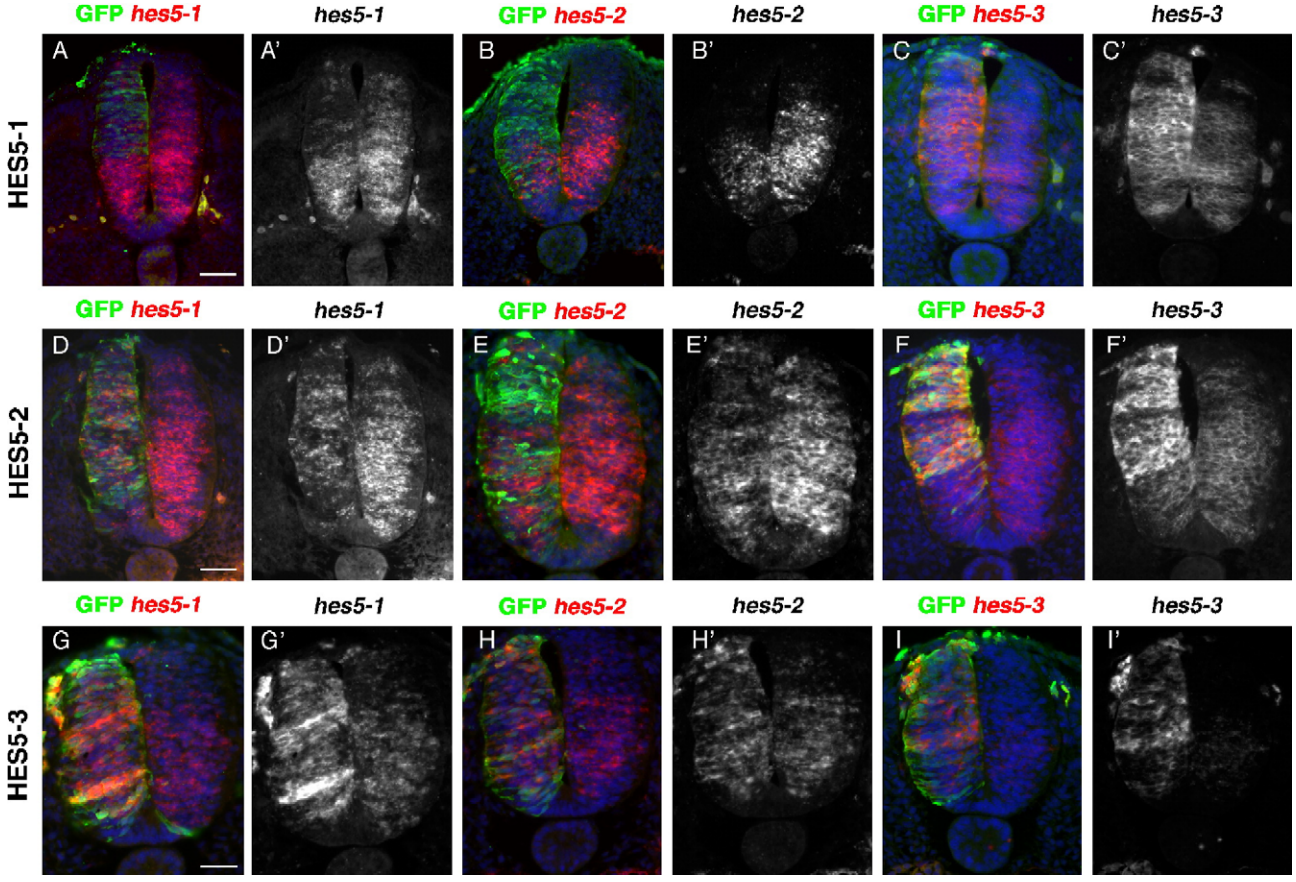

Fig. 7. Cross-regulation between the chick *hes5* genes. Electroporated cells are shown in green due to the expression of GFP reporter. Embryos were collected 24 h after electroporation. Overexpression of HES5-1 results in the down-regulation of the endogenous *hes5-1* (A,A′) and *hes5-2* genes (B,B′), while it leads to up-regulation of *hes5-3* (C,C′). Similarly, overexpression of HES5-2 results in the down-regulation of the endogenous *hes5-2* (D,D′) and *hes5-1* genes (E,E′) and in up-regulation of *hes5-3* (F,F′). On the contrary, overexpression of HES5-3 results in the up-regulation of three *hes5* genes (G–I, G′–I′). Scale bars = 50 μm.

expression leads to a repression of the three *hes5* genes (Figs. 9A–C′), while overexpression of the HES6-2:VP16 fusion has the opposite effect, producing a marked increase on transcription of the three *hes5* genes (Figs. 9D–F′). Together, these results indicate that the HES6-2 protein recognizes the promoter of the *hes5* genes and negatively regulates their transcription, supporting the model that *hes6-2* functions as a central node on the network of *hes5* regulation.

hes6-2 cooperates with the proneural genes to promote neuronal differentiation

The above results suggest that *hes6-2* acts as a negative regulator of Notch signaling by repressing transcription of *hes5* genes. This activity may be important in cells leaving the proliferative zone of the neural epithelium, where *hes6-2* expression reaches its peak and may function to reduce Notch signaling and facilitate differentiation into neurons.

To test this idea, we first asked whether the activity of *hes6-2* is sufficient to promote neuronal differentiation. Our results show that overexpression of *hes6-2*, despite producing a marked decrease in *hes5* expression, leads to a block in neuronal differentiation, as shown by down-regulation of *Delta1* and *ngn1* expression, and a decrease on Tuj1-positive neurons (Figs. 10E–F′ and data not shown). Thus, *hes6-2* activity is not enough to promote neuronal differentiation per se, suggesting that the presence of high levels of proneural activity is, most likely, the decisive factor for neuronal commitment.

Indeed, overexpression of proneural proteins (NGN1 or NGN2) in the chick neural tube is enough to drive cells into differentiation, as shown by the increase in *Delta-1* expressing cells and by the subsequent increase in Tuj1-positive neurons, 24 h after electroporation (Figs. 10C–D′ and data not shown). The increase in *Delta1* expression is accompanied by up-regulation of *hes6-2* (Figs. 10A–B), indicating that this gene may be a target of proneural genes in nascent neurons. This finding raises the hypothesis that *hes6-2*, although unable to drive neuronal differentiation alone, may function as part of the proneural program, cooperating with the proneural proteins to push cells into differentiation.

To test this hypothesis, the embryonic neural tube was electroporated simultaneously with expression vectors containing cDNAs encoding HES6-2 and NGN2. In addition, another group of embryos was electroporated with expression vectors for NGN-2 and HES5-1, and the effects on neurogenesis were compared. The results indicate that simultaneous overexpression of NGN-2 and HES6-2 has indeed a synergistic effect on neuronal differentiation (Figs. 10I–J′), as shown by the higher number of *Delta1*-expressing cells and Tuj1-positive neurons (45.4 ± 6% of electroporated cells are also Tuj1-positive, $n = 2076$), when compared with the overexpression of NGN-2 alone (30.6 ± 5% of electroporated cells are Tuj1-positive, $n = 1868$; $P < 0.05$)(Figs. 10C–D′). By contrast, HES5-1 has an antagonistic effect on NGN-2 activity, as overexpression of NGN-2 and HES5-1 together leads to a decrease on neuronal production (13.7 ± 6% of electroporated cells are Tuj-positive, $n = 1297$; $P < 0,05$) (Figs. 10G–H′). Thus, these results indicate that *hes6-2* cooperates with the proneural genes during neuronal commitment, presumably by inhibiting the Notch effectors encoded by the *hes5* genes in nascent neurons and, thereby, promoting their differentiation.

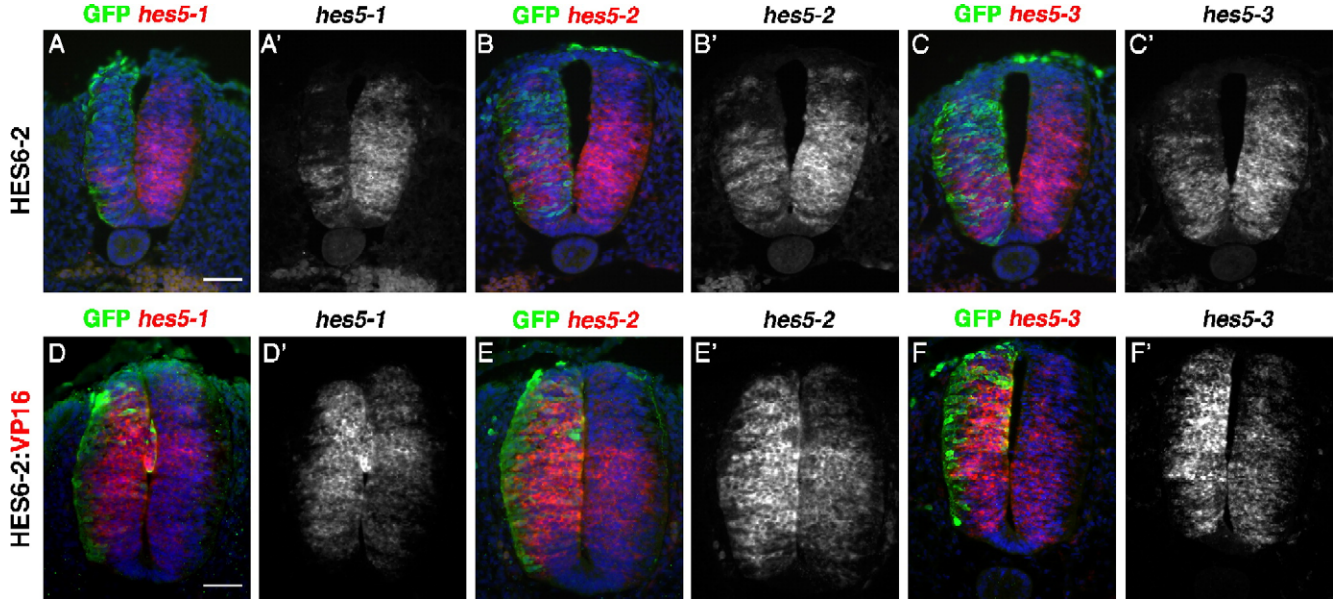

Fig. 9. *hes6-2* is a common repressor of the three *hes5* genes. Overexpression of HES6-2 represses transcription of *hes5-1* (A,A′), *hes5-2* (B,B′) and *hes5-3* (C,C′). On the contrary, overexpression of HES6-2:VP16 activates transcription of the three *hes5* genes (D–F, D′–F′). Embryos were collected 24 h after electroporation. Electroporated cells are shown in green due to the expression of GFP reporter. Scale bars = 50 μm.

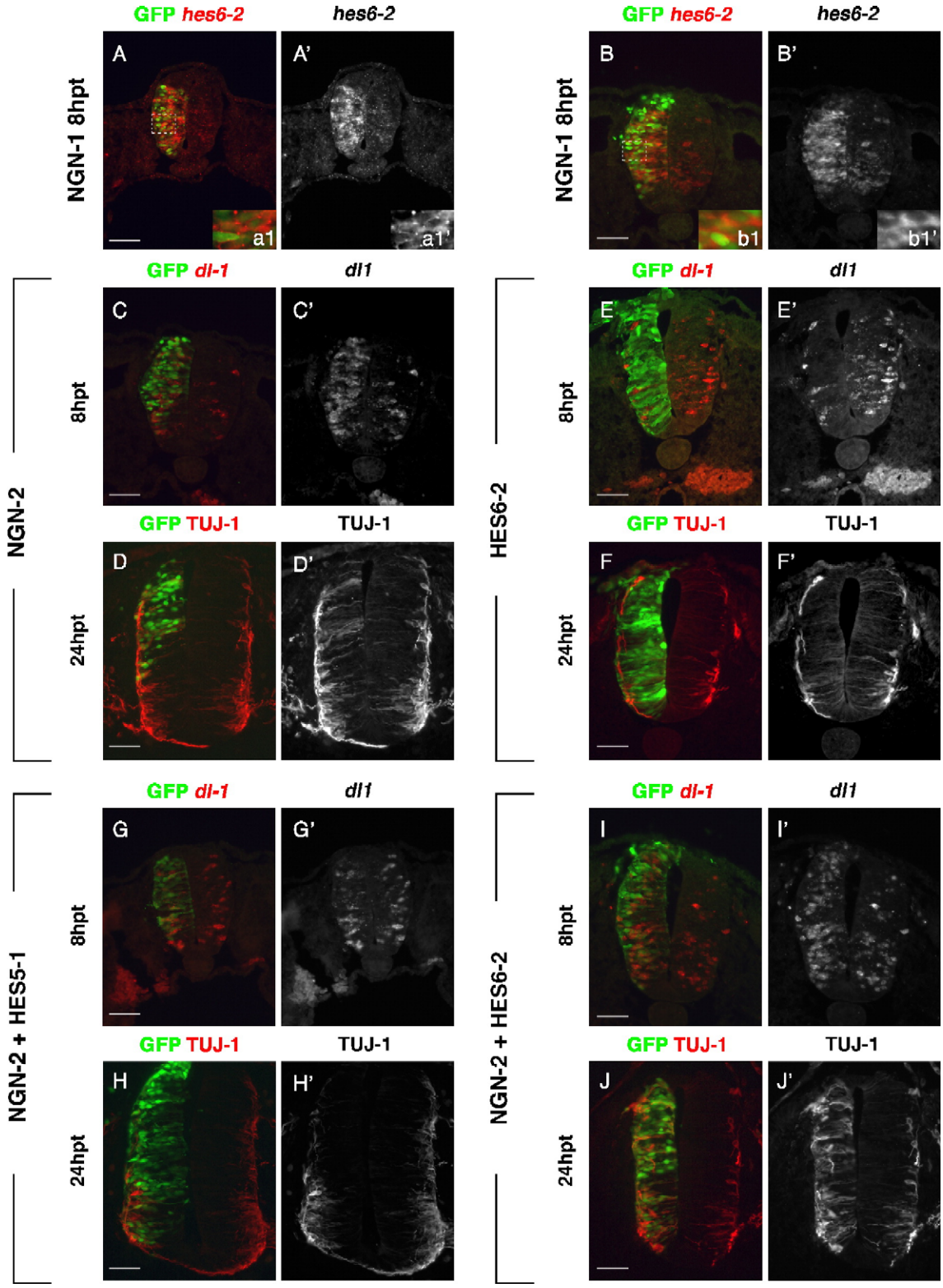

Fig. 10. The proneural genes activate *hes6-2* and, together, these genes promote neuronal differentiation. (A,B) Neurogenins activate *hes6-2* transcription, in a cell-autonomous manner, as shown after overexpression of either NGN-1 (A,A′) or NGN-2 (B,B′). Scale bars = 50 μm. The white box indicates zoomed region (a1, b1). This is accompanied by an increase in *Delta-1* transcription (C,C′) and Tuj-1+ cells (D,D′), indicating that neurogenesis is promoted by proneural proteins. On its own, overexpression of HES6-2 alone does not promote neuronal differentiation, leading instead to a down-regulation of the neuronal marker Tuj-1 (F,F′) and a decrease in the number of *Delta-1* expressing cells (E,E′). However, simultaneous expression of NGN-2 with HES6-2 induces the expression of *Delta-1* (I,I′) and the appearance of Tuj-1+ cells (J,J′) in a synergistic manner, when compared with NGN-2 alone. By contrast, overexpression of NGN-2 with HES5-1 results in an antagonistic effect: 8 h after electroporation, expression of *Delta-1* (G,G′) is down-regulated, and 24 h after electroporation, a decrease in the number of Tuj-1+ cells is observed (H,H′). Electroporated cells are shown in green due to the expression of GFP reporter. Scale bars = 50 μm.

Discussion

In this work, we describe four chick *hes* genes that are expressed in the developing nervous system: three *hes5*-like genes (*hes5-1*, *hes5-2* and *hes5-3*) and one *hes6*-like (*hes6-2*). All four genes are expressed in the ventricular zone of the embryonic neuroepithelium, where neural progenitors are located and where the *Notch1* receptor is expressed. We show that Notch signaling positively regulates the *hes5* genes but reduces expression of *hes6-2*. The four chick *hes* genes appear to be cross-regulated: each *hes5* gene is able to repress *hes6-2*, and all three *hes5* genes seem to be repressed by *hes6-2*. We propose that the function of this *hes5*/*hes6* circuitry is a conserved feature of the Notch pathway, modulating the response of neuroepithelial cells to Notch signals at different phases of their development.

A simple repertoire of hes genes in the chick

Our cloning work and the analysis of the available genome (Release 23.1a.1) and EST databases (Boardman et al., 2002) indicate that the chick contains 5 genes encoding bHLH-O proteins with homology to the *Drosophila* E(spl) proteins (Fig. 1). Three of these genes, named *hes5-1*, *hes5-2* and *hes5-3*, encode highly related proteins with strong homology to mammalian HES5 proteins (Akazawa et al., 1992; Takebayashi et al., 1995), while the other two genes encode proteins with high homology to mammalian HES6 (Bae et al., 2000; Koyano-Nakagawa et al., 2000; Pissarra et al., 2000) and were named *hes6-1* and *hes6-2*. Completion of the chick genome shall reveal if it contains further members of the *hes* gene family, but analysis of the large collection of chick ESTs now available suggests the existence of only the five *E(spl)*-like genes here reported, plus the two *hairy* homologues previously described (*chairy-1* and *chairy-2* (Jouve et al., 2000; Palmeirim et al., 1997)). In comparison, the zebrafish and pufferfish genomes have a much higher number of *hes* genes, at least 19 (Gajewski and Voolstra, 2002; Sieger et al., 2004), probably as a result of their genome duplication.

Analysis of the chick genome reveals that the three *hes5* genes are clustered on a 20 kb region of DNA in chromosome 21 (Supplementary Fig. 1), flanking the *fang1* gene. A similar cluster of three *hes5*-like genes is present in the pufferfish genome (Release 23.2c.1), located also in close proximity to the fugu *fang1* gene (data not shown), suggesting that the *hes5* cluster has been conserved from teleosts (a similar cluster is present in zebrafish) to avians. In mammals, however, a single *hes5* gene flanks the *fang1* gene, implying that the other two genes have evolved differently. Actually, two other *hes* genes–*hes2* and *hes3*–are present near *hes5* at the tip of Chromosome 4 in mouse (Nishimura et al., 1998), and Chromosome 1 in humans, within a 3 Mb region. This might indicate that the *hes2* and *hes3* genes derive from the ancestral *hes5* cluster but have been dispersed in the chromosome, with their promoter and coding sequences evolving so rapidly that they cannot be ascribed to the *hes5* sub-family anymore. In addition, the mouse *hes2* and *hes3* promoters are unable to respond to Notch activation (Nishimura et al., 1998), suggesting that these genes have lost their capacity to function as effectors of the Notch pathway. This highlights a surprisingly rapid evolution of the Notch pathway circuitry in mammals, which contrasts to the established conservation of its function in various cell fate decision processes.

Notch signaling regulates differently the expression of the chick hes genes

In *Drosophila,* the *E(spl)* genes are direct targets and effectors of Notch signaling in the embryonic nervous system (reviewed in (Bray, 1998). The mouse *hes5* gene, a homologue of the *Drosophila E(spl)* genes, is also one of the known targets of Notch signaling in the developing CNS, as shown by the strong reduction of *hes5* transcription in *Notch1* knock-out mice (de la Pompa et al., 1997; Lutolf et al., 2002). Our results, from experiments involving both gain- and loss-of-function assays for Notch signaling, show that the *hes5* genes are also Notch targets in the developing chick CNS, being positively regulated by activation of the Notch receptor (Fig. 5). This regulation is likely to involve direct binding of the Su(H)/CSL-NICD complex to the promoter regions of the *hes5* genes, which contain various putative Su(H)/CSL binding sites (Supplementary Fig. 1). Our experiments further indicate that the chick *hes5* genes are also effectors of Notch signaling, as their overexpression in the developing neural tube mimics the effects of Notch activation during neurogenesis, i.e., inhibition of neuronal differentiation and repression of the known target genes *Delta1* and *ngn1* (Fig. 6).

The *hes6-2* gene, in contrast, is not positively regulated by Notch signaling, being instead repressed when the pathway is activated in the chick neural tube. Conversely, when Notch signaling is reduced, transcription of *hes6-2* seems to increase. Another vertebrate *hes* gene, *Danio rerio her3*, has also been shown to be repressed, rather than activated, by Notch signaling (Hans et al., 2004). Both the zebrafish *her3* and the chick *hes6-2* genes contain Su(H)/CSL binding motifs in their promoters, however, they seem to be insufficient to drive transcriptional activation of these genes when Notch signaling occurs. Two hypotheses can be advanced to explain this finding. First, other transcriptional activators, in addition to the Su(H)/CSL-NICD complex, might be needed to effectively promote *hes6-2* activation in the neural tube, the proneural bHLH proteins being good candidates to play this role. Indeed, our results show that Neurogenins are able to activate *hes6-2* transcription when ectopically expressed in the neural tube (Figs. 10A–B′), supporting a positive role for the proneural bHLH proteins in *hes6-2* regulation. Similarly, in *Drosophila*, the Ac and Sc proneural proteins were shown to cooperate with the Su(H)/NICD complex to activate transcription of some of

the *E(spl)* genes (Cooper et al., 2000; Kramatschek and Campos-Ortega, 1994; Nellesen et al., 1999).

A second mechanism to explain why *hes6-2* is not activated by the Notch pathway, despite the presence of Su(H)/CSL binding motifs in its promoter, might involve the counteracting activity of transcriptional repressors that prevent activation by the Su(H)/CSL-NICD complex in the developing CNS. Our finding that each of the 3 *hes5* genes can repress *hes6-2* transcription raises the hypothesis that the HES5 proteins might be directly responsible for the *hes6-2* repression in Notch-responding cells through binding to the N- and E-boxes (class B/C) present in the *hes6-2* promoter. It is even possible that the *hes6-2* gene is initially induced by Notch signaling, in parallel with the 3 *hes5* genes, but is quickly down-regulated by the activity of these repressors. This hypothesis could not be assessed in our electroporation assays because, at the time when the GFP reporter becomes visible after electroporation, transcription of the NICD transgene and rapid accumulation of the downstream *hes5* effectors have certainly been underway for sometime already, leading to the detectable repression of *hes6-2*. Nevertheless, the presence of Su(H)/CSL binding motifs in both the *hes5* and *hes6-2* promoters opens the possibility that all 4 *hes* genes are equally activated by Notch signaling, with *hes6-2* being swiftly repressed by the abundant HES5 proteins.

A cascade of hes activity in neural progenitors

Our analysis of the regulation of the four *hes* genes expressed in the chick developing neural tube reveals the existence of negative auto-regulatory mechanisms, as well as cross-regulatory interactions between the *hes5* and *hes6-2* genes. Our results led us to postulate that *hes6-2* functions as a common repressor of the *hes5* genes, being itself also repressed by these genes (Fig. 8). In addition, we propose that this circuit of negative feedback regulation between the *hes5* and *hes6-2* genes might play a key role during neurogenesis, modulating Notch activity in both neural progenitors and nascent neurons.

In a simple scenario, when Notch is activated in a neural progenitor, in response to a Delta signal from a neighboring cell, a fast and massive transcription of the three *hes5* genes will follow. As Notch effectors, their activity will be essential to implement the decision to stay as a neural progenitor by repressing the proneural genes (and also *hes6-2*). Later on, negative auto-regulation of *hes5-1* and *hes5-2* would lead to a down-regulation of their own expression, with only *hes5-3* remaining functional. At this point, *hes6-2* might become more active (because their repressors are now less abundant) and would eventually suppress *hes5-3* activity and terminate Notch signaling. Negative auto-regulation of *hes6-2* (data not shown) would finally close a cycle of Notch activity and the cell can again embark on a new process of cell fate decision. This would involve a choice between continuing as a neural progenitor (which requires a new cycle of Notch activity) or committing to neuronal differentiation (which involves a definitive release from Notch signaling). Therefore, neural progenitors go through cyclic bursts of Notch activity, until they finally commit to differentiation (or instead switch to another fate, like glial progenitor (Gaiano and Fishell, 2002)), and we propose that the *hes5*/*hes6-2* circuitry of negative feedback regulation plays a central role in this mechanism.

The existence of cycles of Notch activity in neural progenitors is also supported by the findings of Frade and colleagues (Murciano et al., 2002), who reported that transcription of the *Notch1* gene is switched off when neural progenitors enter S-phase, restarting later to allow the cells to interact with their neighbors and decide their fate. These cycles of Notch activity in neural progenitors might be similar to the cycles described in cells of the presomitic mesoderm (Dale et al., 2003), which also seem to rely on negative feedback of *hes* genes (Lewis, 2003), in the case of the chick, the *hairy1* and *hairy2* genes (Jouve et al., 2000; Palmeirim et al., 1997). Whether the cyclic Notch activity has a "clock-like" function in neuroepithelial cells, like it seems to have during somitogenesis, is an interesting question that merits further investigation.

The role of hes6-2 during neuronal commitment

Our analysis of *hes6-2* expression during chick spinal cord development reveals that this gene is expressed at two different phases of neurogenesis: in neural progenitors located in the ventricular zone, close to the apical region of the neural epithelium, and in nascent neurons entering differentiation, located more basally (Figs. 4D–J). Expression is higher in the latter, which shows also high levels of *Delta1* expression (Henrique et al., 1995; Myat et al., 1996). This raises the hypothesis that *hes6-2*, apart from the potential role in neural progenitors discussed above, could also function in cells committing to differentiation, ensuring that these cells are fully released from Notch signaling and can become neurons.

It is known that the commitment to neuronal differentiation involves the activity of the proneural bHLH proteins, which trigger a cascade of events leading to cell cycle exit of neural progenitors and full differentiation into neurons (reviewed in (Bertrand et al., 2002; Ross et al., 2003). Our results indicate that *hes6-2* is a possible target of the proneural bHLH proteins in nascent neurons. The repressor activity of HES6-2 might be crucial to block any *hes5*-mediated Notch activity in these cells, but this does not seem to be enough to drive neuronal differentiation by itself, as overexpression of HES6-2 in the chick neural tube does not result in increased neurogenesis. However, simultaneous overexpression of NGN2 and HES6-2 leads to a clear increase in neuronal production (Figs. 10J–J′), indicating that *hes6-2* cooperates with the proneural genes to promote neurogenesis in the chick spinal cord. In contrast, HES5 proteins seem to antagonize NGN's proneural activity, as

simultaneous overexpression of NGN-2 and HES5-1 results in little or no effect on neuronal production (Figs. 10G–H). Together, these results indicate that *hes6-2* functions in nascent neurons to reinforce the decision to enter neuronal differentiation by suppressing the inhibitory activity of the *hes5* genes.

In both mouse and *Xenopus*, *hes6* homologues are regulated by proneural genes and were shown to promote neurogenesis, but only in regions of the neural plate where the proneural genes are already expressed (Bae et al., 2000; Koyano-Nakagawa et al., 2000), indicating some conservation of *hes6* function in vertebrate neural development. However, in contrast to our findings in the chick, the function of *hes6* in mouse and *Xenopus* does not seem to involve transcriptional repression of Notch effectors. Instead, *hes6* has been shown to inhibit *hes1* activity, through the formation of HES1:HES6 heterodimers that are unable to repress the normal HES1 targets. This correlates to the fact that, contrarily to mHES1, the mouse HES6 protein cannot bind to N-boxes due to its shorter loop region in the bHLH domain. Furthermore, HES1:HES6 heterodimers seem more prone to proteolytic degradation, for which phosphorylation of a specific serine residue in mHES6 (Ser183) seems to be crucial (Gratton et al., 2003). In the case of the chick, not only HES6-2 lacks an equivalent serine residue, but also its loop region is 2 amino acids longer than that of mHES6 (10 in HES6-2, 8 in mHES6 and 13 in mHES1), raising the possibility that HES6-2 may also have N-box binding activity. Furthermore, the expression of chick *hairy-2*, which encodes the chick HES1 protein (Jouve et al., 2000), does not correlate with Notch activity in the chick spinal cord, so it is unlikely that HES6-2 functions during neurogenesis by controlling HES1 activity. Instead, our results indicate that HES6-2 has the capacity to directly repress the transcription of the chick *hes5* genes and might, in this way, modulate Notch activity. In addition, although not addressed in this work, it is also possible that HES6-2 forms inactive heterodimers with the chick HES5 proteins, further hindering their activity as Notch effectors.

In mammals, no interaction between *hes5* and *hes6* were reported yet, but it is possible that *hes6* also controls *hes5* activity during mammalian neural development. The two genes have very similar expression in the developing neural tube (Bae et al., 2000; Hatakeyama et al., 2004; Koyano-Nakagawa et al., 2000; Pissarra et al., 2000; Takebayashi et al., 1995) and *hes5* is clearly a main Notch effector during mammalian neurogenesis (de la Pompa et al., 1997; Lutolf et al., 2002; Ohtsuka et al., 1999). Furthermore, mouse *hes6* was shown to promote neuronal differentiation in various assays (Bae et al., 2000; Koyano-Nakagawa et al., 2000) and it is unlikely that this activity is uniquely mediated by the interaction with *hes1*, whose expression in the developing neural tube is rather restricted (Hatakeyama et al., 2004). Therefore, although the molecular details may vary in different cells, or between different animals, *hes6* seems to have a conserved function during vertebrate neurogenesis, as a negative regulator of Notch signaling.

Conclusion

In vertebrates, the Notch pathway has conserved functions in developmental processes as different as neurogenesis and somitogenesis, even if the components and regulatory mechanisms might reveal some variability between different species. This functional flexibility is a consequence of the robustness of the Notch pathway, which leads invariably to a stable, and simple, outcome: making two cells (or two groups of cells) adopt distinct developmental decisions. This robustness was proposed to arise from the existence of several interlaced negative feedback loops, inter- and intra-cellular, that amplify minor differences in the cells' potential and ensures that they stably adopt different decisions (Meir et al., 2002).

One of these negative feedback loops is described in this paper, involving the circuitry of *hes5* and *hes6* activity during neurogenesis. Although the molecular details might be different in chick and mouse, the function of this *hes5*/*hes6* circuitry seems to be a conserved feature of Notch signaling in vertebrate neural development. We show that, in nascent neurons, a *hes6* gene represses the Notch effectors encoded by *hes5* genes, cooperating with the proneural proteins to drive these cells into neuronal differentiation. We also propose that the design of the *hes5*/*hes6* circuitry supports the generation of pulses of Notch activity in neural progenitors, which are responsible for the maintenance of these cells within the neuroepithelium. In this process, the *hes5* genes act first as effectors of the Notch pathway to prevent these cells from embarking on neuronal differentiation, after which *hes6* comes into action to repress *hes5* activity and terminate Notch signaling. As a result, neural progenitors are driven back into a "neither-on-nor-off state" at the end of each pulse of Notch activity, being able to start afresh a new cell fate decision process. Neurogenesis in the vertebrate neural tube can thus be viewed as a reiterative process where cells go through successive events of cell fate decision, mediated by the Notch pathway, until all progenitors are exhausted or move into a different competence state.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.03.017.

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NEURONAL PRODUCTION *IN VITRO* FROM EMBRYONIC STEM CELLS

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ABSTRACT

Cell therapy in the nervous system is a promising strategy to cure diseases like Parkinson's or for nerve regeneration in spinal cord lesions. However, it requires the *ex-vivo* generation of neurons or their immediate progenitors in sufficient numbers, and of the correct neuronal type, which can then be used for transplantation. To achieve this, an efficient method for the *in vitro* production of neurons was established, starting with Embryonic Stem (ES) cells. We show that this method mimics several steps of the neurogenesis process in the developing embryo, with cultured cells being able to organize in 3D structures that resemble embryonic neural tubes. This method might prove to be extremely useful to generate differentiated neurons for future transplantation studies in the mammalian brain.

INTRODUCTION

One of the most puzzling natural phenomena is the creation of a complex multicellular organism from a single totipotent cell, the zygote. For many decades, the process of embryogenesis has been a subject of intense research. The accumulated knowledge has allowed the recent emergence of several strategies to[1] achieve the *in vitro* production of differentiated cells, tissues and organs, for therapeutic purposes (1). In this new field, named Regenerative Medicine, a particular type of cell - stem cell, has a fundamental role. Stem cells are characterized by their ability to self-renew and to generate [2]differentiated progeny, being able to functionally reconstitute a given tissue *in vivo*.

Throughout embryonic development, several stem cells emerge that differ in their differentiation potential. They can be found in embryonic tissues, fetal

tissues (e.g. Embryonic Germ (EG) cells, fetal multipotent cells) and adult tissues (e.g. Hematopoietic Stem Cells - HSCs). An important discovery involved the characterization of the so-called Embryonic Stem (ES) cells, which were first isolated from mouse blastocysts (2, 3), one of the earliest stages of embryonic development. These cells are pluripotent, i.e., able to differentiate into cells from the three germ layers, and can be cultured for long periods of time without loosing this ability. In contrast, adult stem cells are more limited in their potential and can be found in specialized tissues in the adult organism, such as the brain, being able to self-renew and differentiate only into cells from the originating tissue.

Given their exclusive properties, stem cells are promising candidates for tissue engineering, cellular therapies and drug screening (4). The *in vitro* reconstitution of neurogenesis, involving the production of neuronal precursors and/or differentiated neuronal subtypes, is one of the most sought-for processes. Successful attempts have been made to achieve in vitro neuronal differentiation from ES cells, either by embryoid body (EB) formation in the presence of retinoic acid (5), by co-culture with stroma/conditioned medium (6, 7), or by monolayer differentiation (8). However, as ES cells are pluripotential and readily differentiate into almost any cell type, lineage selection is usually essential to ensure homogeneity of the differentiated population (9). Neuronal differentiation from neural stem cells (NSCs), either adult or embryonic, has also been achieved and later tested in transplantation studies (10). However, clonal propagation of neural-stem (NS)-derived precursors is limited and a switch from neuronal to mostly glial fate occurs during prolonged culture of these cells.

A successful strategy to achieve production of neuronal precursors *in vitro* must take into account what is known about neurogenesis in the vertebrate embryo and the regulatory events involved in the process. Neural induction happens early during embryonic life and involves both FGF signaling and the inhibition of BMP signaling by SMAD1 phosphorylation (11, 12). Neurogenesis begins when ectoderm cells receive these induction signals coming from the underlying notochord, forming a new embryonic tissue, the neuroepithelium, a thickened epithelial sheet where cells form a tightly-packed monolayer with constricted apical surfaces and elongated fusiform cell bodies. Within the neuroepithelium, cells start to express *Sox1*, a Sry-related transcription factor specific to early commitment stage of

neurogenesis (13). Neuroepithelium then folds into a tube-like structure, the neural tube, where the concerted action of anterio-posterior and dorso-ventral patterning processes leads to the regionalization of the major subunits of neural tube, such as forebrain, midbrain, hindbrain and spinal cord, and their subsequent subdivision.

Neuroepithelial cells show a marked apico-basal polarity, which has both structural and functional importance (Fig. 1). The apical domain is located at the luminal surface and is delineated by the presence of apical protein complexes, like the PAR polarity complex (14), as well as by the presence of junctional structures where N-cadherin and β-catenin accumulate (15). Centrosomes also localize apically in neuroepithelial cells, which enter mitosis close to apical surface due to the characteristic interkinetic nuclear movement (16). This particular organization of neural tube is important for the coordinated production of neurons and glia. Neighboring neuroepithelial cells signal to each other through the interaction of the Delta ligand and the Notch receptor, resulting in the inhibition of differentiation of the cells adjacent to newborn neurons, which will later accumulate at the basal portion of the neural tube and migrate to dorsal root ganglia (17). This process of lateral inhibition, mediated by the Notch pathway, is responsible for the maintenance of neural progenitors throughout the process of neurogenesis and, consequently, for the timely production of the right number of neurons at each time of embryonic development (18).

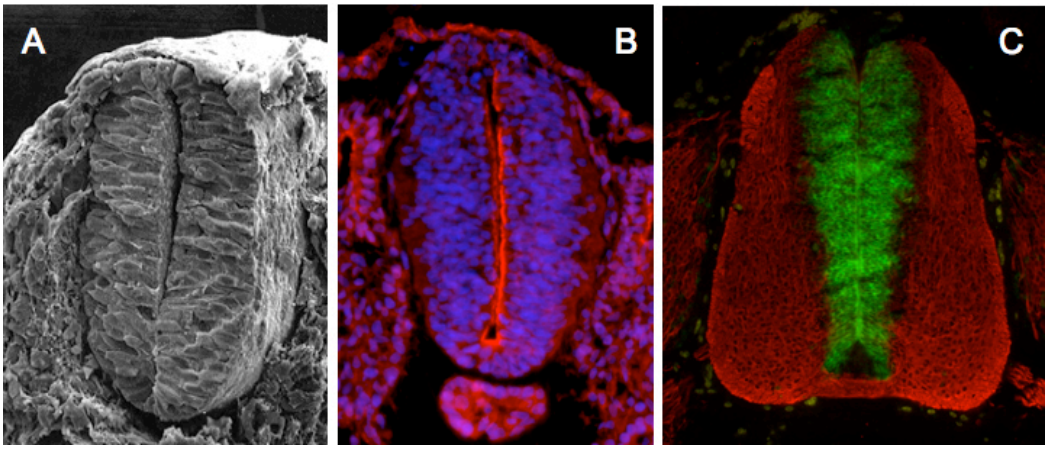

FIGURE 1 **A**, Scanning electron microscope image of the transverse section through the E9 mouse embryo, revealing the closed neural tube. B and C: E2 and E3 chick neural tube, respectively. **B**, β-catenin staining in red, nuclei in blue. **C**, *hes5-3 in situ* hybridization (green), delimiting progenitor zone, and Tuj1 immunostaining (red) for nascent neurons located more basally in the neural tube.

MATERIALS AND METHODS

The 46C ES line (8) was used along this work and was kindly provided by Dr. Austin Smith (Edinburgh University, U.K.). It contains the coding sequence of GFP inserted in the *sox1* gene and has been used successfully to follow neural commitment (8). N2B27 and RHB culture media were obtained from StemCellSciences Co. (U.K.). FGF-2 was obtained from Peprotech. Antibodies were obtained from Upstate Biotech. and Santa Cruz Biotech. (USA). Immunofluorescence studies were performed as described (19).

RESULTS AND DISCUSSION

The simplest way to reconstitute neural commitment *in vitro,* and achieve efficient neuronal production, relies upon monolayer differentiation of ES cells, a method developed by Ying and co-workers (8). In this method, ES cells are cultured in defined medium which does not contain serum and is thus free from BMP-imposed inhibition of neural fate. Prior to initiate neural differentiation, cells are grown overnight in a dense culture, allowing establishment of multiple intercellular contacts. These dense cultures are then replated at low density in defined serum-free medium (N2B27), which contains N2 supplement (insulin, apo-transferrin, sodium selenite, progesterone and putrescine), and B27 supplement, containing retinoic acid. Though none of these components, with the exception of apo-transferrin, is essential for neuronal commitment (20), their combinatorial effect results in up to 90% neuronal commitment by the sixth day of continuous culture, as measured by the activity of the Sox1-GFP knock-in allele present in the 46C ES cell line (Fig. 2).

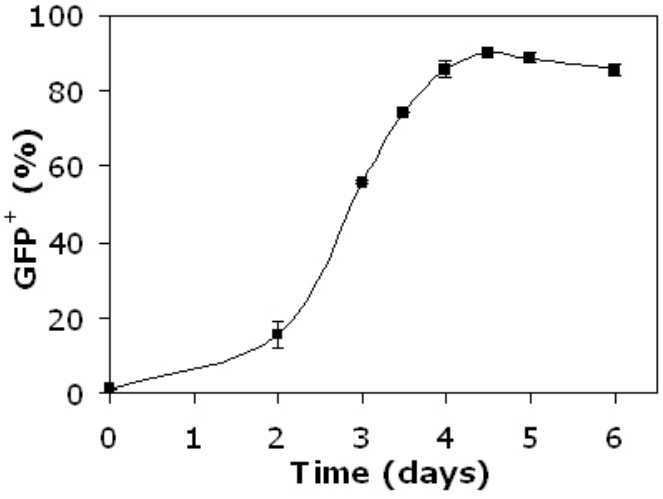

FIGURE 2
Percentage of GFP-expressing cells during monolayer differentiation. The use of this specific cell line allows FACS-based monitoring of the dynamics of neuronal commitment.

Most interesting, by day 5-6 in monolayer culture, the commited neural progenitors form either rounded clusters of GFP-positive cells, or extended sheets with patchy GFP distribution. Immunostaining for apical markers, like the zona occludens protein ZO-1, reveals that GFP-positive patches of cells are organized in rosette-like structures resembling small neural tubes, with well-defined apical domains, around which GFP-positive neural progenitors are organized (Fig. 3).

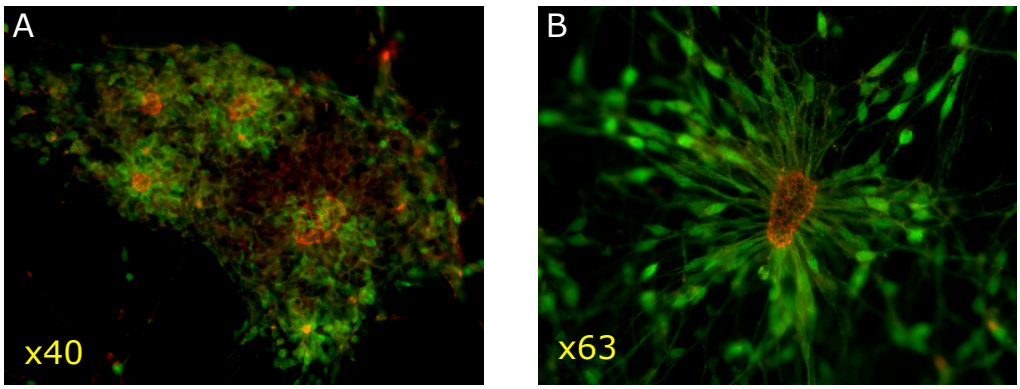

FIGURE 3. **A**, An example of day 6-monolayer culture of 46C cells, stained with ZO-1 antibody (red), and GFP (green). **B**, Rosette structure of neural precursors, formed after replating of day 6-monolayer culture onto poly-D-lysine/laminin-coated dish.

This suggests that neuroepithelial cells are able to achieve a correct apical polarity in the monolayer differentiation conditions employed in these experiments. To confirm this, we have performed a detailed characterization of the rosette-like structures obtained during the monoloayer differentiation protocol, using immunofluorescence localization of several known apical proteins. As shown in figure 4, neuroepithelial cells within rosettes have a polarized distribution of junctional components like N-cadherin and □-catenin, which appear to localize close to the luminal region of such rosettes. The PAR polarity complex is also localized at the same luminal region, confirming that this region constitute the apical domain of rosette's neuroepithelial cells. This is also confirmed by the localization of centrosomes at the region below the apical domain, and by the localized occurrence of mitotic figures in the same region, as it normally happens in the embryonic neural tube. Concurrently, differentiated neurons, detected by the Tuj1 and HU antibodies, are present outside the rosette structures, mimicking their normal migration from the neural tube ventricular region.

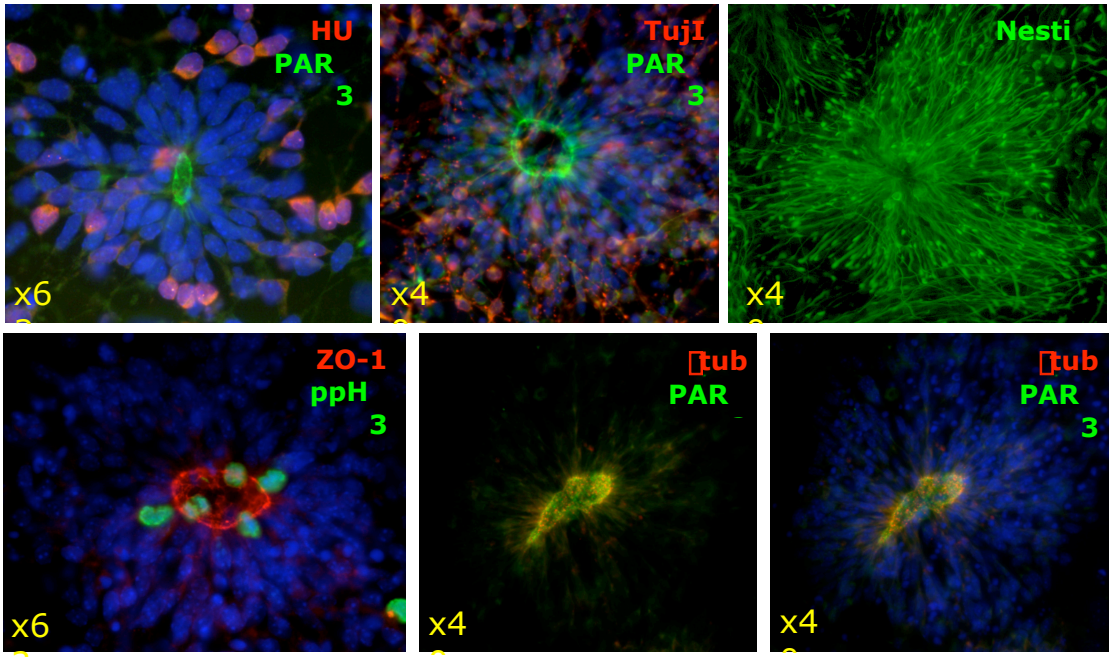

FIGURE 4 Neural tube-like structures formed by S25 ES cells after 12 days in monolayer culture. After the initial 6 day-monolayer culture, cells were replated on poly-D-lysine/laminin coated coverslips and cultured in the same conditions for 6 days more, then fixed and stained with indicated antibodies. S25 ES cell line bears a recombinant Sox2-geo allele and shows the same efficiency of neural commitment in monolayer culture as 46C line, as estimated by Nestin immunofluorescence.

In summary, the monolayer differentiation method constitutes an excellent approach to study neurogenesis *in vitro*, as it permits to reconstruct, at least partially, the tridimensional organization of the embryonic neural tube. We have found that several independent ES cell lines show a similar behaviour during *in vitro* differentiation, indicating that this processes is universal and must be important to achieve normal neuronal commitment and differentiation. Furthermore, as neural progenitors can be found only within the rosette structures, while neurons migrate out of these structures, we propose that a proper epithelial organization is important for neuronal commitment in vitro, as well as to achieve an efficient neuronal production.

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