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SCRATCH2 na diferenciação neural em embriões e em células-tronco

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SCRATCH2 in embryonic and stem cell neural differentiation

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RESUMO

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Os genes *SCRATCH* codificam fatores de transcrição que pertence à superfamília SNAIL e participam do desenvolvimento neural. Membros desta superfamília têm sido descritos como repressores da transcrição. Estruturalmente, apresentam um domínio altamente conservado presente na região N-terminal chamado de SNAG e o domínio *zinc-finger*, na região C-terminal. Além disso, os membros da família SCRATCH apresentam o domínio SCRATCH, que também é altamente conservado. Durante o desenvolvimento de embriões de galinha, SCRATCH2 é expresso em células precursoras neuronais embrionárias em início diferenciação no do tubo neural. Em camundongos, SCRATCH2 é expresso em precursores da glia radial (PGR) e em progenitores intermediários (PI) modulando a migração e diferenciação neuronal. Consistente com o seu papel como fator de transcrição, SCRATCH2 está presente no núcleo das células. A retenção nuclear de SCRATCH2 é controlada pelo domínio *zinc-finger*, enquanto sua atividade repressora encontra-se na região N-terminal. Ao contrário dos membros da família SNAIL, a atividade repressora de SCRATCH2 não é mediada pelo domínio SNAG. No caso de SCRATCH2, a atividade repressora é modulada pelo domínio SCRATCH. A análise da sequência de SCRATCH2, através da comparação de homologia, identificou uma sequência conservada presente na região N-terminal que apresenta dois resíduos fosforiláveis, tirosina$_{77}$ (Y77) e serina$_{78}$ (S78). A sequência contendo estes resíduos é completamente conservada entre os homólogos de SCRATCH2 em galinha, humano, rato e camundongo. Mutação em Y77 ou S78 reduz a capacidade repressora de SCRATCH2. A superexpressão destes mutantes em células HEK293T e embriões de galinha detectou a presença da proteína no núcleo, demonstrando que a redução na repressão de transcrição não é devida a uma mudança na localização subcelular. A substituição concomitante de ambos os resíduos, Y77 e S78, resgata a função normal de SCRATCH2. A análise *in silico*, que prediz o efeito dos nossas mutações sobre a exposição dos sítios de ligação de proteínas, indica que a região entre os domínios SNAG e SCRATCH apresenta uma grande quantidade de alterações. Isto sugere que a substituição de resíduos Y77 e S78 pode modificar a conformação secundária e terciária da proteína e interferir na sua função. Nós verificamos também, a função de SCRATCH2 no contexto de corticogênese de células-tronco embrionárias de camudongo. Durante este processo, os genes *Pax6>Nng>Tbr2>Tbr1* são expressos sequencialmente para originar PGR, PI e, finalmente, neurônios pós-mitóticos. *Scratch2* é gradualmente expresso durante este processo e seu padrão de expressão se assemelha ao de *Tbr2*. A remoção de *Scratch2* por edição genômica via CRISPR/Cas9 resulta no aumento da expressão *Pax6*, *Tbr2* e *Ngn1*. Em contraste, os níveis de *Tbr1* diminuem. Esses dados sugerem que SCRATCH2 atua na manutenção de PI, via *Pax6*, e participa do início da diferenciação neural.

Palavras-chave: Células-tronco embrionárias. Corticogênese. Embrião de galinha. Fator de transcrição. Neurogênese. SCRATCH2.

ABSTRACT

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SCRATCH genes code zinc-finger transcription factors that belongs to the SNAIL superfamily and participate in neural development. This superfamily members have been describe as transcriptional repressors. Structurally, they presented a highly conserved N-terminal SNAG and C-terminal zinc-finger domains. Additionally, SCRATCH family members present the SCRATCH domain that is also highly conserved. SCRATCH is specifically expressed in neural tissues, being found in early postmitotic neural progenitors. During chicken embryos development, SCRATCH2 is expressed in embryonic neuronal precursor cells in early differentiation in the neural tube. In mice, SCRATCH2 is expressed in radial glia progenitos (RGP) and intermediate progenitors (IP) modulating the neuronal migration and differentiation. Consistent with its role as a transcription factor, SCRATCH2 is expressed in the nucleus of chicken neural tube cells. Nuclear retention is controlled by the zinc-finger domain, while its repressor activity relies on the N-terminal region. Unlike SNAIL family members, SCRATCH2 transcriptional repression is not mediated by SNAG domain. In SCRATCH2 case, the repressor activity is modulated by the SCRATCH domain. An analysis of SCRATCH2 through homology comparison identified a N-terminal conserved sequence containing two phosphorylatable residues, tyrosine$_{77}$ (Y77) and serine$_{78}$ (S78). The sequence containing these residues is completely conserved between the homologues of SCRATCH2 in chicken, human, rat and mice. All single mutants forms reduced SCRATCH2 repressor ability. Overexpression of cSCRATCH2 single point mutants in HEK293T and chicken embryos remain in the nucleus demonstrating that the lack of transcriptional repression is not due a subcellular localization shift. Concomitant replacement of both residues, Y77 and S78, rescue the normal function. An *in silico* analysis that predicts the effect of our mutations on the exposure to binding sites demonstrated that the linker region between SNAG and SCRATCH domains presents the higher amount of changes. This suggests that the replacement of residues Y77 and S78 might modify the protein conformation interfering in its function. We also verified the SCRATCH2 function during mouse embryonic stem cell (mESC) differentiation into cortical neurons. During this process, *Pax6>Nng>Tbr2>Tbr1* genes are sequentially expressed to form RGP, IP and finally differentiating postmitotic cells. *Scratch2* is gradually expressed in this process and its expression pattern resembles that of *Tbr2*. Elimination of *Scratch2* expression by editing its genomic locus with the CRISPR/Cas9 system leads to an upregulation of *Pax6*, *Tbr2* and *Nng1*. In contrast, *Tbr1* levels are downregulated. This data suggests that SCRATCH2 plays a role maintaining the IP pool, acting via *Pax6*, thereby regulating the onset of neural differentiation.

Keywords: Chicken embryo. Corticogenesis. Embryonic stem-cells. Neurogenesis. SCRATCH2. Transcription factor.

1 INTRODUCTION

1.1 NEUROGENESIS AND NEURAL DIFFERENTIATION

The developmental process of neurulation involves a series of coordinated morphological movements that result in formation of the neural tube. The neural tube gives rise to vertebrate central nervous system originating the brain and spinal cord. Abnormalities in this process can lead to neural tube defects with devastating effects on nervous system function. The embryonic precursor of the neural tube is the neural plate, or neuroepithelium, a thickened region of ectoderm on the dorsal surface of the early embryo. The neural plate undergoes rostrocaudal lengthening, mediolateral narrowing and further apico–basal thickening, except in the midline where it becomes anchored to the underlying notochord and midline neuroepithelial cells shorten and become wedge-shaped (Colas, Schoenwolf, 2001). After that, the neural plate starts to bend forming the neural folds, which fuses giving rise to the neural tube.

The cephalic portion of the neural tube originates the brain while the caudal portion originates the spinal cord. The neural crest cells, located in the neural plate border, delaminate and migrate to form the peripheral nervous system as well as a variety of other tissues (Figure 1). Neurulation requires both intrinsic and extrinsic forces acting in concert. Intrinsic forces arise within the neural plate and drive neural plate shaping and furrowing, whereas extrinsic forces arise outside the neural plate and drive neural plate folding and neural groove closure.

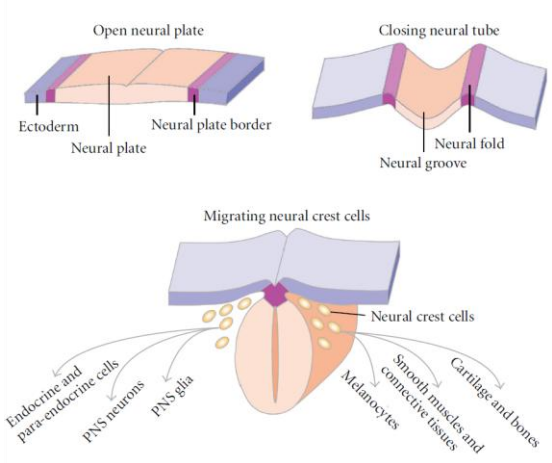

Figure 1 - Neurulation process. The neural plate bends forming the neural groove and the neural fold. The neural plate border cells are called neural crest

cells. The neural fold fuses giving rise to the neural tube. Near the time of neural tube closure (depending on the species), the neural crest cells delaminate from the neural tube and migrate along defined pathways. Figure source: Wislet-Gendebien et al., 2011.

The central nervous system contains a wide variety of neuronal and glia types. The neurons are organized in layers and clusters, each presenting different functions and connections. The cell proliferation in the neural tube occurs in the luminal side of the cell layers, in a region known as the ventricular zone (VZ). In the developing spinal cord, after exiting the cells cycle, the postmitotic cells migrate toward the marginal zone (MZ). During the migration, they refine their identity to become mature neurons. Thus, the posterior neural tube can be divided functionally in concentrically arranged zones around the lumen: a VZ with proliferative progenitors, an intermediate zone (IZ) with postmitotic and undifferentiated cells and a MZ, or mantle zone, containing the differentiated neurons.

The mammalian cerebral cortex is a complex and stratified brain structure segregated into six horizontal layers and its origin reflects the complexity of combining cell migration with the sequential expression of a combination transcription factors. Each cortex layers contain different populations of neurons with distinct functions. During corticogenesis in mammals (from embryonic day 11 (E11) to E19 in the mouse) two germinal compartments: the VZ and the subventricular zone (SVZ), line the cerebral ventricles and generate neural cells. Neurons of the cerebral cortex arise in the VZ at the surface of the lateral ventricles. Initially, the proliferative layer of the mouse cortex forms the VZ. Next, it divides giving rise to the SVZ. Both zones harbor neural progenitors. Progenitors in the VZ will form the lower/deeper layers of neurons in the cortical plate, while progenitors in the SVZ will originate the upper layer of neurons (Frantz et al., 1994). Newborn neurons migrate towards the margin of the cerebral wall to form the primordial plexiform layer or preplate (PP). This zone is then split into the superficial MZ and the deeper subplate (SP) by the arrival of the cortical plate (CP) cells (Figure 2). Most of the neuroblasts generated in the VZ migrate outward to form the CP at the outer surface of the brain.

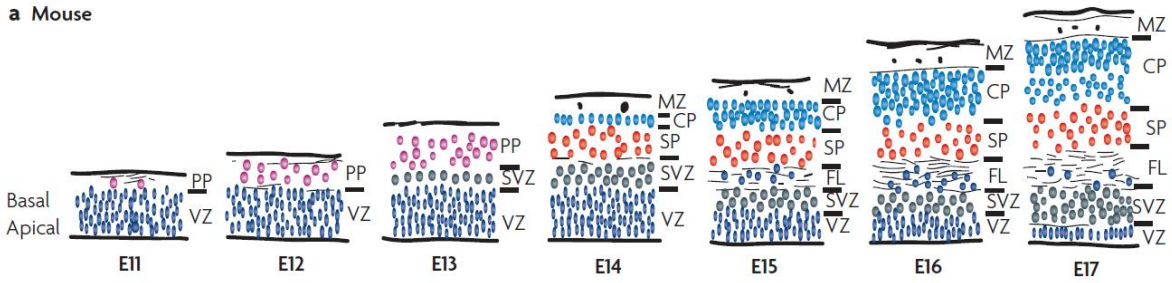

Figure 2 - Compartments and zones of mouse developing cortex. In mouse, corticogenesis lasts 8 days, from embryonic day 11 (E11) to E19. Neurons formed in the ventricular zone (VZ) migrate to their final locations in one of the six layers of the cortex. The VZ harbors the neural progenitors cells called radial glia progenitors (RGPs). Postmitotic cells migrate from the VZ towards the margin of the cerebral wall forming the preplate (PP). Later in development, divisions of RGP produce cells called intermediate progenitors (IP) that detach from the ventricular surface and aggregate in a zone overlying the VZ, the subventricular zone (SVZ). The SVZ serves as an additional proliferative compartment. Then, the PP is divided into the superficial marginal zone (MZ) and the deeper subplate (SP) by the arrival of the cortical plate (CP) cells. Adapted figure from Dehay and Kennedy, 2007.

The adult cortex is a complex structure consisting of six continuous layers. The neurons are layered according to their birth day. At specific stages of development, different populations of neurons permanently stop dividing and leave the VZ. This event is such a defining characteristic of a neuron that the day of its final cell cycle is known as the neuron's "birthday". The neurons with the earliest birthdays form the layers closest to the ventricule. The migration of newborn neurons into the CP occurs in an inside-first, outside-last manner; early-born neurons form the deep layers, whereas later-born neurons migrate past older neurons to form more superficial layers (Figure 3) (Berry , Rogers, 1965; Rakic, 1974). Neurons destined for the SP are generated first, followed by those destined for the deep layers (L6 and L5), and finally, those destined for the upper layers (L4, L3 and L2) close to the pial surface. The migration of young neurons from the VZ to the CP is largely dependent on radial glia (Misson et al., 1991; Rakic, 1990).

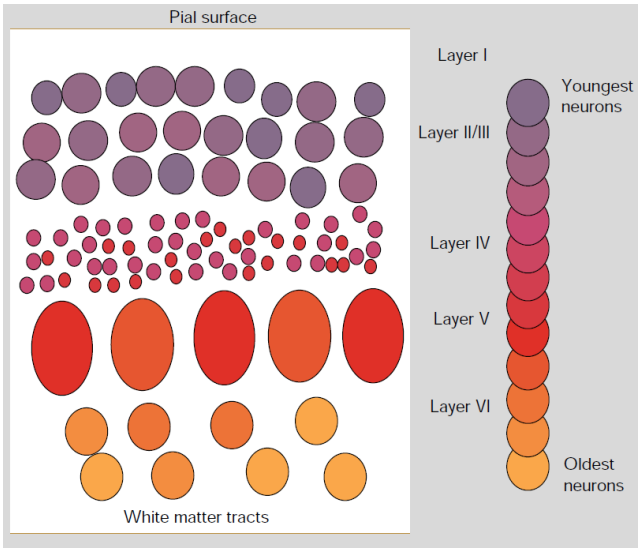

Figure 3 - Cortical layers are sequentially generated in an 'inside-out' way. The neocortex is composed by six different layers, each contain a specific neuronal cell types. The layers are grouped from outside (pial surface) to inside (white matter). The newly arriving cells migrate radially past through the existing neurons before reaching the cortical plate. Thus, the youngest neurons are found closer to the pial surface while oldest neurons are located close to the white matter. Adapted figure from Gilmore and Herrup 1997.

Cortical neurons are generated from three types of precursor cells: radial glial progenitors (RGPs), which are restricted to the VZ, short neural precursors (SNPs) and intermediate progenitor cells (IPs) (Figure 4).

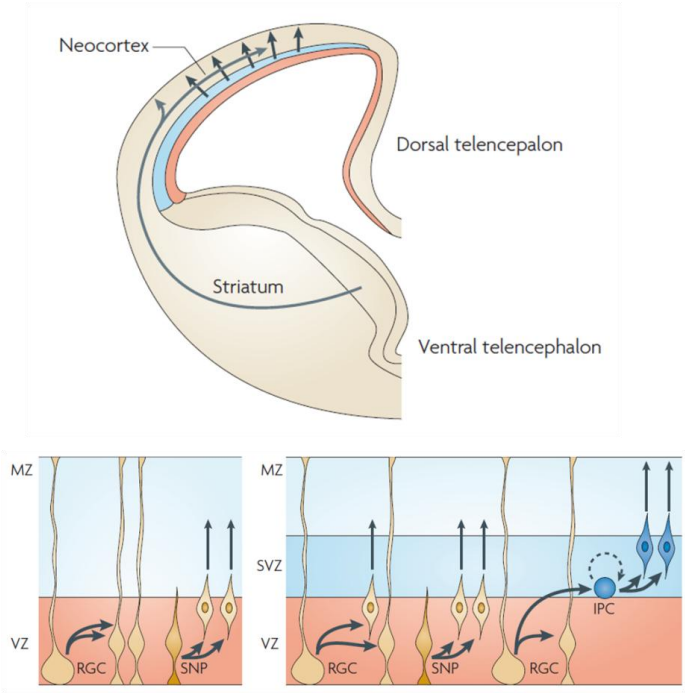

Figure 4 - Mouse cortical progenitor cells. Cortical neurons are generated from three types of precursors: radial glia progenitors (RGPs), short neural

precursors (SNPs) and intermediate progenitor cells (IPs). RGPs and SNPs divide at the apical surface of the ventricular zone (VZ). RGCs can self-renew or undergo asymmetrical division giving rise to the intermediate progenitors (IPs). SNPs are committed neural precursors. IPCs divide away from the ventricular surface in the VZ and in the subventricular zone (SVZ). Most of the IPs undergo neural differentiation, however a small fraction self renew (dotted arrow). Adapted figure from Dehay and Kennedy, 2007.

RGPs and SNPs divide at apical surface of the VZ. SNPs are committed to generate neurons. Through asymmetric cells division, RPGs give rise to IPs. IPs divide in the apical region in VZ and SVZ undergoing few more cell divisions and then centrifugally migrate to their adult location in the cortex (Dehay and Kennedy 2007).

1.2 SCRATCH FAMILY OF TRANSCRIPTION FACTORS

SCRATCH genes code zinc-finger transcription factors that belongs to the SNAIL superfamily. All members of the SNAIL superfamily share a similar organization and also distinct signatures, which classified them in the different families. SNAIL superfamily proteins present a highly conserved C-terminal half and a divergent N-terminal half, with some conserved domains (Nieto, 2002).

In the C-terminal domain is found four to six zinc-fingers. These zinc-fingers are C2H2 type presenting two conserved cysteines and histidines that coordinate the zinc ion. The fingers are structurally composed of two β-strands followed by an α-helix and function as sequence-specific DNA-binding motifs. The number of zinc-fingers varies among SNAIL superfamily members, which may reflect the variations in the length of DNA being recognized (Chiang, Ayyanathan, 2013; Nieto, 2002). Alignments of the five zinc fingers DNA binding domains identified that the first, second and last zinc fingers are specific of each subgroup, whereas the third and fourth zinc fingers present an overall conservation throughout SNAIL superfamily (Kerner et al., 2009).

SNAIL superfamily zinc-fingers recognizes and binds specifically to a hexanucleotide sequence (CAGGTG) known as E-box (Mauhin et al., 1993; Nieto, 2002), which is also recognized by bHLH transcription factors. hSCRATCH1 shares the capacity of other SNAIL family members to bind to E-box enhancer motifs

(Nakakura et al., 2001). Once binding to the E-box consensus site, SNAIL family members act as transcriptional repressors. The repressor activity depends not only on the zinc-finger region, but also on at least two different motifs that are found in the N-terminal region (Hemavathy et al., 2000; Hou et al., 2008; Lin et al., 2010; Molina-Ortiz et al., 2012; Montoya-Durango et al., 2008).

The N-terminal region of SNAIL superfamily members is more divergent, but all members present a conserved domain called SNAG. SNAG is conserved in all vertebrate SNAIL and SCRATCH proteins. It is composed of eight amino acids (MPRSFLVK) and function as a transcription repressor domain (Ayyanathan et al., 2007; Chiang, Ayyanathan et al., 2013; Ferrari-Amorotti et al., 2013). In SNAIL2, removal of SNAG domain impairs the recruitment of the co-repressor proteins affecting its repressor activity (Molina-Ortiz et al., 2012). In other words, the SNAG domain plays a role recruiting co-repressors to regulate transcriptional repressor of SNAIL1 and SNAIL2. (Ayyanathan et al., 2007; Chang et al., 2013; Ferrari-Amorotti et al., 2013; Lin et al., 2010; Molina-Ortiz et al., 2012; Nieto, 2002).

The SNAIL superfamily can be subdivided into two related and independent groups: the SNAIL and SCRATCH families (Manzanares et al., 2001). SNAIL superfamily was divided because new family members in other organisms emerged. This new genes were more similar to Drosophila SCRATCH (dSCRATCH) and its ortholog in Caenorhabditis elegans (CES-1) than any SNAIL gene (Nieto, 2002). According to SCRATCH family phylogenic history proposed by Barrallo-Gimeno and Nieto (2002) based on phylogentic analysis from placozoans to humans, the duplication of a single *SNAIL* gene in the metazoan ancestor originated two other genes, *SNAIL* and *SCRATCH* (Barrallo-Gimeno, Nieto 2005; Barrallo-Gimeno, Nieto 2009; Nieto, 2002). Both genes underwent independent duplications in Cnidarian and Bilaterian. Subsequent tandem gene duplication of a bilateria ancestor gave rise to three other members of SCRATCH and SNAIL family in Droshophila (SCRATCH, SCRATCH-LIKE1 and SCRATCH-LIKE2; ESCARGOT, SNAIL and WORNIU) (Figure 5).

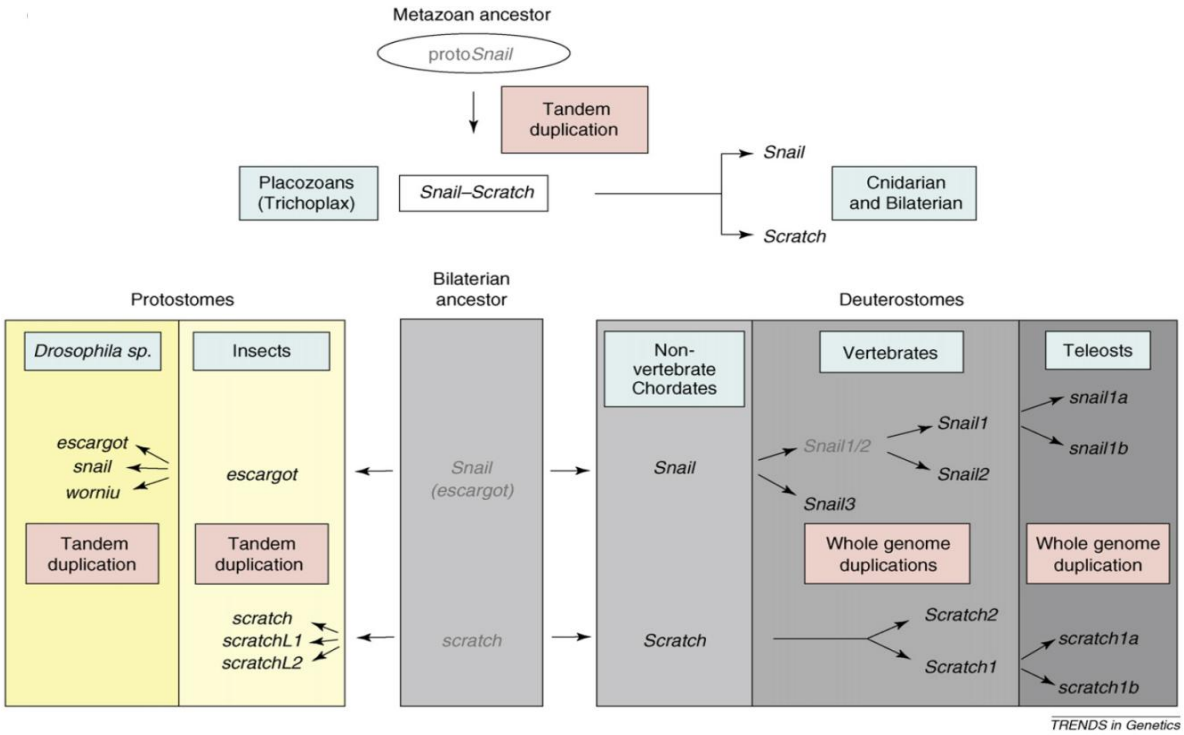

Figure 5 - Origin and evolution of *SCRATCH2* gene. An ancestor gene was duplicated during Metazoan evolution giving rise to *SCRATCH* and *SNAIL* genes. Later, independent duplications in Cnindarian and Bilaterian originated several family members in each group. The whole genome duplication in the vertebrate lineage gave rise to SCRATCH1 and SCRATCH2. Ancestral genes are represented in grey. Figure source: Barrallo-Gimeno, Nieto, 2009.

The founding member of the SNAIL family in vertebrates was isolated from *Drosophila melanogaster*. In vertebrates, the whole genome went through two duplications. The first genome duplication gave rise to the SNAIL1/SNAIL2 and SNAIL3 ancestor. This event also originated two novel member of SCRATCH family: SCRATCH1 and SCRATCH2. SNAIL 1 and SNAIL 2 (also called SLUG - Barrallo-Gimeno, Nieto, 2005) arose from a subsequently genome duplication of the SNAIL1/SNAIL2 and SNAIL3 ancestor (Barrallo-Gimeno, Nieto, 2009). An additional genome duplication in the teleost lineage originated a duplicated SNAIL and SCRATCH genes, SCRATCH1a/b and SNAIL1a/b (Figure 5).

In chicken genome, only SCRATCH2 is found according to NCBI database. Our laboratory has cloned the full-length chicken orthologue of SCRATCH2 (cSCRATCH2). The resulting full-length clone has 831 nucleotides (JN982016) and encodes for 276 amino acids (Vieceli et al., 2013) (Figure 6).

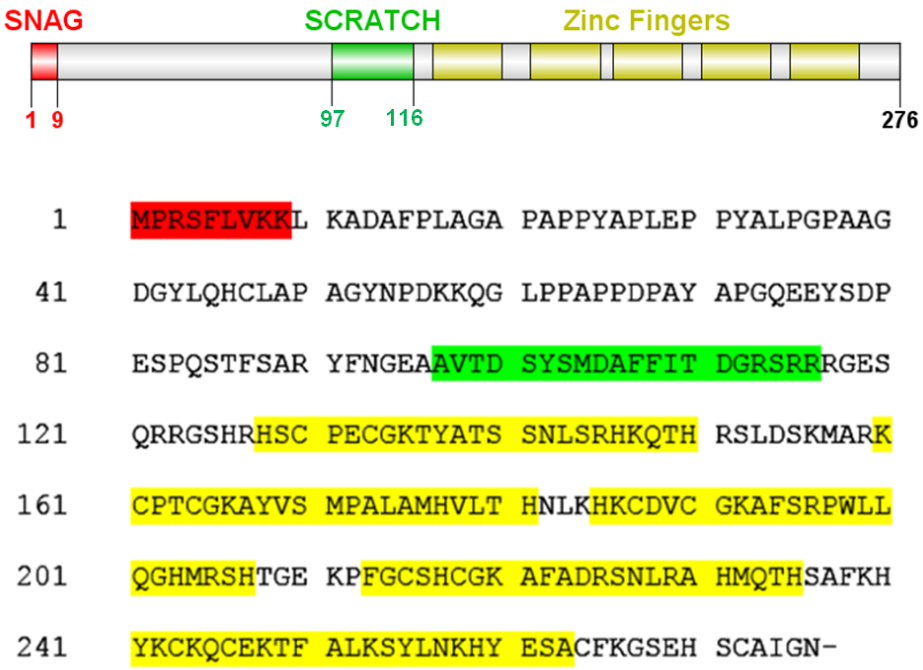

Figure 6 - Schematic representation of the chicken SCRATCH2 protein structure. cSCRATCH2 protein has 276 amino acids and presents three conserved domains. The different domains are represented with a color code. In the N-terminal is found the SNAG domain, 1 to 9 amino acids (red) and the SCRATCH domain, 97 to 116 amino acids (green). C-terminal harbors the zinc-finger domain (yellow) composed by five zinc-fingers motifs. The first zinc-finger motif comprises amino acids 128-150, the second 160-181, the third 185-207, the fourth 213-235 and the fifth 241-263. Bottom diagram shows the protein coding sequence translation and the position of each domain within it.

In hSCRATCH1, the SNAG domain is not necessary to the repressor activity (Nakakura et al., 2001). However, little is known about SNAG domain function in other SCRATCH family proteins.

In the linker region between the SNAG and zinc-fingers domain of SNAIL superfamily members, we find two highly conserved amino acid stretches specific to SCRATCH and SNAIL families. These specific stretches are called SCRATCH and SLUG domains, respectively (Chiang, Ayyanathan et al., 2013; Kerner et al., 2009; Nieto, 2002). In chicken, the SCRATCH domain corresponds to amino acids 97-116 (Figure 6). A recent study has shown that the SLUG domain is necessary for efficient SNAIL2-mediated repression. Deletion of it can impair repression of E-cadherin promoter (Molina-Ortiz et al., 2012). In contrast, to the date, the function of SCRATCH domain is unknown.

SNAIL genes plays multiple roles during development controlling neural crest specification and delamination, mesoderm specification, left-right asymmetry and triggering the epithelial to mesenchymal transition (EMT). They are also implicated in cancer metastasis (Barralo-Gimeno, Nieto 2005; Isaac et al., 1997; Manzanares et al., 1993; Mayor et al., 1995; Nieto et al., 1994; Nieto, 2002; Swami 2009). On the other hand, SCRATCH proteins are exclusively expressed in the developing nervous system and are involved with neurogenesis.

1.3 SCRATCH EXPRESSION PATTERN AND ITS ROLE IN NEUROGENESIS

SCRATCH gene was first identified and described in Drosophila (dSCRATCH), where it is expressed in dividing neuroblasts and persists in postmitotic neurons (Roark et al., 1995). The gene is called SCRATCH due to the scratched eyes phenotype resulting from the gene mutation. Drosophila SCRATCH null mutant presents a reduced number of photoreceptors in the eye and deformations in the ommatidia, suggesting that SCRATCH expression promotes neurogenesis. dSCRATCH ectopic expression in this experimental model results in formation of extra neurons. Furthermore, increased expression of SCRATCH ortholog of *Caenorhabditis elegans* (CES-1) expression blocked apoptosis of specific neuronal populations: the sister cells of serotoninergic neurosecretory motor neurons and I2, leading to the appearance of supranumerary neurons, further suggesting that it regulates the genes required for programmed cell deaths (Ellis, Horvitz, 1991). Likewise, SCRATCH2 knockdown increased the rate of apoptosis in neural tube in zebrafish embryos. SCRATCH2 is required to newly differentiated neurons survival by repressing Puma expression (Rodriguez-Aznar, Nieto, 2011).

In chicken embryos, SCRATCH1 is absent and SCRATCH2 is expressed in the whole extent of the neural tube (Figure 7), in cranial ganglia and nasal placode (Vieceli et al., 2013). During development, cSCRATCH2 expression is first detected at stage HH15 in few cells of the hindbrain and nasal placode. Around stage HH19, its expression domain expands comprising the metencephalon, mesencephalon, myelencephalon and posterior neural tube. In the posterior neural tube, which will give rise to the spinal cord, cSCRATCH2 is expressed in postmitotic cells within the IZ. Its expression pattern overlaps with expression of the postmitotic transcription factor NEUROM and partially overlaps with the external perimeter of

NGN2, a neural progenitor cell marker (Figure 7, Vieceli et al., 2013). Together, these data define cSCRATCH-positive cells as a subset of immediately postmitotic neural progenitors.

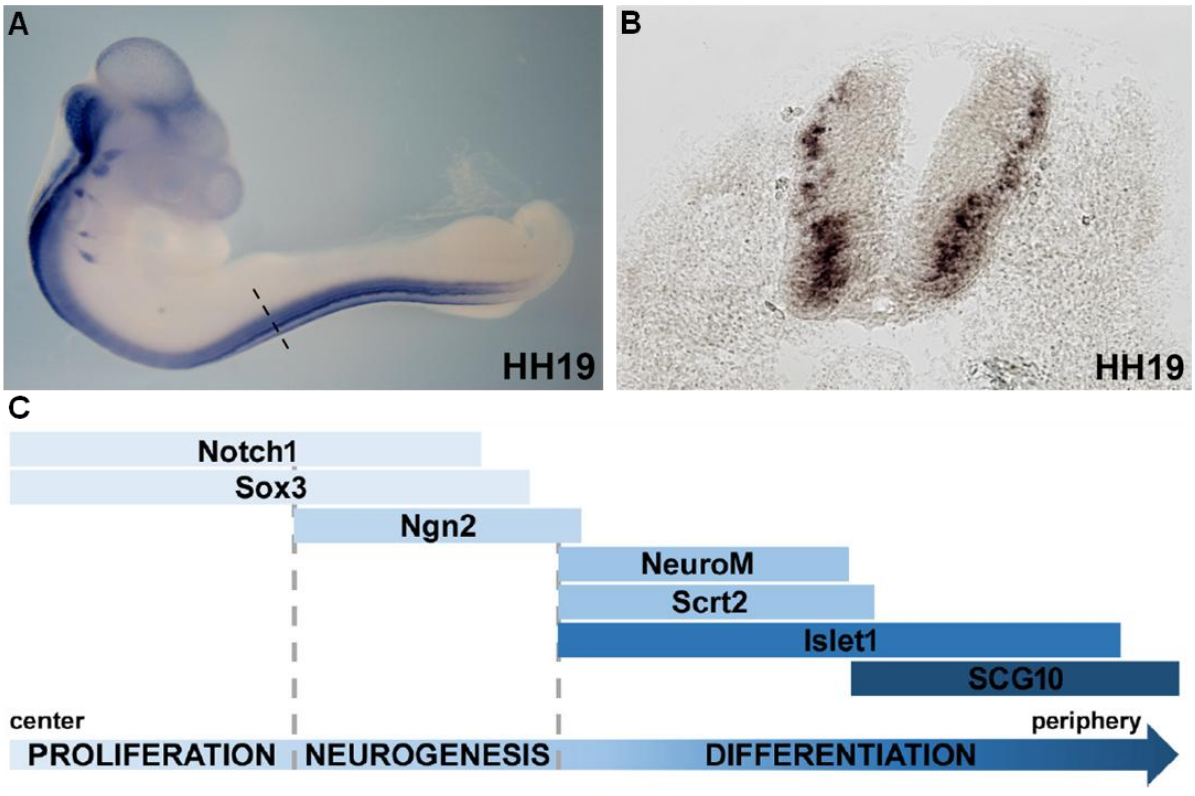

Figure 7 - cSCRATCH2 expression pattern during development. (A) cSCRATCH2 is expressed along the whole neural tube as seen in the whole mount *in situ* hybridization at stage HH19. (B) A cross section of the trunk neural tube shows that cSCRATCH2 is expressed in a intermediate zone. (C) Bottom diagram represents a possible timeline of cSCRATCH2 expression comparing to known neurogenic markers during neurogenesis and differentiation in the spinal cord. Adapted figure from Vieceli et al., 2013.

In mice, two forms of SCRATCH were identified: *Scratch1* (m*Scrt1*) and *Scratch2* (m*Scrt2*). Both are predominantly expressed in the brain and spinal cord appearing in newly differentiating postmitotic neurons. Its expression persists since the onset of postmitotic cells differentiation until the first postnatal day, when m*Scrt2* is became absent in areas where there is no neurogenesis. During mouse development, high levels of SCRATCH start to be detected around E10.5-E11.5 (Itoh et al., 2013; Marín, Nieto, 2006). In these embryonic days m*Scrt* is expressed throughout the brain, from the forebrain to the hindbrain. At E12.5, the expression in all brain regions was more evident due to the progressive thickening of the mantle

layer. m*Scrt* is not detected in premigratory neural crest cells, however both forms are found in some crest derivatives, such as the cranial and dorsal root ganglia as well as in the Schwann cells of the vomeronasal nerve. As development progresses, its expression is significantly downregulated. In mouse adult brain, m*Scrt* expression is restricted to a few isolated neurons in the dentate gyrus of the hippocampus (Marín, Nieto, 2006).

In the mouse developing cortex, m*Scrt2* is expressed in subsets of mitotic and neurogenic RGP at the apical surface of the VZ, IPs at SVZ/IZ and differentiating neurons located in the upper SVZ and IZ border. It is not detected in proliferating cells present in VZ. In VZ, m*Scrt2* expression is relatively low, as expected, since it is expressed in cells that have already exited the cell cycle. In this region, m*Scrt2* expression co-localizes with the expression of PAX6 while in the SVZ/IZ, it co-localizes with the expression of NEUROD1, a marker of early differentiating neurons and partially with the IP marker TBR2. Conditional activation of transgenic m*Scrt2* in mice cortical progenitors promotes neuronal differentiation by favoring the direct mode of neurogenesis of RGPs. In other words, m*Scrt2* favors the generation of neurons directly from RGPs. The high levels of m*Scrt2* induce premature cell cycle exit leading to neurogenic divisions of the progenitors, and diminishing the IP pool. Thus, neuronal amplification via indirect IP neurogenesis is extenuated, leading to a mild postnatal reduction of cortical thickness (Figure 8) (Paul et al., 2012). Corroborating this data, *in vivo* overexpression of m*Scrt2* suppressed the generation of IPs from RGPs and caused a delay in the radial migration of upper layer neurons toward the CP (Itoh et al., 2013). Once RGPs cells commit to becoming neurons or IPs, they delaminate and start migration toward the pial surface. During migration, the cells express different genes and acquire their identity. In this context, m*Scrt* induces the delamination by repressing E-cadherin promoter (Itoh et al., 2013). Together, these expression data strongly suggest that m*Scrt2* may play a role in the initiation and/or modulation of neuronal differentiation as wells as in the onset of neural migration.

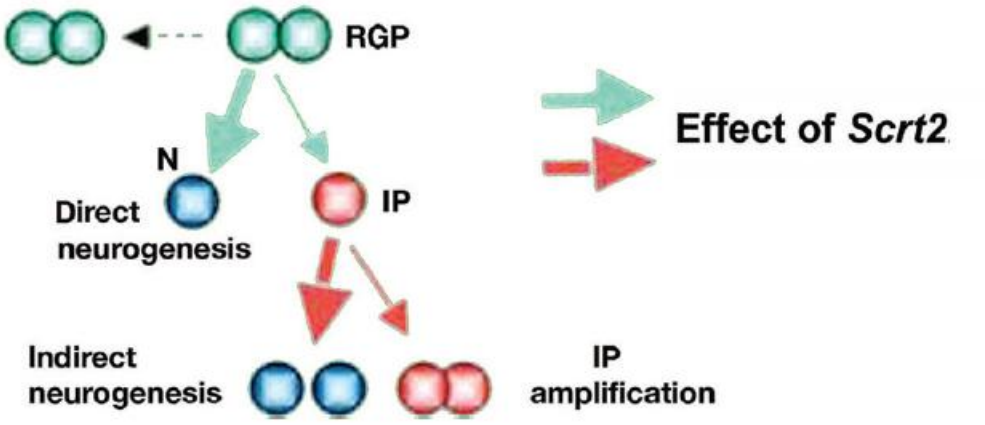

Figure 8 - Schematic illustration of m*Scrt2* overexpression effect in neural progenitors cells. High levels of m*Scrt2* favor the asymmetric divisions of RGPs (thick green arrow) generating excess of neurons, while it does not affect the symmetric division (green cells). The pool of IPs reduces and the cells preferentially undergo differentiation (thick red arrow). Figure source: Paul et al., 2012.

m*Scrt1* expression is also detected in mouse P19 embryonal carcinoma cells induced to neural differentiation. Additionally, overexpression of hSCRATCH1 in this cell line leads to neural differentiation (Nakakura et al., 2001). This indicate that SCRATCH1 might influence neuronal differentiation.

Taken together, the data from these various animal models strongly suggest that SCRATCH function is related to the differentiation of postmitotic neurons.

5 CONCLUSION

Our findings highlight the role of distinct conserved domains relevant to SCRATCH2 subcellular localization and function. We demonstrated that SCRATCH2 nuclear retention is controlled by its zinc-finger domain, present in the C-terminal region. In the SCRATCH2 branch of the SCRATCH family, the SNAG domain role as a repressor domain was lost. Here, we show that the transcriptional repression function of SCRATCH2 is mediated by the SCRATCH domain. In addition, our study identified two phosphorylatable amino acids present in a conserved sequence at the N-terminal portion, Y77 and S78. Single mutations in these residues reduced the repressor capacity of SCRATCH2 while double mutation rescued its function. Thus, we suggest that the residues identity is important for SCRATCH2 normal activity.

In this study, we also investigated SCRATCH2 function during mESC differentiation into cortical neurons. First, we compared *Scratch2* temporal expression profile with known markers of cortical development. Our results point that *Scratch2* expression profile is similar to *Tbr2*, an intermediate progenitor marker. Then, to better understand its role in mESC cortical differentiation, we removed *Scratch2* expression by editing its genomic locus with the CRISPR/Cas9 system. We observed that the elimination of *Scratch2* expression leads to an upregulation of the early neurogenic markers *Pax6, Tbr2* and *Ngn1*. In contrast, the levels of the postmitotic neurons marker, *Tbr1,* is downregulated. Therefore, we suggest that *Scratch2* plays a role maintaining the intermediate progenitos pool, acting via *Pax6*, thereby regulating the onset of neural differentiation.

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