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# Regulation of Dll4 expression in cells committing to differentiation in the V2 domain of the developing spinal cord

Dissertação orientada por:

Catarina Ramos, PhD, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa

Rita Zilhão, PhD, Faculdade de Ciências da Universidade de Lisboa

Joana Matos das Neves

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## Abstract

During spinal cord (SC) embryonic development, several excitatory and inhibitory interneurons (INs) are generated but the molecular mechanisms underlying such cell diversity remain poorly understood. One of the mechanisms that has been involved in the generation of neuronal diversity is the cell-cell signalling mediated by the Notch pathway. Particular attention has been given to the V2 domain of SC since: i) instead of one ligand (as observed in the remaining domains), two Notch ligands (Delta-like 1 and Delta-like 4) are expressed and ii) three molecularly distinct subtypes of INs, V2a, V2b and V2c, are generated from apparently common progenitors. How *Delta-like 4 (Dll4)* expression is regulated and how IN specification is controlled in the V2 domain are the main focus of this thesis. To address these questions, the chick embryo was used as model organism.

Using available databases and bioinformatics tools, we compared chick and mouse *Dll4* promoter sequences. We identified preferred E-boxes for the binding of Mash1 and Neurogenins, two of the main proneural bHLH proteins, predicting that these proteins may regulate *Dll4* expression. To test this hypothesis, we overexpressed Mash1, NGN1 and NGN2 in the chick developing SC. We show that Mash1 and NGN1 are able to activate *Dll4* expression whereas NGN2 is not. As proneural proteins are involved in IN specification, we analysed the number of V2a INs after overexpressing these proteins. We show that while Mash1 represses V2a IN fate, NGN1 and NGN2 promote this fate. Moreover, as HES proteins (another bHLH protein family member) have been shown to bind to E-boxes, we tested if HES6-2 could regulate *Dll4* expression and found that it might act as a repressor of *Dll4*. To further investigate how *Dll4* expression is regulated, we generated a fluorescent reporter using the 3310bp sequence localized upstream of the *Dll4* coding region as a promoter sequence. This reporter failed to reflect endogenous *Dll4* expression, indicating that for the accurate expression of *Dll4,* there must be essential regulatory sequences outside the 3310bp upstream of the *Dll4* coding region.

This thesis presents new evidences on how *Dll4* expression can be regulated by different proneural proteins and HES6-2 protein and what may be their contribution to V2 interneuron specification. This study provides new insights on how neurogenesis is controlled in the V2 domain of the developing SC.

**Keywords:** Chick, Neurogenesis, Spinal Cord, V2 domain, Notch Signalling, Delta-like 4, Proneural proteins, HES6-2 protein

## Resumo

O Sistema Nervoso Central é um sistema muito complexo sendo constituído por um grande número e variedade de células - neurónios e células da glia - as quais são, na sua grande maioria, produzidas durante o desenvolvimento embrionário. Estas células têm de ser geradas no momento e posição correctos, de forma a interagirem entre si e a formarem circuitos funcionais. Durante o desenvolvimento da espinal medula, um grande número de interneurónios excitatórios e inibitórios são gerados, mas os mecanismos moleculares subjacentes a esta diversidade celular são ainda pouco conhecidos. No entanto, sabe-se que a via de sinalização Notch desempenha um papel essencial durante o desenvolvimento do Sistema Nervoso Central.

A via de sinalização Notch é um sistema de comunicação célula-a-célula que ocorre através do contacto directo entre duas proteínas membranares: o receptor Notch e os seus ligandos (Delta ou Serrate). Quando um ligando de uma célula interage com o receptor de outra célula desencadeia uma série de clivagens proteolíticas que levam à libertação do domínio intracelular do receptor Notch (NICD). O NICD é translocado para o núcleo onde se associa a outros factores (CSL e Mastermind) para activar a expressão de genes alvo, como os genes *Hes*. Na ausência de NICD, a proteína CSL actua como repressor transcricional dos mesmos genes.

Actualmente, a função melhor caracterizada da via de sinalização Notch é a manutenção de progenitores neurais durante a neurogénese. A decisão de uma célula permanecer como progenitor ou diferenciar é controlada pelo balanço de dois tipos de factores de transcrição: proteínas proneurais, que promovem a diferenciação, ou proteínas HES, que reprimem a diferenciação neural. Uma célula ao expressar elevados níveis de proteínas proneurais vai iniciar o processo de diferenciação. Como estas proteínas activam a expressão de ligandos Notch, esta célula vai expressar elevados níveis de ligandos e vai activar a via Notch nas células vizinhas. Como consequência da activação desta via, estas células vão expressar elevados níveis de proteínas HES e vão permanecer como progenitores. Desta forma, a célula que expressa o ligando Notch diferencia-se em neurónio mas simultaneamente assegura que as células vizinhas se mantenham como progenitores. Visto que a neurogénese ocorre durante uma larga janela temporal, a manutenção de uma população de progenitores permite que estas células sejam expostas a diferentes estímulos e, como tal, diferenciem em diferentes neurónios durante o desenvolvimento.

Evidências recentes mostram que a via Notch está também envolvida na especificação de células neurais, nomeadamente na especificação de interneurónios no domínio V2 da espinal medula. Neste domínio, três interneurónios, V2a, V2b e V2c, são produzidos a partir de progenitores comuns. Na ausência de sinalização Notch, apenas os interneurónios V2a

são produzidos, o que indica que a sinalização Notch é necessária para a produção dos interneurónios V2b (os interneurónios V2c foram identificados recentemente e, como tal, não existem muitos dados disponíveis sobre esta linhagem). Curiosamente, nesta fase do desenvolvimento, este é o único domínio onde se sabe que diferentes interneurónios são simultaneamente produzidos e o único domínio onde dois ligandos da via Notch são expressos: Delta-like 1 (DLL1) e Delta-like 4 (DLL4). Pensa-se que o ligando DLL1 esteja envolvido na activação da via Notch e consequente manutenção de progenitores, enquanto o ligando DLL4 estará provavelmente envolvido na especificação dos interneurónios.

Como é que a expressão de *Dll4* é regulada no domínio V2 da espinal medula e como é que a especificação de interneurónios é controlada neste domínio são os principais temas investigados nesta tese.

O organismo modelo utilizado para estudar estas questões foi o embrião de galinha.

Recorrendo à técnica de hibridação *in situ*, mapeámos a expressão de *Dll4* na espinal medula durante o desenvolvimento embrionário da galinha. O mRNA deste gene não foi detectado em células neurais da espinal medula no dia embrionário 2 (E2) nem no E3. No entanto, foi detectado no E4 na zona ventricular do domínio V2. Inesperadamente, observámos expressão de *Dll4* em vários domínios dorsais e ventrais no E6, o que sugere que este ligando poderá estar envolvido na manutenção de progenitores ou na especificação de interneurónios produzidos mais tarde no desenvolvimento embrionário da galinha.

Visto que as sequências importantes para a regulação da expressão de um gene tendem a ser conservadas entre diferentes espécies, utilizámos ferramentas bioinformáticas e bases de dados disponíveis para comparar as sequências do promotor do gene *Dll4* de ratinho e galinha. Esta análise revelou que o nível total de semelhança destas sequências entre as duas espécies é baixo, tendo sido apenas possível identificar 5 regiões com semelhança superior a 60%. Nestas regiões conservadas foram identificadas E-boxes, sequências consenso às quais se ligam proteínas proneurais e proteínas HES. Uma análise mais detalhada permitiu identificar sequências consenso específicas para a ligação das proteínas proneurais Mash1 e Neurogeninas (NGN), o que sugere que estas proteínas possam estar directamente implicadas na regulação da expressão de *Dll4*.

Para testar esta hipótese, as proteínas Mash1, NGN1 e NGN2 foram sobre-expressas na espinal medula de embriões de galinha no estádio HH17-18 pela electroporação *in ovo* de plasmídeos que codificam estas proteínas. Utilizando a técnica de hibridação *in situ* foi possível observar que tanto Mash1 como NGN1 promovem a expressão ectópica de *Dll4* e, como tal, regulam positivamente a sua expressão. No entanto, após a sobre-expressão de NGN2, não se verificou nenhuma alteração na expressão endógena de *Dll4*, indicando que esta proteína proneural não será um factor importante no controlo da expressão deste gene.

Visto que as proteínas proneurais controlam a especificação de neurónios durante o desenvolvimento de Sistema Nervoso Central, analisámos se depois da sobre-expressão destas proteínas o número de interneurónios V2a produzidos é alterado. Após a sobre-expressão de Mash1, observámos uma diminuição no número de interneurónios V2a, indicando que Mash1 reprime, directa ou indirectamente, a produção destes interneurónios. Contrariamente, observámos que, após a sobre-expressão de NGN1 e NGN2, o número de interneurónios V2a aumenta, o que sugere que as proteínas NGN1 e NGN2 promovem directamente a diferenciação de interneurónios V2a.

As proteínas HES também se ligam a E-boxes e, como tal, podem estar envolvidas na regulação da expressão de *Dll4*. Visto que a proteína HES6-2 actua como repressor negativo da via Notch e é expressa ao longo do eixo dorso-ventral da espinal medula, esta proteína pode estar envolvida na regulação da expressão de *Dll4* impedindo a sua expressão não só fora do domínio V2 no E4, mas também nos interneurónios V2b. A proteína HES6-2 contém um domínio repressor (WRPW) responsável pela sua actividade repressora. Para testar se esta proteína funciona como repressor transcricional do gene *Dll4*, utilizámos uma forma dominante negativa, na qual o domínio repressor WRPW foi substituído por um domínio transactivador - VP16. Esta proteína (HES6-2:VP16), em vez de reprimir, activa a transcrição dos genes alvo. HES6-2:VP16 foi sobre-expressa na espinal medula de galinha no estádio H17-18 pela electroporação *in ovo* de um plasmídeo que codifica esta proteína. Utilizando a técnica de hibridação *in situ*, observámos expressão ectópica de *Dll4*, indicando que a proteína HES6-2 reconhece o promotor deste gene e regula negativamente a sua expressão.

Paralelamente, gerámos um repórter fluorescente da expressão de *Dll4*. No entanto, utilizando a sequência de 3310pb localizada a montante da região codificante do gene *Dll4* de galinha como sequência promotora, não conseguimos gerar um repórter cuja expressão do gene repórter (*Venus*) reflicta a expressão endógena do gene *Dll4*. Como tal, poderão existir sequências regulatórias necessárias para a correcta expressão de *Dll4* (nos estádios analisados) que não se encontram localizadas nos 3310pb a montante da região codificante deste gene. Sabe-se que as regiões regulatórias podem estar localizadas vários kb a montante do promoter, em intrões ou mesmo a jusante da região codificante. Como tal, é necessário fazer uma análise mais alargada do *locus Dll4* para tentar identificar novas regiões conservadas que possam conter sequências regulatórias necessárias à correcta expressão do gene *Dll4*.

Este estudo apresenta novas evidências sobre a função que diferentes proteínas proneurais e a proteína HES6-2 têm na regulação da expressão de *Dll4* e na especificação

de interneurónios no domínio V2. Estes resultados fornecem dados para uma futura análise detalhada de como a neurogénese se processa neste domínio.

**Palavras-chave:** Galinha, Neurogénese, Espinal medula, Domínio V2, Sinalização Notch, Delta-like 4, Proteínas proneurais, Proteína HES6-2

## Abbreviations

AP – Anterior-Posterior
ASC - Achaete-Scute
ATO - Atonal
BAC – Bacterial artificial chromosome
bHLH – basic Helix-Loop-Helix
BMP - Bone Morphogenetic Protein
bp – base pair
CNS – Central Nervous System
CSL - CBF1, Suppressor of Hairless, Lag-1
DIG - Digoxigenin
*Dll – Delta-like*
DMSO – Dymethyl sulfoxide
DNA – Deoxyribonucleic acid
DSL - Delta/Serrate/Lag-2
DV – Dorsal-Ventral
E – Embryonic day
FBS – Fetal Bovine Serum
FGF – Fibroblast Growth Factor
HES - Hairy and Enhancer of Split homologues
HH stage – Hamburger and Hamilton stage
IN – Interneuron
kb – kilobase
LB – Luria Bertani bacterial medium
min - minutes
MZ – Mantle Zone
NGN - Neurogenin
NICD – Notch Intracellular Domain
NLS – Nuclear localization signal
o/n – Overnight
pb – pares de bases
PCR – Polymerase Chain Reaction
RA – Retinoic Acid
RNA – Ribonucleic acid
RT – Room Temperature
Shh - Sonic Hedgehog
SOB – Super Optimal Broth
VEGF – Vascular endothelial growth factor
VZ – ventricular zone

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# 1. Introduction

The Central Nervous System (CNS) is composed by a huge number and variety of neurons and glia cells which are mainly produced during embryonic development. These cells must be generated in the correct number and position in order to accurately interact with each other and assemble into a functional network. Therefore, the formation of the CNS must be strictly controlled.

## 1.1. Vertebrate neurogenesis

It is known that neurogenesis occurs over a long developmental time window and that the pool of progenitor cells that gives rise to all neuronal cell types is relatively small. Thus, mechanisms must exist to ensure that this pool is maintained throughout embryonic development, being exposed to different environmental cues and giving rise to different cell types at different times (early and late cell fates). The maintenance of progenitor cells depends mainly on the balance between proliferation events and commitment to cell differentiation, which occurs upon withdrawal of cells from the cell cycle.

In the vertebrate neural tube, the rudiment of the CNS, neural progenitors are localized in the ventricular zone (VZ), the inner most layer, whereas differentiating neurons accumulate in the outer region, known as mantle zone (MZ) (Fig. 1).

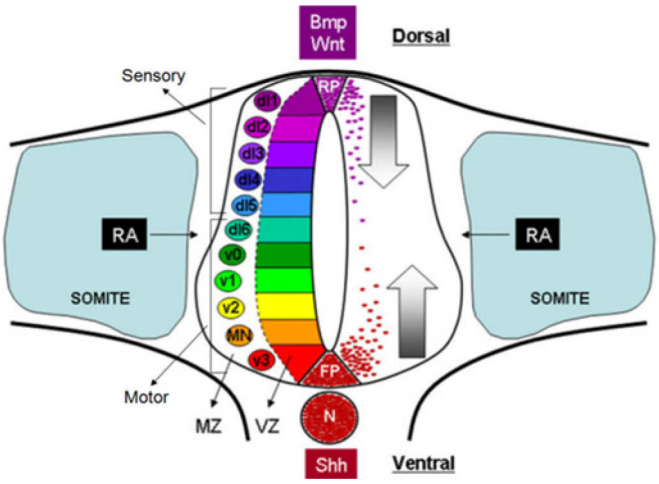


**Figure 1 – Diagram of a transverse section of the neural tube.** The progenitors localized in the ventricular zone (VZ) differentiate into post-mitotic neurons that accumulate in the mantle zone (MZ). Different populations of neuronal progenitors and their correspondent post-mitotic derivates are distributed in a specific order along the dorsal-ventral axis. This patterning is established by the action of gradients of Shh, secreted from the notochord (N) and the floor plate (FP), and Wnts and BMPs, produced by the roof plate (RP) and the dorsal epidermis. The Retinoic Acid (RA) produced by the adjacent somites is also involved in DV and AP patterning of the developing spinal cord. *Adapted from Ulloa and Martí, 2010.*

Within the VZ, neural progenitors are organized in a polarized neuroepithelium with each cell extending through the entire width of the epithelium. The neural progenitors are bond at the apical (near the central lumen) and basal surfaces of the neuroepithelium but their nuclei migrate along the axis of the cell accordingly to the cell cycle phase: M-phase nuclei are at the apical side while cells in S-phase have their nuclei close to the basal surface. During G1 and G2 phases, the nuclei migrate between these two opposing positions, in a movement known as interkinetic nuclear migration (reviewed in[1]). After division, each of the daughter cells either repeats or exits the cell cycle. Neural progenitors that re-enter the cell cycle remain in the neuroepithelium[2]. If the cell exits cell cycle, it loses apical and basal attachments and starts the differentiation process, migrating out of the VZ and entering the

MZ[2]. One of the most important mechanisms involved in this decision – proliferation versus differentiation – is the Notch signalling pathway (described in detail in Section 1.3.).

### 1.2. Generation of different neuronal subtypes along the DV axis of the spinal cord

For the CNS to be functionally assembled not only the balance between progenitors and differentiating neurons must be controlled but also the position where specific neurons are generated must be tightly regulated. After neural tube formation (reviewed in[3]), neural progenitors acquire distinct characteristics and different fates according to their positions along the anterior-posterior (AP) and dorsal-ventral (DV) axes of this structure. The neural tube will originate the brain anteriorly (from which the forebrain, midbrain and hindbrain will be formed) and the spinal cord posteriorly.

Neural progenitors of the neural tube normally develop anterior identity and differentiate into forebrain neurons[4]. The remaining neural progenitors along the AP axis of the neural tube need to be kept in an undifferentiated state in order to gradually acquire different identities and to differentiate into midbrain, hindbrain and spinal cord neurons. The anterior secretion of retinoic acid (RA), which promotes neuronal differentiation, and the posterior secretion of FGF, which represses neuronal differentiation, by surrounding mesodermal tissues is responsible for the generation of the CNS in a rostral-to-caudal sequence allowing progenitors to gradually differentiate in the correct moment and position[5,6].

Neural progenitors that give rise to spinal cord neurons, not only acquire an AP identity but also a DV identity. Along the DV axis of the developing spinal cord, neural progenitors are subdivided into eleven molecularly distinct progenitor domains, from which different cell types are generated. Initially, this subdivision into six dorsal domains and five ventral domains results from the activity of three secreted signalling molecules: Sonic Hedgehog (Shh) produced ventrally by the notochord and the floor plate, and Bone Morphogenetic Protein (BMP)-family members and Wnt produced dorsally by the roof plate and the dorsal epidermis (reviewed in[7,8,9], Fig. 1). It is known that the combination of different levels of these morphogenes induces the expression of specific combinations of transcription factors, known as patterning proteins.

The patterning proteins expressed in each domain provide specific positional identities, activating region-specific differentiation programmes and, therefore, specifying the identity of neurons that derive from individual progenitor populations (reviewed in[10], Fig. 2). To restrict developmental programmes to particular domains, many patterning proteins repress transcription factors of the adjacent progenitor populations in order to define boundaries between domains (reviewed in[10]). As a consequence, different subtypes of neurons will be specified in different domains, in a highly organized and reproducible manner. Moreover, the way neurons wire in the neuronal circuits reflects their embryonic specification, meaning that

neurons derived from common domains will connect into specific circuits. In fact, neurons that process sensory input reside always in the dorsal spinal cord whereas circuits involved in motor output are concentrated ventrally (reviewed in[8], Fig. 1).

Region-specific differentiation programmes, induced by different patterning proteins, involve the expression of unique combinations of proneural transcription factors, which play a central role in the differentiation of neural progenitors into neurons (reviewed in[10], Fig. 2). The function of proneural proteins in interneuron specification in one ventral domain of the spinal cord, the V2 domain, is part of the central theme of this thesis.

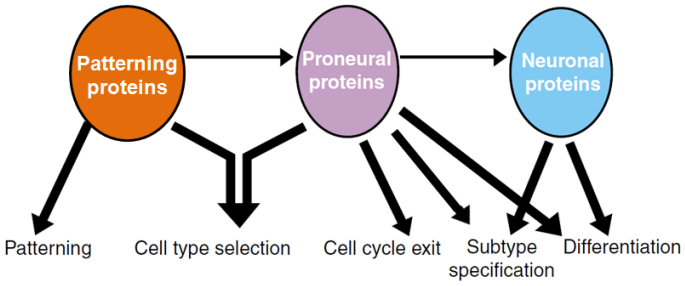


**Figure 2 – The differentiation of neural progenitors into post-mitotic neurons involves transcriptional cascades.** Patterning proteins induce the expression of proneural proteins, which in turn induce the expression of neuronal homeodomain proteins and neuronal differentiation bHLH proteins. These factors regulate different phases of neural development. *Adapted from Guillemot, 2007.*

### 1.2.1. Proneural Proteins

The proneural transcription factors contain a Helix-Loop-Helix (HLH) domain that allows these proteins to dimerize and, subsequently, to bind DNA through their basic domain. These proteins, which act normally as transcriptional activators but can also act as transcriptional repressors, bind DNA as heterodimeric complexes that are formed with ubiquitously expressed E proteins (belonging to the bHLH family). These heterodimers specifically bind to DNA consensus sequences known as E-boxes (CANNTG). Interestingly, a comparison of E-box sequences in the promoters of various target genes revealed that the sequence specificity goes beyond the four conserved bases of the core E-box, a fact that is likely due to the interaction of proneural proteins with other co-factors (reviewed in[11]).

The proneural proteins were first identified in *Drosophila* and were divided into two families: the Atonal (ATO) and Achaete-scute (ASC) families (reviewed in [11]). In vertebrates, many genes have been found to encode proteins related to these families: the vertebrate ASC family includes Ash1 which is present in all species analyzed (e.g. Mash1 in mouse, Cash1 in chick and Zash1 in zebrafish) and three other that have each been found in only one class of vertebrates (Mash2 in mammals, Xash3 in *Xenopus* and Cash4 in chick). The number of vertebrate proteins related to *Drosophila* ATO family is larger, but only two of them (Math1 and Math5 in mouse) have a bHLH domain similar enough to that of ATO to be considered as orthologues (reviewed in[11]). Other vertebrate ATO-related proteins can be grouped into distinct families, e.g., the Neurogenin (NGN) family, the NeuroD family and the Olig family (reviewed in[11]).

The proneural proteins promote cell cycle exit, commitment to neurogenesis and neuronal differentiation (Fig. 2). However, the expression of proneural genes in neural

progenitors is transient. In the case of vertebrate spinal cord, proneural genes are down-regulated before the progenitor exits the VZ (reviewed in[11]). Thus, the ability of proneural proteins to promote neuronal differentiation must depend on the induction of expression of downstream regulatory genes: proneural proteins promote neuronal subtype specification and terminal differentiation of post-mitotic neuronal cells by regulating the expression of neuronal homeodomain proteins and neuronal differentiation bHLH proteins[10] (Fig. 2).

Altogether, and as a result of specific differentiation programs, different domains are defined by the combination of transcription factors being expressed. This thesis is focused on a particular domain, the V2 domain, where several bHLH proteins are expressed.

#### 1.2.2. bHLH proteins in the V2 domain

In the V2 domain, the patterning proteins Nkx6.1, Irx3 and Pax6 confer the molecular identity to V2 progenitors[12]. Acting downstream of these proteins, a combination of proneural and neuronal proteins (intrinsic cues) will play a fundamental role in the differentiation of three subtypes of interneurons, named V2a, V2b and V2c[13,14]. However, how each protein is involved in conferring competence and identity to V2 cells is still far from being understood.

Available data suggest that Mash1 is expressed in V2 progenitors[13] but how it contributes to V2 interneuron specification is still controversial: Parras et al.[15] reported a decrease in V2a interneurons in Mash1-mutant mice while the opposite result is reported by Li et al.[16]. NGN1 and NGN2 are also expressed in V2 cells[17,18] but how their expression might influence V2 cell fate specification is still under study.

Moreover, other bHLH proteins, like Scl and bHLHb5, which are also involved in interneuron specification, have been reported to be expressed in the V2 domain[13,19]. In fact, bHLHb5 has been recently associated with V2a lineage specification, acting cell-autonomously to promote this fate[19], whereas Scl is directly involved in V2b interneuron development[13].

### 1.3. Notch pathway in vertebrate neurogenesis

Notch signalling is one of the most important signalling pathways regulating development of metazoans. This pathway is implicated in probably all developmental programs (e.g. neural development, body segmentation, embryonic haematopoiesis, etc) controlling many biological functions, such as apoptosis, cell proliferation or differentiation and lineage decisions throughout embryonic development (reviewed in[20]).

#### 1.3.1. The core components of the Notch pathway

The Notch signalling is an evolutionary conserved cell-cell communication system that occurs through direct contact between cell surface proteins, the Notch receptor and its ligand. Despite being a highly conserved pathway, the number of Notch components is variable between species.

- Notch receptors: The Notch receptor is a type I transmembrane protein that accumulates at the plasma membrane as a heterodimer, composed by the Notch extracellular domain and a membrane bound intracellular domain, which are formed in the trans-Golgi as the result of a proteolytic cleavage (at site S1) (reviewed in[21], Fig. 3). Whereas *Drosophila* presents only one Notch receptor, four Notch receptors (Notch1, Notch2, Notch3 and Notch4) have been identified in mammals. In chick, two Notch receptors (Notch1 and Notch2) were described (reviewed in[22]). The overall structure of both extracellular and intracellular domains of the Notch receptor is conserved between different receptors and different species (reviewed in[21]).
- Notch ligands: The Notch ligands belong to two protein families: Delta and Serrate (Jagged in mammals). A related ligand was identified in *C. Elegans* and named Lag-2. The DSL (Delta/Serrate/Lag-2) ligands are type I transmembrane proteins. While the overall structure of the extracellular domain of these proteins is conserved between different ligands and different species, little conservation is observed in the intracellular domain of different DSL ligands (reviewed in[21]). In *Drosophila*, two Notch ligands exist, Delta and Serrate, while in chick four ligands were identified, Delta-like 1 (DLL1), Delta-like 4 (DLL4), Serrate1 and Serrate2. In mammals, besides these four, another ligand, Delta-like 3 (DLL3) was identified (reviewed in[22]).

### 1.3.2. Activation of the Notch pathway

Notch signalling is activated upon cell-to-cell contact as a result of the interaction between a DSL ligand and a Notch receptor expressed on a neighbouring cell. The interaction of these proteins leads to a conformational change in the receptor and, as a consequence, three successive proteolytic cleavages take place (at sites S2, S3 and S4) (Fig. 3). As a result of these cleavages, the Notch intracellular domain (NICD) is released and translocated into the nucleus. The NICD binds to the CSL transcription factor (human CBF1, fly Supressor of Hairless, worm Lag-1) and the Mastermind co-activator (MAM), forming a tripartite nuclear complex which recruits other factors. In the absence of NICD, the transcription factor CSL is part of a transcriptional repressor complex that represses genes containing promoters with CSL-binding sites, whereas in the presence of NICD it activates the transcription of the same genes (reviewed in[22], Fig. 3).

### 1.3.3. Notch target genes

There are many putative CSL-binding sites throughout the genome but it is not clear which actually represent Notch targets. In vertebrates, the best characterized Notch target genes encode transcriptional repressors belonging to the Hairy and Enhancer of Split homologues (HES) or HES related families (reviewed in[20]). These proteins are basic helix-loop-helix-Orange (bHLH-O) transcriptional repressors. The HLH domain allows the homo- and heterodimerization of these proteins, which then bind to consensus DNA sequences (E-

box: CANNTG or N-box: CACNAG) through their basic domain. After binding DNA, its C-terminal WRPW motif recruits co-repressor factors, which lead to the transcriptional repression of target genes, such as proneural genes (reviewed in[23]). In addition to a mechanism dependent on DNA-binding, bHLH-O proteins can inhibit transcription by directly interacting and forming heterodimers with bHLH activator proteins, such as proneural proteins, through their HLH domain. This interaction will prevent the bHLH activators from binding to the DNA and thereby, from activating transcription (reviewed in[23]).

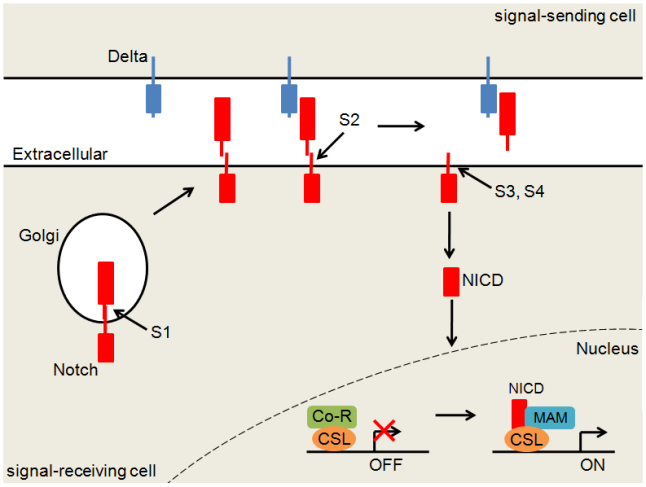


**Figure 3 – Notch pathway activation.** Delta ligand at the surface of the signal-sending cell binds to the Notch extracellular domain present in the neighbouring cell. Upon ligand-receptor interaction, three proteolytic cleavages occur, releasing NICD. NICD is translocated into the nucleus where it associates with CSL and MAM, displacing the co-repressor (Co-R) and triggering a switch from repression to activation.

### 1.3.4. Main functions of Notch signalling pathway during vertebrate CNS development

- Maintenance of neural progenitors – Lateral Inhibition

Lateral inhibition is one of main processes through which Notch signalling acts to promote cell diversity within an equivalence group, a group of cells that share a similar developmental potential. This process ensures that two interacting cells do not acquire the same fate: inhibitory signals which prevent the acquisition of a primary fate are sent by two neighbouring cells, but at the end one of the two signalling cells acquires this fate whereas the second acquires an alternative fate. In the particular case of neural progenitor maintenance, one of the cells starts the process of neuronal differentiation (primary fate) while the other remains as progenitor (alternative fate).

The choice of remaining as a neural progenitor or to differentiate into a neuron is controlled by the balance between two different sets of transcription factors: proneural bHLH proteins, which drive progenitors into neuronal differentiation, and HES proteins, which repress neuronal differentiation and therefore maintain cells as progenitors. However, due to lateral inhibition mediated by Notch signalling, the fate of each neural progenitor depends on the fates that its neighbours acquire. Neural progenitors in the VZ of the neural tube express proneural proteins and have therefore the ability to mature into neurons. In addition, all these cells express Notch ligands and receptors. Stochastic variations in gene expression cause one cell to express higher levels of proneural proteins and to start the differentiation process. As proneural proteins positively control the expression of Notch ligands, the expression of

these ligands will be enhanced in this cell (reviewed in[11]). This cell triggers the process of lateral inhibition by activating Notch signalling in neighbouring cells expressing Notch receptors. The activation of this pathway in the surrounding neural progenitors leads to an increase in the levels of HES proteins being produced, which will repress the expression of proneural proteins in these cells. The decrease of proneural genes expression prevents neural progenitors from differentiating prematurely into neurons (reviewed in[11]). Lateral inhibition mediated by Notch signalling thus provides a feedback mechanism to control the production of neurons[24]. If neurons are being produced in excessive number, it will result in an excess of inhibitory signal (Notch signalling) which will prevent further differentiation. Low production of neurons results in the opposite effect. Lateral inhibition therefore maintains a pool of neural progenitors throughout neurogenesis, which allows these cells to be exposed to different environmental cues and to differentiate into different neurons during development. Moreover, as neurons and glia cells are produced from the same progenitors, the maintenance of these cells by Notch signalling allows the production of glia cells at the correct developmental stage (after neurogenesis)[20].

- Cell fate specification

Notch signalling has been also implicated in specifying neuronal fates. In fact, it was suggested that Notch signalling might have not only an indirect role in glia cell fate specification (by the maintenance of progenitors) but also an instructive character, being directly involved in the production of glia cells (reviewed in[20]). Another example of cell fate specification controlled by Notch signalling occurs within the V2 domain of the developing spinal cord, where V2 progenitors give rise, simultaneously, to three different neuronal subtypes (V2a, V2b and V2c interneurons (INs)). It was shown that Notch signalling is necessary for the specification of V2b INs, since in the absence of Notch activity only V2a INs are generated[13] (described in detail in Section 1.4.2.).

## 1.4. Notch ligands in the developing spinal cord

### 1.4.1. Expression pattern of Notch ligands during early stages of neurogenesis in the developing spinal cord

In the developing spinal cord, different Notch ligands are expressed. DLL1 is expressed in the VZ of most domains, being only absent from the two domains where Jagged1 is expressed (V1 and dl6 domains)[25,26,27].

DLL4 ligand has a very curious pattern of expression. In fact, it has received special attention not due to its expression in the CNS but due to its expression in blood vessels during angiogenesis and its function in controlling vascular growth[28,29] (described in Section 1.4.3.). Within the CNS, *Dll4* is specifically expressed in two regions: the developing retina (which develops from the forebrain) and the V2 domain of the developing spinal cord. Interestingly, within these structures, *Dll4* is only expressed in a small number of cells.

Moreover, in both regions, *Dll4*-expressing cells correspond to cells that are starting the process of neuronal differentiation[30,31]. To understand how *Dll4* expression is regulated in the V2 domain of the developing spinal cord is the main focus of this thesis.

### 1.4.2. Notch ligands in the V2 domain: DLL1 and DLL4

The V2 domain is one of the five ventral domains of the spinal cord where three subtypes of interneurons (INs), V2a, V2b and V2c, are generated. In mice, these INs are produced after embryonic day 10 (E10) while in chick the production starts at HH21 (Hamburger and Hamilton stage[32]) (E3,5)[13]. It is worth to mention that V2a and V2b INs, which are specified from apparently common V2 progenitors over the same period of time, have opposite physiological functions: while V2a are excitatory INs, V2b INs are inhibitory[9,33]. It is known that V2a INs are involved in left-right motor coordination in the adult mice but the function of V2b INs is still unknown[34]. The V2c INs were only recently described to be present in mouse spinal cord and not much is known regarding the function of these cells[14]. Moreover, it was suggested that other V2-derived cells are produced from this domain[14].

In the V2 domain, *Dll1* and *Dll4* are expressed in the same population of differentiating neurons[35] (Fig. 4). The expression of two Notch ligands in the VZ of the V2 domain is an exception to what seems to be a general rule exhibited in the other domains of the developing spinal cord, where a single Notch ligand seems to be sufficient to regulate neurogenesis[27].

Within the VZ of the V2 domain, cells that start the differentiation process express high levels of proneural proteins and, as a consequence, high levels of DLL1, enabling Notch activation in neighbouring cells and preventing them from following the same fate[35] (Fig. 4). This is in agreement with studies on Presenilin1 and Notch1 mutants, where a neurogenic phenotype was observed as a consequence of the excessive differentiation of V2 progenitors[13,36].

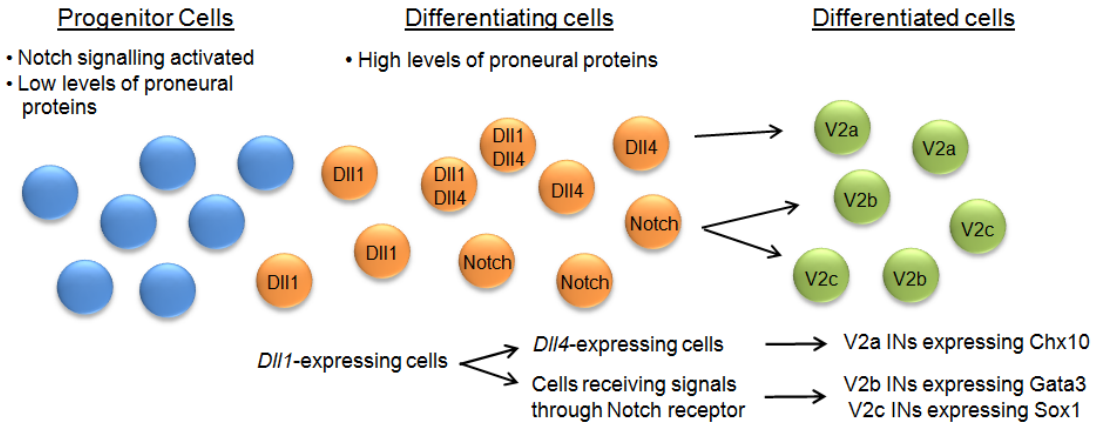


**Figure 4 – Expression of Notch ligands in the V2 domain of the developing spinal cord.** Cells starting the differentiation process express high levels of proneural proteins and, consequently, high levels of *Dll1*. These cells activate Notch signalling in the neighbouring cells which will remain as progenitors. Some of the *Dll1*-expressing cells will express *Dll4* and will differentiate into V2a INs while other V2 cells receive signals through the Notch receptor and differentiate into V2b and V2c INs.

As development takes place, *Dll1*-expressing cells migrate out of the VZ and can acquire, as previously mentioned, three different fates (Fig. 4). Some of the V2 cells committing to differentiation will express high levels of *Dll4* and will differentiate into V2a INs, characterized by the expression of the homeodomain transcription factor Chx10, the Lim homeodomain factor Lhx3 and the bHLHb5 protein[13,19,37](and unpublished data from our laboratory). The remaining V2 cells committing to differentiation, which do not express *Dll4*, will instead generate V2b INs, characterized by the expression of Scl and the zinc finger transcription factors Gata2 and Gata3[13], and V2c INs, which derive from Gata3-expressing cells and are characterized by the expression of the transcription factor Sox1[14](and unpublished data from our laboratory).

Although it is well accepted that Notch signalling plays a crucial role in V2 cell fate specification, how each Notch ligand, DLL1 and DLL4, mediates this process is still under study. The analyses of Dll1-mutant mice reveal that V2a and V2b INs are both generated (even though the number of V2a INs increases) whereas in Presenilin and Notch1 mutant all V2 progenitors differentiate into V2a INs, at expense of the V2b fate[13,35,38]. This suggests that Notch signalling is required for the generation of V2b INs and that DLL1 may be dispensable for the V2a-V2b binary decision which is most likely controlled by DLL4-mediated Notch signalling[35](and unpublished data from our laboratory). In agreement, overexpression of DLL4 in the chick spinal cord increases the number of V2b INs and decreases the number of V2a INs whereas overexpression of DLL1 does not significantly affect the number of V2a and V2b INs[13].

### 1.4.3. Regulation of *Dll4* expression

Existing data point to the fact that DLL4 exerts an important role during interneuron specification in the V2 domain. Therefore, *Dll4* expression must be tightly regulated for the appropriate number and type of V2 INs to be produced. However, regulation of *Dll4* expression during spinal cord development is still poorly understood. I will summarize the available data on how expression of this ligand is regulated in another developmental context and what is known regarding the regulation of expression of Delta ligands in the CNS.

Regulation of *Dll4* expression has been extensively studied in endothelial cells since Notch signalling mediated by DLL4 has been implicated in vascular growth. It is known that the vascular endothelial growth factor (VEGF) activates the expression of *Dll4*. In turn, DLL4 is able to activate its own expression in neighbouring cells in a process that is dependent on Notch signalling: NICD activates the expression of *Dll4*, activation that is dependent on the CSL-binding sites present in the promoter of this gene. The ability of the NICD to specifically induce the expression of *Dll4* provides a mechanism by which Notch signalling is propagated between communicating cells with initially a limited amount of ligand, in a positive feed-forward mechanism[39]. Coordinated activation of Notch signalling produces a wave of *Dll4*

expression in endothelial cells, preventing a situation in which only a subpopulation of cells express high levels of *Dll4*. In contrast, in the V2 domain of the developing spinal cord, *Dll4* is expressed in a salt-and-pepper pattern, meaning that cells expressing high levels of *Dll4* are surrounded by cells expressing low levels of this gene, situation that is maintained as result of lateral inhibition. Therefore, the mechanism behind *Dll4* expression in the V2 domain is most likely different from the mechanism controlling *Dll4* expression in endothelial cells.

The best candidates to regulate *Dll4* expression in the CNS are proneural proteins. In *Drosophila*, several proneural bHLH proteins such as Achaete, Scute and Lethal of scute, have been shown to induce the expression of *Delta*[40]. In zebrafish, expression of *DeltaD* (one of the four zebrafish Delta homologues) in the brain and spinal cord is regulated by NGN and Zash1[41]. The homologous proneural proteins were reported to directly regulate *Dll1* expression in the mouse spinal cord and brain[42]. In another model, the chick retina, Cash1 was suggested as a potential regulator of *Dll1* expression, whereas NGN2, NeuroD4 and/or Atoh7 were proposed as better candidates to regulate *Dll4* expression[43].

In the chick developing spinal cord, the proneural protein Cash1 is involved in the regulation of *Dll4* expression[13]. However, in the mouse spinal cord, Mash1 is not necessary for *Dll4* expression as Mash1-null mice show normal expression of *Dll4* in the V2 domain. In fact, it is believed that another transcription factor, Foxn4, is necessary for the expression of *Dll4* in mouse, since *Dll4* expression is abolished in Foxn4-null mice[38].

Altogether, these findings suggest that, as occurs in *Drosophila*, proneural proteins control the expression of Delta ligands in zebrafish, chick and mouse. Moreover, it suggests that although different mechanisms are used to regulate the expression of these ligands in different species and different tissues, all share the same purpose, to control the number and type of cells produced.

## 2. Aims

The main aim of this thesis is to understand how *Dll4* expression is regulated in the developing spinal cord. For this purpose, we analysed and compared chick and mouse *Dll4* promoter sequences in order to identify the most conserved regions which may contain putative binding sites for transcription factors regulating *Dll4* expression. To identify the minimal promoter sequence necessary for correct *Dll4* expression, we designed several expression vectors containing different portions of the most proximal sequence upstream of the chick *Dll4* gene driving expression of a reporter gene (*Venus*). In addition, as proneural proteins are the best candidates for inducing *Dll4* expression, we overexpressed different proneural proteins in the chick developing spinal cord to test this hypothesis. Finally, V2 IN specification was also addressed in these conditions. The chick embryo was used as a model organism in these studies due to its accessibility, easy handling and simplicity in terms of genetic manipulation.

# 3. Materials and Methods

## 3.1. Bioinformatics Analysis

Chick and mouse DNA sequences, with accession numbers ENSGALG00000008514 and ENSMUSG00000027314, respectively, were obtained from the Ensembl (http://www.ensembl.org/index.html) database.

Alignments between chick and mouse sequences, as well as the identification of the most conserved regions, were performed with ClustalW (http://align.genome.jp/), LaLIGN (http://www.ch.embnet.org/software/LALIGN_form.html) and SPIN programme (a component of the Staden Package, http://staden.sourceforge.net/). Minimal promoter prediction was performed with Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html).

Primer sequences were designed and analysed using Netprimer (http://www.premierbiosoft.com/netprimer/index.html). Sequencing results were analysed with ClustalW alignments, by comparing the sequencing data with the expected sequence. Sequencing ab1 files were visualized with Trev (a component of the Staden Package).

## 3.2. Bacterial Artificial Chromosome (BAC)

A BAC containing chick *Dll4* locus (CH261-184P22) was used as a template to amplify the *Dll4* promoter sequence necessary for the generation of *Dll4* reporter vector (Section 3.3.). The BAC was digested with two enzymes and the product of these digestions was analysed to confirm if the BAC had the correct sequence (data not shown). BAC purification, restriction digestions and Pulse Field Gel Electrophoresis were performed as described in Annex A.

## 3.3. Molecular cloning strategy

Molecular cloning techniques were used to produce several plasmids: some were generated in order to obtain a *Dll4* reporter while others were generated for the overexpression of proneural proteins (Mash1, NGN1 or NGN2). All newly generated plasmid constructs were verified by 3 independent restriction digestions. Whenever Polymerase Chain Reactions were involved in the generation of these plasmids, the final plasmid sequences were also confirmed by DNA sequencing (Stabvida).

3.3.1. Polymerase Chain Reaction (PCR): To generate inserts for cloning in DNA vectors, PCR primers were designed for the specific target sequence. Primers used during the course of this work for PCR (and/or for sequencing) were synthesized by Frilabo and are listed in Annex A, Table 1. PCR reactions are described in detail in Annex A.

3.3.2. Restriction digestions: Enzymatic restriction of DNA was performed for approximately 1-2 hour using commercially available restriction enzymes and respective buffers (Fermentas, Promega, New England Biolabs). The volume of enzyme used in each reaction never exceeded 10% of the total reaction volume. Addition of BSA to the reaction mixture and the temperature at which it was performed depended on the enzyme used.

3.3.3. Dephosphorylation: To reduce the number of negative clones caused by vector self-ligation, vector backbone was dephosphorylated before the ligation with the insert. Dephosphorylation was performed on 3-10µg of digested vector at 37°C during 1.5 hour using 2U of Calf Intestinal Phosphatase (Promega) with the appropriate buffer in a total volume of 50µL.

3.3.4. DNA precipitation: Precipitation was done by adding to the sample 1/10 of its volume of 3M sodium acetate and then two volumes of 100% ethanol. Mixture was vortexed and incubated for at least 1 hour at -20°C. After centrifuging for 30 min at maximum speed at RT, the supernatant was discarded, the pellet was washed with 500µL of 70% ethanol and centrifuged for 10 min at maximum speed at RT. After removing the supernatant, the pellet was dried at RT. DNA was resuspended in water, 10mM Tris pH8 or TE buffer.

3.3.5. Analysis and isolation of DNA by agarose gel electrophoresis: Gels were prepared by heating agarose (SeaKem®LE Agarose, Lonza) until complete dissolution in 1x TAE buffer (details in Annex A). Gels were either photographed (analytical gels) or DNA was extracted from them (preparative gels). In the case of the preparative gels, the region of the gel containing the DNA fragment of interest was excised and purified using *Wizard Plus SV Gel and PCR Clean-up System* (Promega), according to the manufacturer's instructions.

3.3.6. DNA Ligation Reactions: Ligation between the vector and the insert was performed overnight (o/n) at 15°C, using 5U of T4 DNA Ligase (Fermentas) and the respective ligation buffer, in a final volume of 10µL. The proportion between the vector and the insert to be cloned was 1:3, based on DNA concentrations assessed by visualizing the fragments after agarose gel electrophoresis.

3.3.7. Plasmid transformation of chemically competent *E. coli* bacteria: For plasmid DNA transformation, 100µL of competent *E. coli* were used. After thawing cells on ice, DNA was incubated with bacteria for 20 min on ice. The mixture was heat-shocked for 45 seconds in a water bath at 42°C and then incubated on ice for 2 min. After adding 900µL of Super Optimal Broth (SOB) medium supplemented with 10mM $MgCl_2$ and 10mM $MgSO_4$, bacteria were incubated with shaking at 37°C for 1 hour. The mixture was centrifuged and most of the supernatant discarded. Cells were resuspended in the remaining volume (100µL), plated on the appropriate selective LB agar media and incubated at 37°C o/n.

3.3.8. Plasmid DNA purification: Plasmid DNA purification was performed using commercially available kits – details in Annex A.

3.3.9. DNA quantification: DNA concentration was determined by spectrophotometry using the *NanoDrop* spectrophotometer (Thermo Scientific) – details in Annex A.

3.3.10. Plasmid constructs generated: (described in detail in Annex A)

- Dll4 reporter (Annex A, Figure 1): 1 – polyA@pKS; 2 – VenusNLSpolyA@pKS; 3 – Dll4(1641)-Venus; 4 – Dll4(3310)-Venus; 5 – Dll4(609)-Venus

- Other constructs: Ngn1@pCAGGS; Ngn2@pCAGGS; Mash1@pCAGGS

### 3.4. Embryo manipulation

Chick embryo was the model used in this study. The plasmid constructs generated were electroporated into the spinal cord of chick embryos, which were then harvested, sectioned and analysed. The number of embryos analysed is shown in Annex A, Table 2.

3.4.1. *In ovo* chick embryo electroporation: Plasmid DNA was injected into the spinal cord of chick embryos at HH 16-17 (different concentrations were used for different plasmids – described in Annex A, Table 3). With the exception of Hes6-2:VP16@pCIG, each plasmid DNA was co-electroporated with mCherry@pCAGGS (0.2µg/µL) in order to visualize the electroporated cells and as a positive control for electroporation efficiency. Fast green was used to stain the injected solution for proper visualization of DNA being injected. Platinum electrodes, distanced 4 mm between anode and cathode, were placed parallel to the spinal cord and embryos were pulsed 4 times (25V/50 ms) using the Electro Square Porator™ ECM830 (BTX). Eggs were sealed, incubated again and after 16, 24 or 36 hours embryos were harvested, the extraembryonic membranes were removed and the embryos were fixed in 4% paraformaldehyde, at 4°C o/n.

3.4.2. Tissue embedding and preparation of cryostat sections: After fixation, embryos were washed twice in PBS1x and transferred to a solution of 30% sucrose in PBS1x for cryoprotection. Embryos were then embedded in a solution containing 7.5% gelatine and 15% sucrose in PBS1x, and frozen in cold isopenthane (-75°C) for 1-3 min. Frozen embedded embryos were stored at -80ºC until sectioned in a cryostat (Leica CM 3050). Embryonic tissue was sectioned (16µm) and collected on Superfrost slides.

### 3.5. *In situ* hybridization

*In situ* hybridization was used to label cells expressing *Dll4* gene in non-electroporated embryos as well as in embryos electroporated with plasmids encoding proneural proteins (Mash1, NGN1 or NGN2) or HES6-2:VP16 protein. This technique allowed me to map the expression of *Dll4* during chick spinal cord embryonic development and to analyse if the electroporated proteins influence *Dll4* expression.

3.5.1. Antisense RNA probe synthesis: Digoxigenin (DIG)-labelled RNA anti-sense probes, complementary to the mRNA of the genes of interest, were synthesized *in vitro* by T7 or T3 RNA polymerase, from plasmid templates containing the cDNAs (details in Annex A).

3.5.2. *In situ* hybridization: *In situ* hybridization on cryostat sections was done by hybridizing DIG-labelled anti-sense RNA probes o/n at 65ºC-70°C (hybridization temperature varied depending on the probe) in a humidified chamber. Probes were diluted (0.1-1µg/ml) in hybridisation buffer and denatured at 70°C for 10 min. After o/n hybridization, sections were washed for 10 min with pre-warmed washing solution at hybridization temperature to remove coverslips and then washed twice with the same solution for 20 min at hybridization

temperature. Sections were washed* and blocked with a solution of 2% Blocking Reagent and 20% heat inactivated sheep serum in TBST, for 1 hour at RT in a humidified chamber. Sections were then incubated with antibodies anti-DIG coupled to Alkaline phosphatase enzyme (Roche, 1:2000 in antibody incubator solution) o/n at 4ºC in a humidified chamber. Sections were washed* and then washed twice for 10 min in NTMT at RT. Staining reaction was performed using BM Purple (Roche), incubating at 37ºC in a humidified chamber until the development of the signal (typically o/n). Lastly, sections were washed twice in PBS1x, counterstained with DAPI for 10 min and mounted with Mowiol® mounting medium.

### 3.6. Immunohistochemistry

In order to analyse expression of the different chick *Dll4* reporter vectors, immunohistochemistry was used to label cells expressing Venus protein after electroporation of the reporter alone or after co-electroporation with plasmids encoding proneural proteins (Mash1, NGN1 or NGN2). This technique was also used to label cells expressing Chx10 protein, a transcription factor that is specifically expressed by V2a interneurons. This labelling allowed me not only to identify the V2 domain, but also to analyse the number of V2a interneurons present when the embryos were harvested.

After gelatine removal with pre-warmed PBS1x on a 37°C water bath, sections were treated with 3% $H_2O_2$ in methanol for 30 min at RT to reduce background by blocking endogenous peroxidase. Sections were then treated with 0.1M Glycine in PBS1x for 10 min at RT to quench paraformaldehyde, permeabilized with 0.5% triton in PBS1x for 10 min at RT, blocked with 10% Fetal Bovine Serum (FBS) in TBST for 1 hour at RT and incubated with primary antibodies (diluted in 10% FBS in TBST) o/n at 4ºC in a humidified chamber. After primary antibody binding, sections were washed* and incubated with secondary antibodies (diluted in 10% FBS in TBST) 1 hour at RT in a humidified chamber. Sections were washed* and then counterstained with DAPI for 10 min. After being washed*, sections were mounted with Mowiol® mounting medium.

The $H_2O_2$:methanol treatment was also used to remove mCherry fluorescence allowing simultaneous labeling of Chx10-positive cells and Venus-positive cells. This treatment was not performed on electroporated embryos when fluorescence was to be preserved.

The antibodies used during the course of this work are described in Annex A. The tests performed to determine the specificity of the anti-GFP antibodies are described in Annex A.

### 3.7. Fixed tissue imaging

Bright field images of fixed sections were acquired using the microscope Leica DM5000B, equipped with a Leica DC500 digital camera. Images of fixed sections with fluorescence were acquired using the microscope Leica DM5000B equipped with a Leica DC350F digital camera. The acquired images were then treated for noise reduction and colour adjustments in Adobe Photoshop Software.

Note: The composition of all solutions used is described in Annex A, Table 4.

* Washed three times with TBST for 10 min at RT.

# 4. Results

## 4.1. *Dll4* expression in the chick developing spinal cord

To map the expression of *Dll4* in the chick developing spinal cord, embryos from E2 (HH12) to E7 (HH31) were analysed. As neurogenesis does not occur simultaneously along the AP axis of spinal cord, all embryos were analysed at the forelimb level.

We did not observe expression of *Dll4* in neural cells of the developing spinal cord of E2 and E3 embryos (Fig. 5, A and B), which is in agreement with previous results[30]. Expression of *Dll4* in neural cells was first detected at E4 in a very restricted region of the ventral spinal cord (as previously described in[30]) (Fig. 5, C). In order to identify in which ventral domain *Dll4* is expressed, we used adjacent sections and performed immunostaining to label cells expressing Chx10 (a transcription factor that is specifically expressed by V2a INs). As expected, *Dll4*-positive cells are localized in the VZ of the V2 domain (Fig.5, C and D).

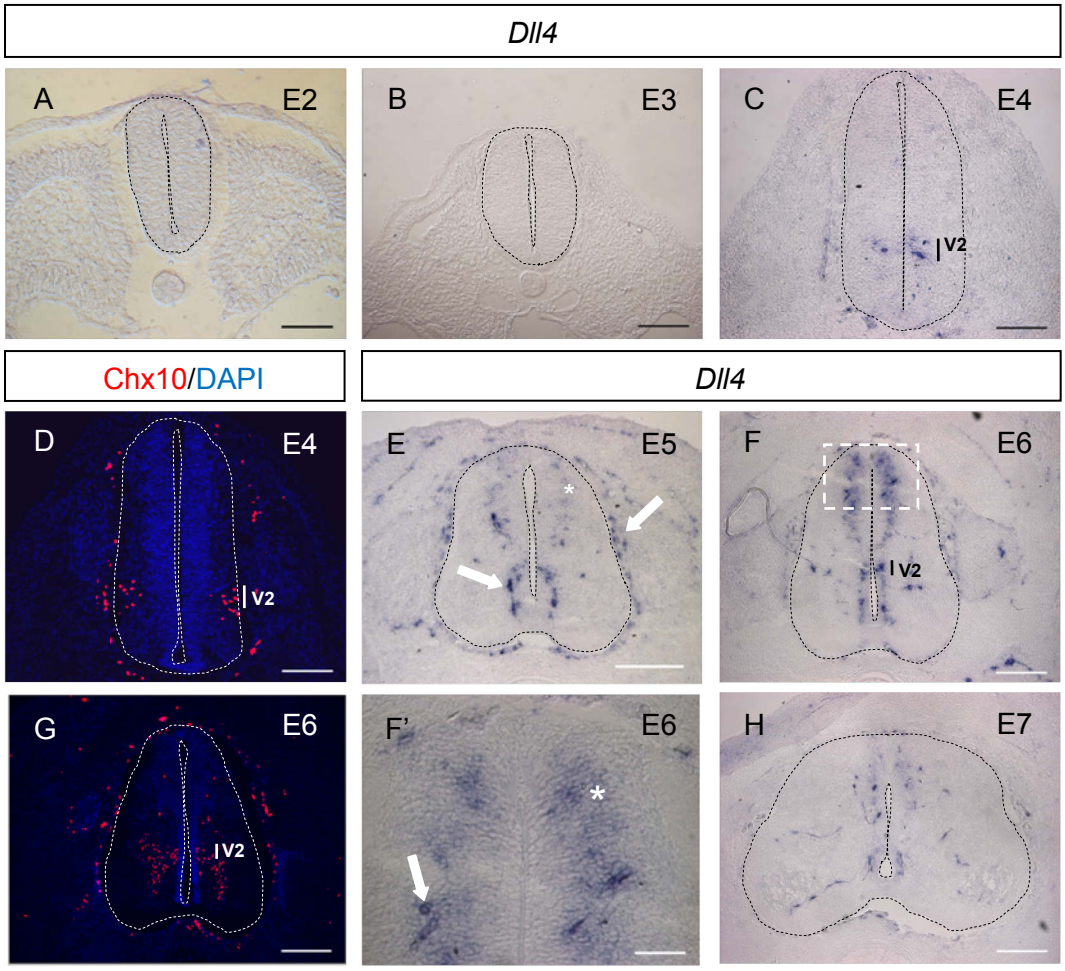


**Figure 5 – *Dll4* expression in the developing spinal cord.** (A, B) - *Dll4* is not expressed at E2 neither at E3. (C) - At E4, *Dll4* is expressed in the VZ of the V2 domain which was identified by the expression of Chx10 (D). (E) - At E5, *Dll4* is strongly expressed in blood vessels (indicated by arrows). At this stage, it is possible to observe a faint expression of *Dll4* in neural cells not only in the V2 domain but also in dorsal domains (indicated by asterisk). (F) - At E6, expression of *Dll4* remains in the blood vessels (indicated by arrow in F') and becomes evident along the DV axis of the spinal cord (indicated by asterisk in F'). At this stage, *Dll4* expression in neural cells is only absent from one region localized above the V2 domain (which was identified by the expression of Chx10 (G)). (H) - At E7, expression of *Dll4* is down-regulated in neural cells along the DV axis of the spinal cord, remaining only in the blood vessels. (F') – magnification of the selected area in (F). Scale bars: A, F' - 50μm; B, C, D - 100μm; E, F, G, H - 200μm.

In E5 embryos, *Dll4* is expressed in blood vessels invading the spinal cord as well as in blood vessels surrounding it (Fig. 5, E – indicated by arrows), observations that are in agreement with previous results[30]. At this stage, we also observed a faint expression of *Dll4* in neural cells outside the V2 domain (Fig. 5, E – indicated with an asterisk). At E6 (Fig. 5, F), *Dll4* is strongly expressed not only in blood vessels (Fig. 5, F' – indicated by an arrow) but also in neural cells along the DV axis of the spinal cord (Fig. 5, F' – indicated with an asterisk), meaning that *Dll4* expression is no longer restricted to the V2 domain. Interestingly, only neural cells in the region immediately above the V2 domain (which was identified by the expression of Chx10 using an adjacent section – Fig. 5, G), do not express *Dll4* (Fig. 5, F). At E7, expression of *Dll4* is down-regulated in neural cells, remaining only in blood vessels (Fig. 5, H).

Since the purpose of this work is to understand how *Dll4* expression is regulated in the V2 domain of the developing spinal cord, all the analyses were performed between E3 and E4, when *Dll4* expression is restricted to the V2 domain (Fig. 5, C).

### 4.2. Promoter analysis

#### 4.2.1. Five conserved regions exist between chick and mouse Dll4 promoter sequences

In order to investigate how *Dll4* expression is regulated in the V2 domain, a promoter analysis was carried out. As important regulatory sequences tend to be conserved across species and as these conserved sequences reflect conserved functions[44], *Dll4* promoter sequences from different species were analysed. Alignment between these sequences enables the identification of the most conserved regions which may contain putative binding sites for transcription factors regulating *Dll4* expression. In 2004, a study of the *Sox2* locus revealed that the phylogenetic distance between chick and mammals is optimal for identifying genetic regulatory elements as conserved sequences[44]. For this study, we followed a similar approach comparing mouse and chick sequences.

Alignment of the 5kb upstream of the *Dll4* coding sequence of chick and mouse revealed that only five regions have a high level of similarity (>60%) (Annex B, Fig. 1). The low overall level of similarity of *Dll4* promoter between chick and mouse suggests that the few regions that are conserved contain the regulatory information necessary for the correct expression of *Dll4*.

The most proximal conserved region contains a motif previously identified by Castro et al. as a Mash1/Brn motif[42] (Annex B, Fig. 1, V). This motif is composed of an E-box CAGCTG and an evolutionary conserved octamer one nucleotide upstream of the E-box. This E-box corresponds to the consensus binding sequence for Mash1 (CAG[C/G]TG)[42] whereas the octamer is a conserved consensus binding site for the POU family of homeodomain proteins, such as Brn proteins. Moreover, another conserved E-box (CAGGTG) was detected upstream of the Mash1/Brn motif (Annex B, Fig. 1, IV). Once again, this E-box corresponds

to the consensus binding sequence for Mash1. The conservation of these two small sequences (Mash1 E-box and Mash1/Brn motif) suggests that Mash1 may directly regulate *Dll4* expression in the chick developing spinal cord.

Within the other three identified conserved regions (localized between 1554bp and 3011bp upstream of the chick *Dll4* coding sequence), several canonical and non-canonical E-boxes can be identified. E-box sequences are potential targets for the binding of proneural proteins and it is known that different proneural proteins have different binding affinities to different E-boxes (reviewed in[11]). As shown in Annex B, Fig. 1 (I, II, III) some of the identified E-boxes are predicted to be the preferred binding site of specific proneural proteins (reviewed in[11]). By analysing these E-boxes we can predict that, as might occur with Mash1, Neurogenins may also regulate *Dll4* expression. In addition, E-box sequences are also potential targets for the binding of HES proteins, suggesting that these proteins might also directly regulate *Dll4* expression. HES proteins may also bind N-boxes (reviewed in[23]) but these were not identified in the conserved regions.

4.2.2. Transcription start point

The available databases do not contain any information regarding *Dll4* transcription start site. Through bioinformatics tools and based on the typical minimal RNA Polymerase II promoter, we predicted the transcription start site of chick *Dll4* gene. The core promoter elements (meaning the shortest sequence at which RNA polymerase II can initiate transcription) are predicted to be localized within the 250bp upstream of the ATG codon. According to Lewis[45], the typical RNA Polymerase II core promoter is composed by a TATA box and an initiator element (Inr) as shown in Fig. 6, A. Upstream of the chick *Dll4* coding region, two different sequences were identified containing two possible TATA boxes and two possible Inr (Fig. 6, B and C). The sequence in Fig. 6, B is the one that most resembles the typical RNA Polymerase II core promoter, the only variation being the presence of a purine localized four nucleotides downstream the transcription start point (marked with an asterisk). The sequence in Fig. 6, C is less similar to the typical minimal promoter as it contains four purines in the initiator element instead of pyrimidines (marked with an asterisk). Therefore, the sequence in Fig. 6, B, localized 208bp upstream of the *Dll4* coding region, is the best candidate to contain the minimal RNA polymerase II promoter region.


**Figure 6 – Predicted minimal promoter sequences in chick Dll4 promoter region.** (A) - The typical minimal RNA polymerase II promoter has a TATA box approximately 20bp upstream of the initiator element (Inr). The Inr has pyrimidines (Y) surrounding the transcription start point. *Adapted from Lewis, 2008*. (B, C) - Predicted minimal promoter sequences in chick *Dll4* promoter region. The differences between these sequences and the typical minimal RNA polymerase II promoter are marked with asterisks.

### 4.3. Mash1 or NGN1 overexpression induces *Dll4* expression in the chick developing spinal cord

Our initial bioinformatics analysis of *Dll4* promoter suggested that Mash1 and Neurogenins may regulate *Dll4* expression. To test this hypothesis, Mash1, NGN1 and NGN2 were overexpressed in the chick developing spinal cord. In these experiments, Cherry protein was used to identify the electroporated half of the spinal cord and as a positive control of electroporation efficiency.

As a negative control, embryos which were not electroporated with plasmids encoding proneural proteins were analysed. In these embryos, *Dll4* expression is detected in the V2 domain in both sides of the spinal cord (Fig. 7, A-C). It is possible to observe some *Dll4*-expressing cells surrounding the spinal cord which correspond to blood vessels (Fig. 7, B - arrow). The erythrocytes present in these blood vessels have auto-fluorescence but can be easily distinguished from V2 Chx10-positive cells or Cherry-positive cells due to their peripheral position (Fig. 7).

Mash1 overexpression results in ectopic *Dll4* expression along the DV axis of the developing spinal cord. Almost all electroporated cells express *Dll4* whereas in the non-electroporated side of the spinal cord *Dll4* is only expressed in the V2 domain (Fig. 7, D-F). Overexpression of NGN1 also induces *Dll4* expression outside of the V2 domain in the electroporated half of the spinal cord whereas in the non-electroporated half *Dll4* expression is restricted to the V2 domain (Fig. 7, G-I). In contrast, overexpression of NGN2 does not have the same effect, as *Dll4* restricted expression is maintained in the V2 domain in both sides of the spinal cord (Fig. 7, J-L). These observations indicate that *Dll4* expression is regulated by Mash1 and NGN1, but not by NGN2.

To determine whether misexpression of these proneural proteins affects V2 interneuron specification, the number of Chx10-postive cells (V2a INs) was analysed in embryos electroporated with plasmids encoding Mash1, NGN1 or NGN2. In none of the cases differences were observed between the electroporated and non-electroporated sides of the spinal cord (Fig. 7). The lack of differences in the number of Chx10-positive cells could be due to the fact that Chx10 is expressed in differentiated cells and these embryos were harvested 16 hours after electroporation. It might be too early for V2a INs to have completed the process of neuronal differentiation and so it could take longer to observed differences.

### 4.4. Mash1 overexpression leads to a decrease in V2a INs whereas NGN1 or NGN2 overexpression has the opposite effect

To test if overexpression of Mash1, NGN1 or NGN2 influences V2 IN specification, embryos electroporated with plasmids encoding these proteins were harvested 36 hours after electroporation and Chx10 expression was analysed.

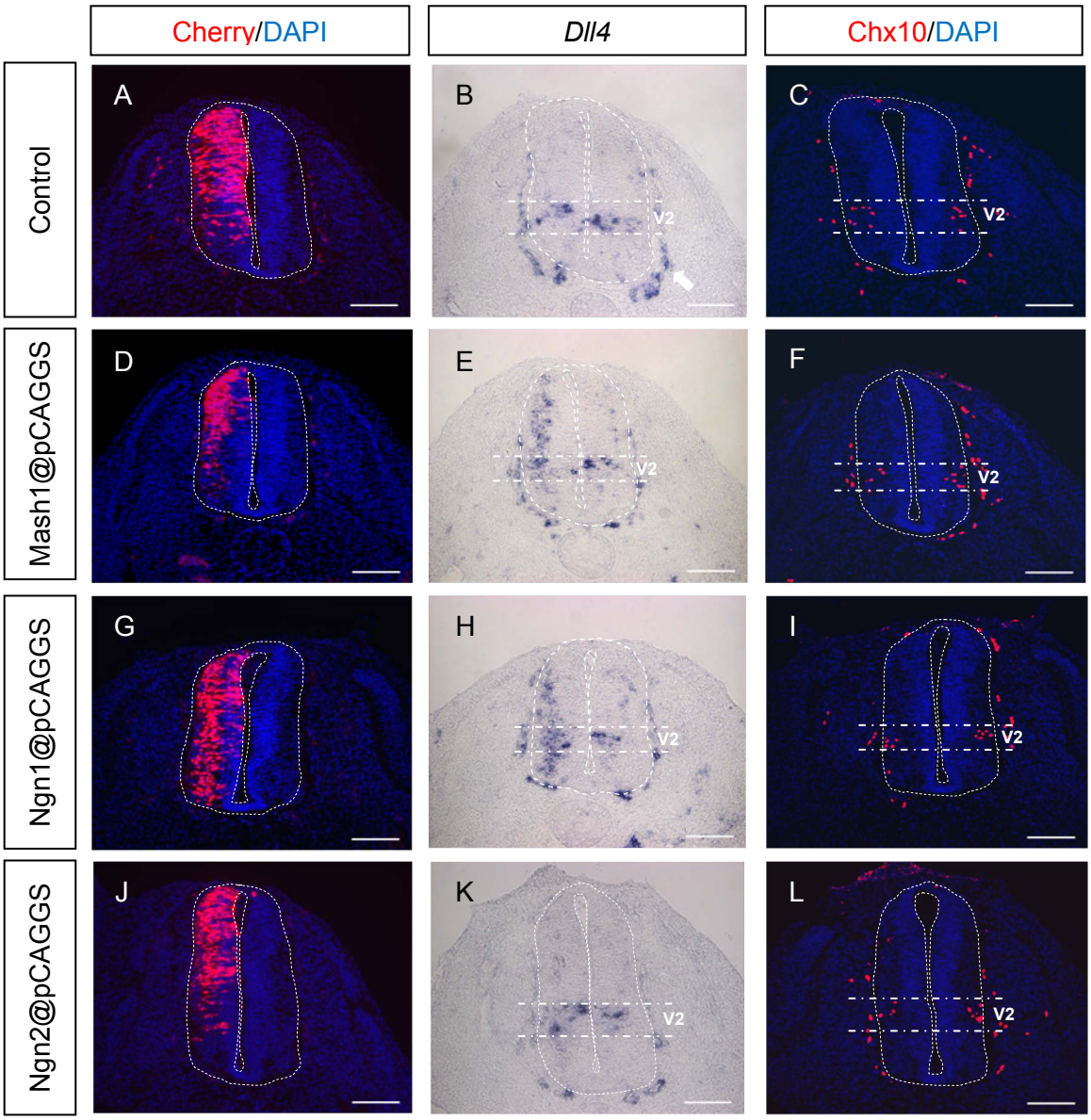


**Figure 7 – *Dll4* expression after electroporation of Mash1@pCAGGS, Ngn1@pCAGGS or Ngn2@pCAGGS.** (A, B, C) – In embryos which were not electroporated with plasmids encoding proneural proteins (control), *Dll4* expression in neural cells is restricted to the V2 domain. *Dll4* is also expressed in blood vessels surrounding the spinal cord (indicated by arrow). (D, E, F) - Mash1 induces *Dll4* expression along the DV axis of the spinal cord. (G, H, I) - *Ngn1* overexpression also promotes the expression of *Dll4* along the DV axis of the spinal cord. (J, K, L) - *Ngn2* overexpression does not induce *Dll4* expression.
In all embryos, Cherry protein was used to identify the electroporated side of the spinal cord and as a positive control of the electroporation efficiency (A, D, G and J). Chx10 expression was used to identify the V2 domain (C, F, I, L) which is defined by the dashed lines. Embryos were harvested 16 hours after electroporation. Scale bars: 100µm.

After Mash1 overexpression, we observed a decrease in the number of Chx10-positive cells in the electroporated half when compared with the non-electroporated half of the spinal cord (Fig. 8, A, B). Moreover, the Chx10-positive cells that appear in the electroporated half correspond to cells that were not electroporated (Chx10-positive/Cherry-negative cells) (Fig. 8, C). After NGN1 or NGN2 overexpression, we observed an increase in the number of Chx10-positive cells in the electroporated half when compared with the non-electroporated half of the spinal cord (Fig. 8, D, E and G, H). In both cases, most of the Chx10-positive cells in the electroporated half correspond to electroporated cells (Cherry-positive cells) (Fig. 8, F

and I). Moreover, it is possible to observe some Chx10-positive cells localized ventrally to the V2 domain (Fig. 8, E and H).

Overall, these observations indicate that Mash1 represses V2a IN fate whereas NGN1 and NGN2 promote this fate.

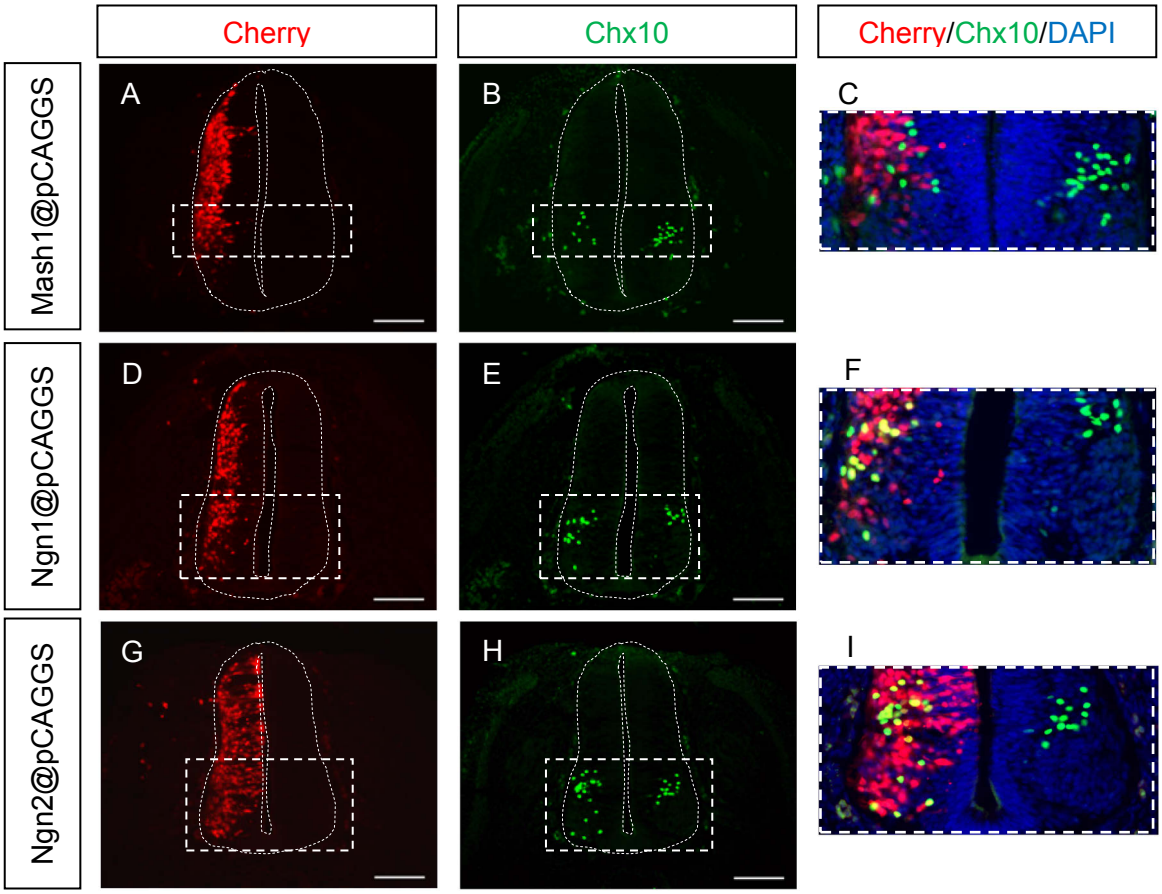


**Figure 8 – Chx10 expression 36 hours after electroporation of Mash1@pCAGGS, Ngn1@pCAGGS or Ngn2@pCAGGS.** (A, B) - Mash1 overexpression leads to a decrease in the number of Chx10-positive cells. (C) - Chx10-positive cells in the electroporated half correspond to cells that were not electroporated (Cherry-negative cells). (D, E) - NGN1 overexpression leads to an increase in the number of Chx10-positive cells. (F) – Most of the Chx10-positive cells in the electroporated half correspond to electroporated cells (Cherry-positive cells). (G, H) - NGN2 leads to an increase in the number of Chx10-positive cells. (I) – Most of the Chx10-positive cells in the electroporated half correspond to electroporated cells (Cherry-positive cells). (C), (F) and (I) are magnifications of the selected areas in (A and B), (D and E) and (G and H). Scale Bars: 100µm.

### 4.5. HES6-2 protein might act as a repressor of *Dll4* expression in the chick developing spinal cord

Some HES proteins were reported to also bind to E-boxes and therefore might be involved in the regulation of *Dll4* expression. As the HES6-2 protein acts as negative repressor of the Notch signalling pathway (an exception to most of HES proteins which act as effectors of this pathway) and is expressed along the DV axis of the spinal cord[46], this protein could repress *Dll4* expression outside the V2 domain between E3 and E4. To test this, we used a dominant-negative form of this protein, in which the repressor domain (WRPW) was replaced by a potent transactivation domain - VP16[46]. This protein (HES6-2:VP16) is expected to bind to the same promoter sites as the normal HES6-2 protein but

activate, rather than repress, transcription of target genes[46]. HES6-2:VP16 overexpression results in strong ectopic expression of *Dll4* along the DV axis of the spinal cord, suggesting that the normal form of HES6-2 acts as a repressor of *Dll4* expression (Fig. 9, A-B).

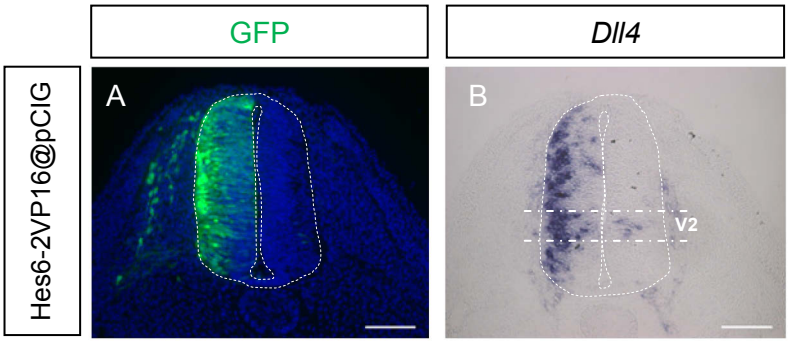


**Figure 9 – *Dll4* expression after electroporation of a plasmid encoding the HES6-2:VP16 protein.** (A) – GFP was used to identify the electroporated side of the spinal cord and as a positive control of the electroporation efficiency. (B) - Dll4 expression was induced by the HES6-2:VP16 protein. Embryos were harvested 24 hours after electroporation. Scale bars: 100µm

### 4.6. Dll4 reporter

To identify the minimal sequence necessary for correct *Dll4* expression in the chick developing spinal cord between E3 and E4, meaning when *Dll4* expression is restricted to the V2 domain, we designed several expression vectors containing different portions of the most proximal sequence upstream of the *Dll4* gene driving expression of a reporter gene.

The sequence upstream of the chick Dll4 coding region was subcloned from a BAC containing the chick *Dll4* locus and fused to a cDNA encoding a fluorescent reporter protein. The reporter protein used was Venus-NLS, which is a variant of the enhanced yellow fluorescent protein (EYFP) fused to a nuclear localization signal (NLS) to facilitate the identification of Venus-expressing cells. The reporter vectors were always co-electroporated with mCherry@pCAGGS, which express a red fluorescent protein under the control of a constitutive promoter, in order to identify the electroporated half of the spinal cord and as a positive control for electroporation efficiency.

#### 4.6.1. In the absence of conserved regions, Venus is expressed in all electroporated cells

The Dll4(609)-Venus vector contains the 609bp sequence upstream of the chick *Dll4* coding region (Fig. 10, A). This vector contains the predicted minimal promoter of *Dll4* but does not contain any conserved regions. After electroporation, Venus-positive cells are detected along the entire DV axis of the spinal cord, meaning that almost all electroporated cells (expressing Cherry) express low levels of Venus (Fig. 10 B-D). This indicates that the most proximal 609bp contain the minimal information to promote transcription but do not confer any specificity.

#### 4.6.2. The three most proximal conserved regions restrict Venus expression to two defined regions

To determine if the most proximal 1641bp confer specificity to reporter expression, we analysed the expression of Dll4(1641)-Venus vector. This vector contains the predicted minmal promoter and the three most proximal conserved regions (Fig. 11, A - III, IV and V).

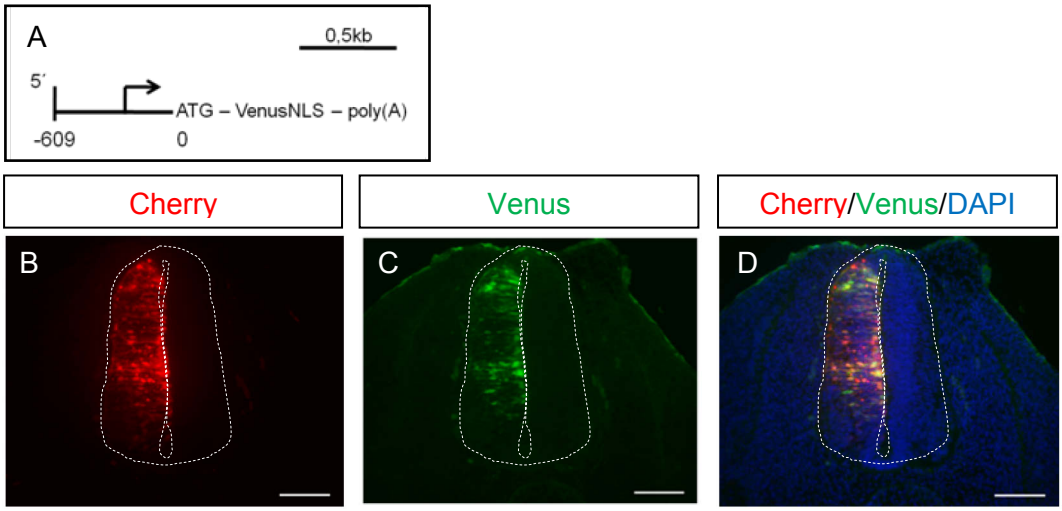


**Figure 10 – Electroporation of Dll4(609)-Venus.** (A) – The vector Dll4(609)-Venus does not contain any conserved sequence. (B) - Cherry was used to identify the electroporated cells and as a positive control of the electroporation efficiency. (C, D) – Venus is expressed in almost all electroporated cells along the DV axis of the spinal cord. Embryos were harvested 16 hours after electroporation. Scale bars: 100μm.

After electroporation, Venus-positive cells are detected in two zones of the spinal cord: one ventral and one dorsal (Fig. 11, B-D). To determine if the ventral region correspond to the V2 domain, we labelled Chx10-expressing cells (V2a INs). In fact, we observed that the ventral Venus-positive cells are localized in the V2 domain (Fig. 11, E-G). Within this domain, we found some Chx10-expressing cells (V2a INs which derive from *Dll4*-expressing cells) that are also Venus-positive, confirming that Venus is expressed in cells that are expressing (or have expressed) *Dll4* (Fig 11, G – arrow). These results show that the Dll4(1641)-Venus reporter is expressed in the correct ventral lineage. Nevertheless, we observed some Venus-positive cells that do not express Chx10 as well as some Chx10-positive cells that do not express Venus (see Discussion – Section 5.4.).

Altogether, these results show that the 1032bp present in the Dll4(1641) vector, which were absent from Dll4(609)-Venus vector, confer specificity to reporter expression, as Venus is no longer expressed in all the electroporated cells (Fig. 11, B-D). However, this sequence is not enought to restrict *Dll4* expression to the V2 domain.

### 4.6.3. The five conserved regions identified in the *Dll4* promoter do not restrict Dll4 expression to the V2 domain

One possible explanation to justify Venus expression in the dorsal region could be that *Dll4* expression needs to be repressed in this region of the spinal cord at this stage and that the binding site for the repressor is not localized within the promoter region of the Dll4(1641)-Venus reporter. To test if the two conserved regions identified within the second promoter fragment (Fig. 12, A – I and II) contain binding sites for repressors of *Dll4* expression in the dorsal region of the spinal cord, the Dll4(3310)-Venus vector was electroporated into the chick spinal cord. Analysis of sections of electroporated embryos shows that Venus is expressed in a similar pattern to the one observed after Dll4(1641)-Venus electroporation

(Fig. 12, B-D). Therefore, the most proximal 3310bp sequence does not contain the necessary information to restrict *Dll4* expression to the V2 domain.

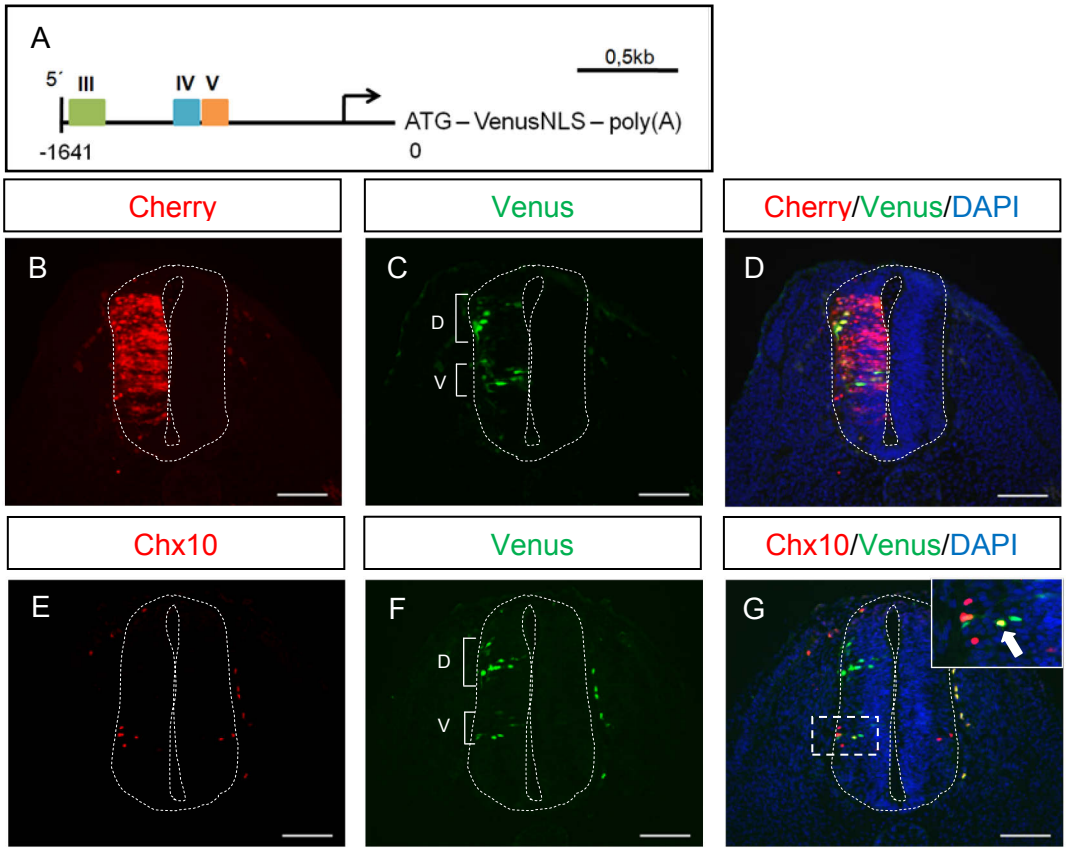


**Figure 11 – Electroporation of Dll4(1641)-Venus.** (A) – The Dll4(1641)-Venus vector contains the three most proximal conserved regions. (B) – Cherry was used to identify the electroporated half of the spinal cord and as a positive control of the electroporation efficiency. (C, D) - Venus-positive cells are localized in two restricted regions: one dorsal and one ventral. (E, F, G) - Using Chx10 expression to identify the V2 domain, it is possible to observe that the ventral Venus-positive cells are localized in the V2 domain. Moreover, that Venus is expressed in the V2a lineage, since it is possible to observe cells expressing Chx10 (V2a IN) and Venus (indicated by an arrow). Embryos were harvested 16 hours after electroporation. Scale bars: 100µm.

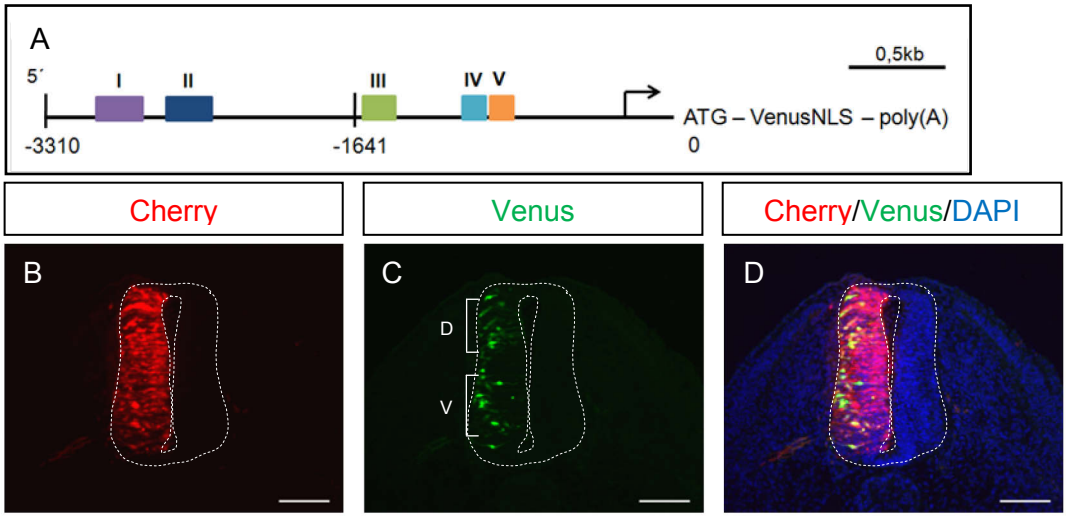


**Figure 12 – Electroporation of Dll4(3310)-Venus.** (A) – The Dll4(3310)-Venus vector contains the five conserved regions. (B) – Cherry was used to identify the electroporated half of the spinal cord and as a positive control of the electroporation efficiency. (C, D) - Venus is expressed in a similar pattern to the one observed after Dll4(1641)-Venus electroporation (Fig. 11). Embryos were harvested 16 hours after electroporation. Scale bars: 100µm.

### 4.6.4. Venus expression is induced by Mash1, NGN1 or NGN2 proteins

Even though the most proximal 1641bp sequence (first fragment) does not contain the necessary information to restrict *Dll4* expression to the V2 domain, it is possible that it contains the sequences necessary for the induction of *Dll4* expression by Mash1 or NGN1. In fact, possible binding sites for Mash1 and NGN1 were identified within the first fragment (Annex B, Fig. 1). To test this hypothesis, Dll4(1641)-Venus was co-electroporated with Mash1 or NGN1. After these co-electroporations, Venus is expressed in the large majority of the electroporated cells (Fig. 13, A-F), and not only in the two restricted regions (as in Fig. 11). These results show that Mash1 and NGN1 are inducing the expression of Venus cell-autonomosly. Since NGN2 does not induce endogenous *Dll4* expression (Fig. 7 J-L), we hypothesized that by co-electroporating Dll4(1641)-Venus with NGN2, expression of the repoter is not increased. However, Venus expression is strongly induced along the whole DV axis of the spinal cord by NGN2 overexpression (Fig. 13, G-I). As Venus expression is activated not only by Mash1 and NGN1 but also by NGN2, this shows that the promoter sequence present in Dll4(1641)-Venus does not contain all regulatory information to confer specificity to the binding of proneural proteins.

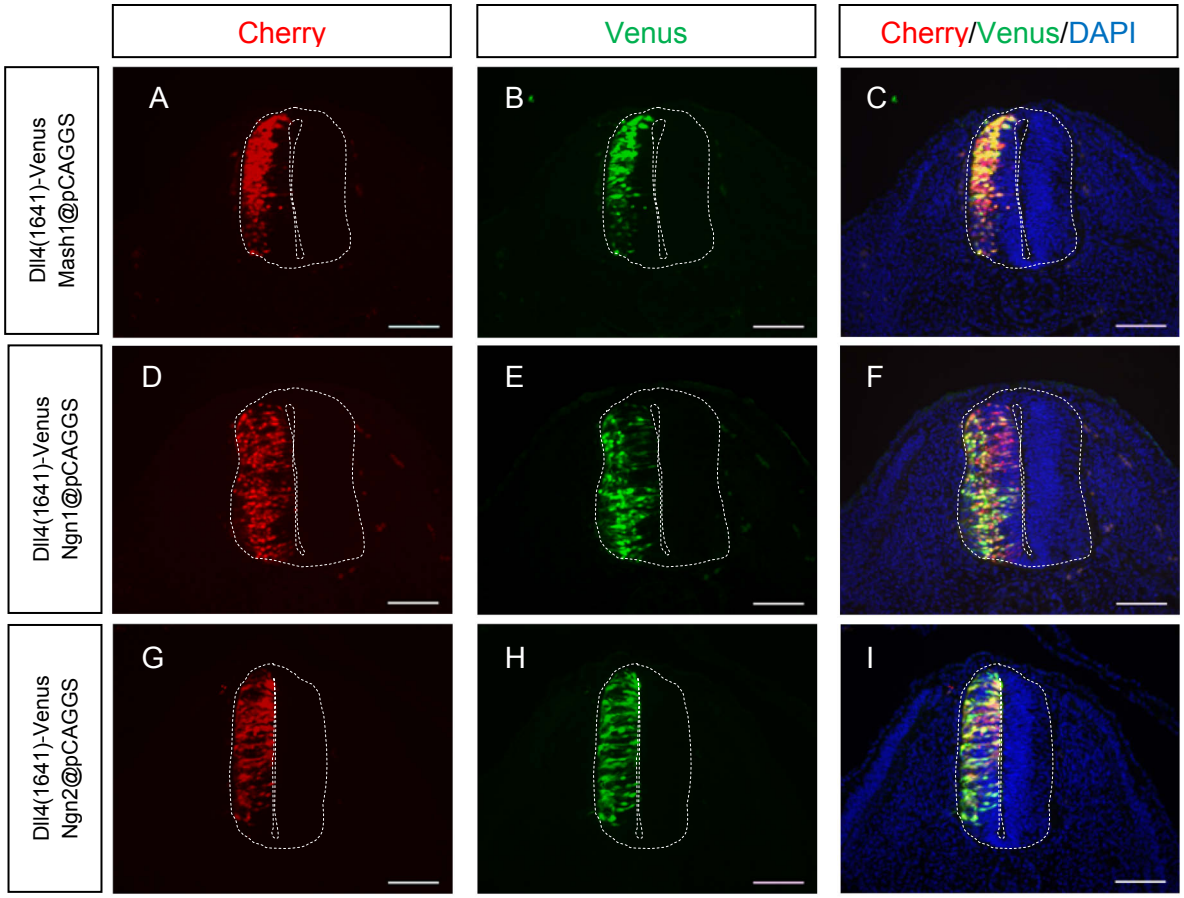


**Figure 13 – Co-electroporations of Dll4(1641)-Venus with plasmids encoding proneural proteins.** (A, B, C) – Mash1 induces Venus expression. (D, E, F) - NGN1 induces Venus expression. (G, H, I) - NGN2 induces Venus expression. In all these embryos, Cherry protein was used to identify the electroporated half of the spinal cord and as a positive control of the electroporation efficiency. Embryos were harvested 16 hours after electroporation. Scale bars: 100µm.

# 5. Discussion

The CNS is a complex system composed by an enormous variety of cells but the mechanisms responsible for the generation of this cell diversity are not fully understood. It is known that Notch signalling is involved in this process and this study presents new data on how the expression of one Notch ligand, DLL4, is regulated and might be involved in interneuron specification in the V2 domain of the chick developing spinal cord.

## 5.1. *Dll4* expression during spinal cord embryonic development

*Dll4* expression was detected in the VZ of the V2 domain at E4, as it was previously reported by others[30]. Interestingly, the results presented in this thesis show that at later stages (between E5 and E7) *Dll4* expression in neural cells is not restricted to the V2 domain. At these stages, *Dll4* is strongly expressed along the DV axis of the spinal cord, being only absent from one narrow region above the V2 domain. Although the role of this ligand between E5 and E7 has not been addressed, one can speculate that *Dll4* broader expression may be related to the specification of late-born neurons. After E5, dorsal neurons are generated from a single dorsal progenitor population (dIL domain). In the dIL domain, excitatory INs (dILB) and inhibitory INs (dILA) are generated simultaneously from the same progenitors and it is known that Notch signalling pathway is involved in dIL INs specification: in its absence, the number of dILB neurons is reduced by more than 40%[47]. DLL1 ligand was reported to be expressed in this region and to be responsible for the activation of Notch signalling[47]. Nevertheless, DLL4 ligand may also play a role in this process.

The V2 domain and this dorsal region have similarities: two Notch ligands (DLL1 and DLL4) are expressed and two subtypes of INs with opposing physiological functions are produced simultaneously from the same progenitors. Therefore, similar to what occurs in the V2 domain of the spinal cord, it is possible that DLL4 ligand is involved in activating Notch signalling in these dorsal progenitors to specify an inhibitory versus excitatory fate from an apparently common pool of progenitors.

## 5.2. Proneural proteins in the V2 domain

### 5.2.1. Bioinformatics analysis

The main aim of this thesis was to understand how *Dll4* expression is regulated in the chick developing spinal cord. Based on the idea that the regulatory mechanisms controlling the expression of homologous genes might be conserved between different species, chick and mouse *Dll4* promoter sequences were compared in order to identify conserved regions to which regulators of *Dll4* expression could bind.

Three conserved sequences believed to be preferred for the binding of Mash1 were identified. In fact, the two most proximal ones (Mash1 E-box and Mash1/Brn motif) were characterized as mouse *Dll1* regulatory regions: it was shown that the binding of Mash1 to the E-box and the cooperative binding of Mash1 and Brn proteins to the conserved

Mash1/Brn motif activates *Dll1* expression[42]. These sequences were also detected in zebrafish *DeltaD* regulatory region believed to be necessary for the activation of *DeltaD* expression by Mash1[41].

In addition, three conserved E-boxes (CAGATG), believed to be preferred for the binding of Neurogenins, were identified. Interestingly, a similar E-box is also present in the *DeltaD* conserved regulatory region believed to be responsive to Neurogenins[41].

Altogether, the bioinformatics analysis suggests that Mash1 and Neurogenins may directly regulate *Dll4* expression.

5.2.2. Mash1 and NGN1 promote *Dll4* expression

In order to test the bioinformatics predictions, we designed a gain-of-function approach where Mash1, NGN1 and NGN2 were overexpressed in the chick developing spinal cord.

We observed that Mash1 is able to induce ectopic expression of *Dll4*, which is in agreement with previous studies showing that Mash1 induces *Dll4* expression[13,38]. Moreover, this is also in agreement with previous reports showing that Mash1 induces expression of genes that contain Mash1/Brn motif and Mash1 E-boxes in the promoter region[42]. Therefore, as *Dll1* and *DeltaD*, we show that *Dll4* gene is positively regulated by Mash1. However, to determine if Mash1 is directly regulating *Dll4* expression through the Mash1 E-boxes and/or Mash1/Brn motif, it would be necessary to mutate these sequences.

In addition, we show that NGN1 also induces *Dll4* expression in the chick developing spinal cord. The same effect was not observed after NGN2 overexpression, indicating that this protein may not be an important factor controlling *Dll4* expression.

Even though NGN1 and NGN2 tend to bind to the same consensus sequences (CANATG)[11], only NGN1 is able to activate *Dll4* expression. This difference could be due to interactions with different co-factors which can affect the interaction of proneural proteins with their DNA-binding sites and/or modulate their transcriptional activity. Also, the nucleotides around the E-box may confer specificity to the binding of proneural proteins and justify this difference.

Overall, we show that *Dll4* expression is regulated by Mash1 and NGN1, but not by NGN2.

5.2.3. V2 interneuron specification

Since proneural proteins regulate neuronal subtype specification, we analysed if overexpression of Mash1, NGN1 or NGN2 affects the number of V2 INs produced in the electroporated half of the spinal cord. However, only the number of V2a INs (Chx10-expressing cells) could be analysed, as none of the available probes or antibodies recognize Gata3 (transcription factor expressed in V2b INs) mRNA or protein, respectively.

After Mash1 overexpression, we observed a decrease in the number of V2a INs produced in the electroporated half of the spinal cord, supporting Li et al. published results[16].

However, how misexpressed Mash1 represses V2a IN fate is not clear yet. Two possibilities can be considered. Mash1 can act cell autonomously to repress V2a fate, as most of Chx10-positive cells that appear in the electroporated side correspond to non-electroporated cells. Alternatively, Mash1 can repress V2a IN fate indirectly by activating *Dll4* expression. Due to the overexpression of Mash1, Dll4 is not expressed in salt-and-pepper pattern but instead neighbour cells express high levels of Dll4. In this scenario, each cell would activate Notch signalling in the neighbour cell and, as a result of this activation, two neighbour cells would differentiate into V2b INs, resulting in a decrease in the number of V2a INs. Nevertheless, it would be necessary to confirm that the number of V2b INs decreases after Mash1 overexpression. This possibility is in agreement with previous reports showing that DLL4 overexpression results in an increase in the number of V2b INs and a decrease in the number of V2a INs[13]. This also indicates that, during the normal process of neurogenesis, DLL4 ligand is not cell-autonomously activating a specific differentiation programme to induce V2a fate.

In contrast to what is observed upon Mash1 overexpression, after NGN1 overexpression the number of V2a INs increases. Interestingly, NGN1 promotes *Dll4* expression but a decrease in the number of V2a INs was not observed. As most of Chx10-positive cells correspond to electroporated cells, one possible explanation is that misexpressed NGN1 can act cell-autonomously to promote the V2a IN fate, independently of Notch signalling activation in these cells.

Although NGN2 overexpression does not affect *Dll4* expression, it affects V2 interneuron specification as the number of V2a INs increases. As most of Chx10-expressing cells correspond to electroporated cells, these results suggest that NGN2 is acting cell-autonomously to promote the V2a IN fate. However, it would be necessary to confirm that the number of V2b INs does not increase after NGN2 overexpression.

After NGN1 and NGN2 overexpression, we observed some Chx10-positive/Cherry-positive cells localizing ventrally to the V2 domain, a position where differentiated V2 INs usually migrate to. It is possible that NGN1 and NGN2 are strongly promoting the V2a IN fate, causing these cells to be further advanced in the differentiation process. Alternatively, NGN1 and NGN2 might be inducing ectopic V2a INs in the MN and/or V3 domains.

To complement these studies, it would be interesting to use loss-of-function techniques, such as mouse conditional knockout, in which our gene of interest is inactivated in a specific region (in our case in the spinal cord) - technique already in use in our laboratory.

### 5.3. HES6-2 might act as a repressor of *Dll4* expression

To determine if HES6-2 protein is a repressor of *Dll4* expression, we electroporated a plasmid encoding a dominant-negative form of this protein (HES6-2:VP16) into the chick

spinal cord. As HES6-2:VP16 protein is able to strongly induce *Dll4* expression, we suggest that HES6-2 might be a potent repressor of *Dll4* expression along the DV axis of the spinal cord.

This finding opened the possibility that the transcription factors needed to initiate *Dll4* transcription might be present in most of the differentiating cells along the DV axis of the spinal cord. In addition, when *Dll4* expression is restricted to some cells in the V2 domain of E4 embryos, HES6-2 protein is expressed along the whole DV axis of the spinal cord[46] and might be repressing *Dll4* expression. Moreover, it is possible that at later stages, between E5 and E7, *Hes6-2* expression is downregulated, allowing the expression of *Dll4* in several dorsal and ventral domains. However, there is no available data regarding *Hes6-2* expression after E4. It would be interesting to map the expression of *Hes6-2* and *Dll4* during spinal cord embryonic development to find out if they have complementary patterns of expression.

The observation that Mash1 and NGN1 are able to induce ectopic *Dll4* expression raises the question of why *Dll4* is not normally expressed in the domains where Mash1 and NGN1 are expressed. The normal expression of *Dll4* might be ensured by the presence of HES6-2 in those domains, which might repress *Dll4* transcription and restrict it to the V2 domain. Nevertheless, when Mash1 or NGN1 are overexpressed, the concentration of these proteins in the cells becomes very high, which might overcome the ability of HES6-2 to repress *Dll4* transcription.

Regarding the V2 domain, unpublished data from our laboratory shows that V2b INs derive from cells that have never expressed *Dll4*. Could it be that HES6-2 protein is expressed in prospective V2b INs preventing these cells from expressing *Dll4*? To answer this question it would be necessary to investigate the expression of *Hes6-2* in the V2 domain by comparing it to the transcription factors specifically expressed in V2a and V2b INs. If *Hes6-2* and V2b INs markers are co-expressed in the same cells, this could provide the mechanism for the biased expression of *Dll4* among V2 progenitors.

Even though the overexpression of HES6-2:VP16 indicates that HES6-2 is a repressor of *Dll4* expression, it is necessary to overexpress the normal HES6-2 protein to confirm that it is able to repress endogenous *Dll4* expression.

### 5.4. Dll4 reporter

To identify the minimal promoter sequence necessary for the correct expression of *Dll4* in the chick developing spinal cord between E3 and E4, we generated several expression vectors containing different portions of the most proximal sequence upstream of the *Dll4* gene driving expression of a reporter gene. These vectors were electroporated into the chick spinal cord and the expression of the reporter was analysed.

The first 609bp upstream of the *Dll4* coding region contain the minimal sequence for RNA polymerase II to initiate transcription but do not confer any specificity, as almost all electroporated cells express the reporter. However, when the three most proximal conserved regions were added to the reporter, the expression of Venus was restricted to two regions, one dorsal and one ventral, meaning that these sequences confer specificity to reporter expression. The ventral region corresponds to the V2 domain, which was identified by the expression of Chx10. We observed cells co-expressing Chx10 (V2a INs which derive from *Dll4*-expressing cells) and Venus, meaning that the reporter is expressed in the *Dll4*-positive lineage. However, we also observed cells expressing Chx10 but which do not express Venus. These cells could correspond to cells that were not electroporated or to V2a cells that were already downregulating *Dll4* expression when the embryos were electroporated. Moreover, cells expressing the reporter that were not Chx10-positive were also observed. These cells could belong to the V2b lineage (*Dll4*-negative cells) or could be prospective V2a INs that have not yet started the expression of Chx10, as this gene is only expressed in differentiated cells. We tested whether these Venus-positive/Chx10-negative cells express Gata3, a transcription factor that is expressed in V2b INs, but none of the available antibodies or probes recognized Gata3 protein or mRNA in the chick embryo, respectively. Further optimizations and/or tools will be needed to exclude the possibility that Venus is also expressed in the V2b lineage.

Interestingly, in the presence of the three most proximal conserved regions, the pattern of expression of the reporter resembles the Mash1 expression pattern previously described by Liu et al.[48], suggesting that the reporter might be responding to endogenous Mash1. Therefore, one possible explanation for the dorsal expression of Venus could be that *Dll4* expression needs to be repressed in this region and that the binding site for the repressor could not be localized within the first fragment. To determine if the regulatory sequences necessary to restrict *Dll4* expression to the V2 domain were localized within the second fragment, this sequence was inserted into the reporter. We observed that even in the presence of the five conserved regions, Venus expression is not restricted to the V2 domain, indicating that other regulatory regions must exist to control *Dll4* expression.

Even though the presence of the three proximal conserved regions do not restrict Venus expression to the V2 domain, we analysed if the sequences responsible for the specificity of the induction of *Dll4* expression by Mash1 and NGN1 are present within the first fragment (1641bp). To do this, we co-electroporated Dll4(1641bp)-Venus vector with Mash1, NGN1 or NGN2. We observed that Venus expression was induced not only by Mash1 and NGN1 (as endogenous *Dll4* expression) but also by NGN2, which does not induce endogenous *Dll4* expression. Although the most proximal 1641bp sequence contains

sequences that respond to proneural proteins, it does not contain the necessary regulatory information to confer specificity in the binding of these proteins.

The low overall level of similarity between sequences upstream of mouse and chick *Dll4* genes suggested that the few conserved regions could contain the regulatory information necessary for the expression of *Dll4*. However, we observed that these sequences are not enough for the correct expression of *Dll4* in the developing spinal cord of chick embryos between E3 and E4. It is known that regulatory sequences might be localized several kb upstream of the promoter sequence as well as in introns or even downstream of the coding region. For example, it was shown that the cis-regulatory regions that direct *DeltaD* gene expression in neuroectodermal and mesodermal tissues are distributed over 12.5kb of genomic DNA encompassing 6kb upstream and 6,5kb downstream of the transcription start site[41]. A broader analysis of *Dll4* locus is therefore necessary to identify more conserved regions, which may contain regulatory sequences important for the correct expression of *Dll4*.

In the ventral spinal cord, the molecular mechanisms by which apparently common progenitors differentiate into interneurons with distinct and specialized properties are poorly understood. The work here presented provides new evidences regarding neurogenesis in the V2 domain: how different proneural proteins and HES6-2 control *Dll4* expression and how this ligand and these proteins might be involved in V2 IN specification.

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# Annexes

## Annex A – Materials and Methods

Bacterial Artificial Chromosome

- BAC purification: An isolated colony was grown o/n with shaking at 37ºC in 10mL of LB with the appropriate antibiotic. Bacteria culture was centrifuged, the supernatant discarded and the pellet ressuspended in 400µL of resuspension buffer (P1, Roche). Then, 600µL of lysis buffer (P2, Roche) was added and, after inverting the tubes to mix, the mixture was incubated 5 minutes at room temperature (RT). After lysis, 600µL of neutralization buffer (P3, Roche) were added, the tubes were again inverted to mix and incubated for 10 min on ice. After centrifuging 10 min at maximum speed at 4ºC, the supernatant was split into 2 tubes and the same volume of isopropanol was added to precipitate the DNA. The pellet was washed with 70% ethanol, centrifuged, dried at RT and resuspended in 50µL of TE buffer.
- BAC restriction digestions: Enzymatic restriction of BAC DNA was performed for 6 hours a 37°C, in a final volume of 40µL with 20µL of DNA, using 5U of *Xho*I (Fermentas) or *Sna*BI (Promega) enzymes and NEBuffer 2 and BSA or NEBuffer 4 and BSA (New England Biolabs), respectively.
- Pulse Field Gel Electrophoresis: To perform pulse field gel electrophoresis a Bio-Rad *Chef-DRII System* was used. Before the run, the electrophoresis chamber was loaded with double-distilled water and the pump and cooling device, set to 14°C, were turned on for cleaning and cooling. After 1 hour, water was replaced by running buffer (0.5x TBE buffer). A 1% agarose gel was prepared and placed in the electrophoresis chamber filled with 0.5x TBE buffer. DNA samples were mixed with loading buffer and electrophoresis was carried out for 14 hours with an initial pulse time of 1 s, a final pulse time of 2 s, and with a field strength of 6V/cm. The BAC DNA was visualized after immersion of the gel in post-staining solution (GelRedNucleic Acid Stain – Biotium, 0.5x) with shaking for 15 min. The size of the DNA fragments was estimated by comparison with linear DNA strands of known molecular weight (1kb Plus DNA Ladder – Invitrogen or Lambda DNA Mono Cut Mix – New England Biolabs).

PCR Reactions: PCR reactions were prepared in a final volume of 25µL (1ng template plasmid DNA, 1x buffer, 0.2mM dCTP, 0.2mM dGTP, 0.2mM dATP, 0.2mM dTTP, 25pmol of each primer and 0.5U of *Phusion* DNA Polymerase - Finnzymes). In some cases, additives (DMSO at 10% or formamide at 5%) were added to the reaction mixtures to improve the product yield. Standard conditions were: an initial denaturation step at 98ºC for 30 seconds, followed by 25 cycles at 98ºC for 15 seconds, annealing at 60ºC for 30 seconds, 72ºC for 45 seconds/kb, followed by 10 min at 72ºC and 5 min at 4ºC. Adjustments in the annealing temperature (Tm) were made for each primer.

**Table 1 –** Oligonucleotides used in several steps of the molecular cloning strategies adopted in this thesis.

| Oligonucleotide | Oligonucleotide sequence (5'-3') | Tm |
|---|---|---|
| cDll4 #1 Rev | CTCCCATGGTCGCGCCGCTCGT | 65°C |
| cDll4 #1 Fwd | GATGCTGCCCGAGGATCCCCAG | 65°C |
| cDll4 #2 Rev | CTGGGGATCCTCGGGCAGCATCT | 57°C |
| cDll4 #2 Fwd | GTGCCGCATCTAGAATTCTTTGTAG | 57°C |
| VenusNEXBNF | ATAAGATAGCGGCCGCATAGATATCATATCTAGAATAGGATCCATACCATGGGAGCAAGGGCGAGGAGC | 65°C |
| NLSSTOPEcoRIR | CGCGAATTCTCAGGGGTCTTCTACCTTTCTCTTCTTTTTTGG | 65°C |
| cDll4889bpBamHIF | TAGGATCCATTACCATACAGCTGAGGGTTTA | n/a |
| cDll42611bpXabIF | TATCTAGACTGTCCCAGTTGGCACGCG | n/a |
| cDll41930bpXabaIF | GATCTAGAGCAGCAGGTGGTTTGTTATATTCC | n/a |
| GFPup | AGCTCGACCAGGATGGGCA | n/a |

Agarose gel electrophoresis: Final agarose concentration depended on the size of DNA to be resolved. RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, 0.2x) or Ethidium Bromide Staining Solution (Sigma, 2µg/mL) were used to visualize DNA. After mixing DNA with loading buffer, electrophoresis was carried out in 1x TAE buffer. The size of the fragments was estimated by comparison with linear DNA strands of known molecular weight (1kb Plus DNA Ladder – Invitrogen).

Plasmid DNA purification: For small scale preparation of plasmid DNA, 2mL of a 3 mL o/n 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 preparations of plasmid DNA, 100mL of the selective LB medium was inoculated with 100µL of plasmid bacterial culture, shaken at 37°C o/n and processed using the *Genopure Plasmid Midi Kit* (Roche), according to the manufacturer's instructions.

DNA quantification: One A260 unit corresponds to 50µg/mL of double stranded DNA[49]. The purity of the nucleic acid preparation was estimated by the ratio between the readings obtained at 260nm and 280nm (pure preparations of DNA show ratio values of 1.8).

Plasmids generated during the course of this work

- *Dll4* reporter (Annex A, Figure 1)

1 – polyA@pKS: The polyadenylation sequence from rabbit β-globin gene was obtained from the pCAGGS vector[50], by digestion with *Hin*dIII and *Eco*RI enzymes. The polyadenylation sequence was inserted into pBluescript II KS vector (Stratagene), digested with the same enzymes.

2 – VenusNLSpolyA@pKS: The VenusNLS coding sequence was amplified by PCR with the VenusNEXBNF and NLSSTOPEcoRIR primers (Annex A, Table 1) using as template a vector already in use in the laboratory containing the VenusNLS sequence. An

n/a – not applied. These primers were only used for sequencing reactions.

*Eco*R*I* restriction site and a stop codon were inserted in the 5' region of the reverse primer (NLSSTOPEcoRIR). *Nco*I, *Bam*HI, *Xba*I, *Eco*RV and *Not*I restriction sites were added in the 5' region of the forward primer (VenusNEXBNF). The insert was digested with *Not*I and *Eco*R*I* enzymes and inserted into polyA@pKS digested with the same enzymes. The *Nco*I, *Bam*HI and *Xba*I resctriction sites added to the forward primer were used to insert the chick *Dll4* promoter region as described next.

3 – Dll4(1641)-Venus: The 1641bp DNA sequence upstream of the chick *Dll4* coding region (hereafter called first fragment) was amplified by PCR from the BAC containing the chick *Dll4* locus using cDll4#1Rev and cDll4#1Fwd primers (Annex A, Table 1). The reverse primer contains two point mutations to create an *Nco*I restriction site, which is necessary to clone this DNA fragment into the VenusNLSpolyA@pKS plasmid. The first fragment was digested with *Nco*I and *Bam*HI enzymes and inserted into VenusNLSpolyA@pKS digested with the same enzymes.

4 – Dll4(3310)-Venus: The DNA sequence between 3310bp and 1641bp upstream of the chick *Dll4* coding region (hereafter called second fragment) was amplified by PCR from the BAC containing the chick *Dll4* locus using the cDll4#2Rev and cDll4#2Fwd primers (Annex A, Table 1). The second fragment was digested with *Xba*I and *Bam*HI enzymes and inserted into Dll4(1641)-Venus digested with the same enzymes.

5 – Dll4(609)-Venus: The Dll4(1641)-Venus was digested with *Not*I enzyme in order to remove the most distal 1032bp of the first fragment and obtain a vector containing only the proximal 609bp of the first fragment. This was possible since *Not*I restriction sites were present in the primer VenusNEXBNF and in the sequence of the first fragment.

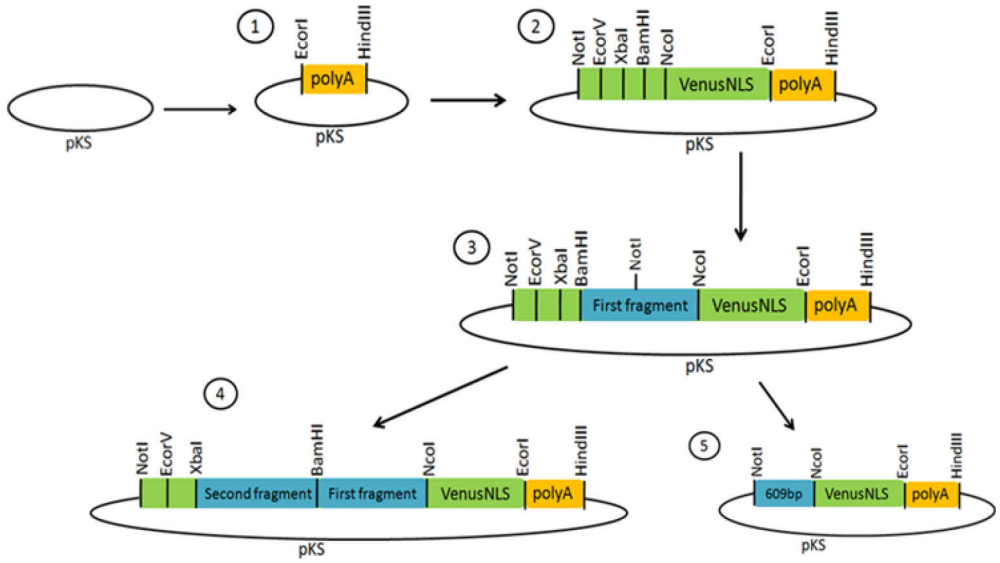


**Figure 1 –** Molecular cloning strategies adopted in this thesis. The pBluescript II KS plasmid was used as backbone vector to construct the Dll4 reporter. The polyadenylation sequence was inserted in the pBluescript II KS (1), followed by the insertion of the VenusNLS coding sequecence (2). The 1640bp upstream of chick Dll4 coding region (first fragment) were inserted upstream of the VenusNLS sequence (3). From this plasmid two other plasmids were obtained: one containing the 3310bp upstream chick Dll4 coding sequence (4), and the other containing only the most proximal 609bp upstream of chick Dll4 coding sequence (5).

- Other constructs:

- Ngn1@pCAGGS: cDNA encoding full-length rat NGN1 protein was obtained by digesting rNgn1@pCIG vector with *Eco*RI enzyme. The resulting cDNA was inserted into pCAGGS vector digested with the same enzyme.

- Ngn2@pCAGGS: cDNA encoding the full-length rat NGN2 protein was obtained by digesting rNgn2@pCIG vector with *Eco*RI enzyme. The resulting cDNA was inserted into pCAGGS vector digested with the same enzyme.

- Mash1@pCAGGS: cDNA encoding the Mash1 protein was obtained by digesting Mash1@pCIG vector with *Xho*I and *Bgl*II enzymes. The resulting cDNA was inserted into pCAGGSMCSKS vector digested with the same enzymes. The pCAGGSMCSKS is a pCAGGS vector that was modified to contain the MCS of pBluescript II KS vector (Evguenia Bekman, unpublished).

**Table 2 –** Number of embryos analysed in each condition.

| Condition | Number of embryos analyzed |
|---|---|
| Embryonic day 2 | 3 |
| Embryonic day 3 | 3 |
| Embryonic day 4 | 3 |
| Embryonic day 5 | 3 |
| Embryonic day 6 | 3 |
| Embryonic day 7 | 3 |
| Mash1@pCAGGS | 7 |
| Ngn1@pCAGGS | 7 |
| Ngn2@pCAGGS | 7 |
| Hes6-2:VP16@pCIG | 3 |
| Dll4(609)-Venus | 4 |
| Dll4(1641)-Venus | 12 |
| Dll4(1641)-Venus + Mash1@pCAGGS | 4 |
| Dll4(1641)-Venus + Ngn1@pCAGGS | 4 |
| Dll4(1641)-Venus + Ngn2@pCAGGS | 4 |
| Dll4(3310)-Venus | 4 |

**Table 3 –** Concentration of plasmids electroporated into chick spinal cord.

| Plasmid | Concentration (µg/µL) |
|---|---|
| Mash1@pCAGGS | 1 |
| Ngn1@pCAGGS | 1 |
| Ngn2@pCAGGS | 1 |
| Hes6-2:VP16@pCIG | 0,5 |
| Dll4(609)-Venus | 0,5 |
| Dll4(1641)-Venus | 0,5 |
| Dll4(3310)-Venus | 0,5 |

Antisense RNA probe synthesis

- DNA template preparation: 10µg of plasmid DNA were linearized, using 50U of the appropriate restriction enzyme in a final volume of 100µL, for 2 hours at 37°C. After confirming complete digestion (by running 5µL in an agarose gel), DNA template was subjected to column purification using *Wizard Plus SV Gel and PCR Clean-up System* (Promega). DNA was precipitated and quantified (as previously described).

- Probe synthesis: Anti-sense transcripts were produced using 1µg of linearized plasmid DNA and 20U of RNA polymerase in the presence of 30mM DTT, 1x DIG-NTP mix (1mM ATP, CTG, GTP, 0.65mM UTP and 0.35mM DIG-UTP), 40U of RNase inhibitor (Roche) and 1x Transcription Buffer (Stratagene), in a final volume of 25µL. After 3 hours of incubation at 37ºC, the sample was precipitated by adding 20.5µL of RNAse-free water, 2µL of 0.5M EDTA (pH8.0), 2.5µL of 8M LiCl, 150µL of ethanol and 1µL of glycogen and incubated o/n at -20ºC. After centrifugation, the supernatant was discarded, RNA precipitate was washed with 70% ethanol, resuspended in 100µL of 10mM EDTA and stored at -20ºC. To quantify the probe, 2µL were mixed with loading buffer containing formamide and, after denaturing for 10 min at 70ºC, run in agarose gel along with a probe of known concentration.

Antibodies used during the course of this work: Primary antibodies: rabbit anti-GFP (Abcam, 1:500), mouse anti-GFP (Abcam, 1:200), sheep anti-Chx10 (Exalpha, 1:100). Secondary antibodies: goat anti-rabbit (Molecular Probes, 1:400), rabbit anti-mouse (Molecular Probes, 1:200) and donkey anti-sheep (Molecular Probes 1:400).

Tests performed to determine the specificity of the anti-GFP antibodies: The fluorescent reporter protein used was Venus protein which is a variant of EYFP protein and is recognized by the anti-GFP antibody. As previously mentioned, the reporter vectors were always co-electroporated with mCherry@pCAGGS in order to identify the electroporated half of the spinal cord and as a positive control for electroporation efficiency. As the N- and C-terminal regions of mCherry protein are derived from GFP, it was necessary to make sure that the anti-GFP antibodies used do not recognize mCherry protein. Two anti-GFP antibodies were available in our laboratory: rabbit polyclonal anti-GFP and mouse monoclonal anti-GFP. The monoclonal anti-GFP antibody shows no reactivity against mCherry protein, with or without previous $H_2O_2$:methanol treatment (data not shown). As the polyclonal anti-GFP is more sensitive detecting cells expressing low levels of GFP, this antibody would be preferred. However, we observed that after $H_2O_2$:methanol treatment the polyclonal anti-GFP antibody recognizes mCherry (data not shown). Therefore, in the electroporated embryos, whenever the $H_2O_2$:methanol treatment was performed, we used the monoclonal anti-GFP antibody in order to avoid false positives.

**Table 4 –** Composition of the solutions used during the course of this work.

| Solution | Composition |
|---|---|
| 0.5x TBE Buffer | 45mMTris-Borate, 1mM EDTA |
| TE Buffer | 10mM Tris, 1mM EDTA, pH 8 |
| 1x TAE Buffer | 40mM Tris, 1mM EDTA, 0.35% glacial aceticacid |
| Loading Buffer | 60% Glycerol (v/v), 10mM EDTA, 0.2% OrangeG (Sigma) |
| SOB medium | 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl |
| Hybridization Buffer | 1x Salts, 50% deionised formamide, 10%dextran sulphate, 1mg/mL rRNA, 1x Denhardt's solution |
| Washing Solution | 1x SSC, 50% formamide |
| SSC | 0.3M sodium citrate, 3M sodium chloride, pH 7 |
| Blocking Reagent | 10% Boehringer Blocking Reagent (BBR) in maleic acid buffer |
| Maleic acid Buffer | 1M maleic acid, 1.5M NaCl, pH 7.5 |
| Antibody incubator | 2% blocking reagent and 1% heat-inactivated sheep serum in TBST |
| TBST *in situ* hybridization | 150mM NaCl, 10mM KCl, 50mM Tris pH 7.5, 0.1%Tween-20 |
| NTMT | 0.1M NaCl, 0.1M TrisHCl pH 9.5, 0.05M MgCl, 1% Tween-20 |
| Mowiol | 0.1%Mowiol® (Calbiochem), 33% glycerol, 0.1M Tris, pH 8.5 |
| DAPI (Sigma) | 0.15% (w/v) 4',6-diamidino-2-phenylindole |
| TBST Immunohistochemistry | 20mM TrisHCl pH 8, 150mM NaCl, 0.05% Tween-20 |

## Annex B – Results

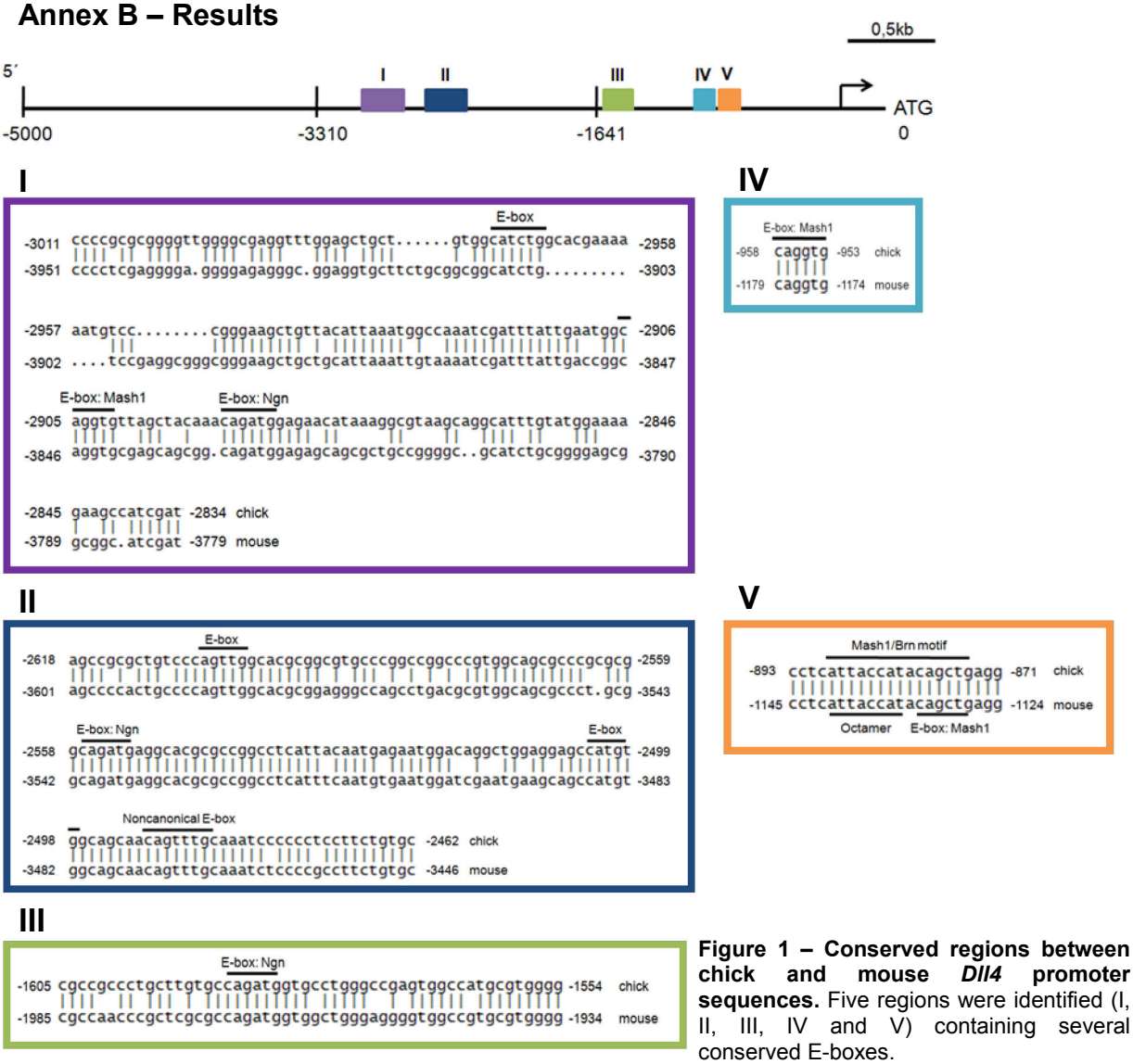


**Figure 1 – Conserved regions between chick and mouse *Dll4* promoter sequences.** Five regions were identified (I, II, III, IV and V) containing several conserved E-boxes.