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The transcription factor chicken *Scratch2* is expressed in a subset of early postmitotic neural progenitors

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ABSTRACT

Scratch proteins are members of the Snail superfamily which have been shown to regulate invertebrate neural development. However, in vertebrates, little is known about the function of Scratch or its relationship to other neural transcription factors. We report the cloning of chicken *Scratch2* (*cScrt2*) and describe its expression pattern in the chick embryo from HH15 through HH29. *cScrt2* was detected in cranial ganglia, the nasal placode and neural tube. At all stages examined, *cScrt2* expression is only detected within a subregion of the intermediate zone of the neural tube. *cScrt2* is also expressed in the developing dorsal root ganglia from HH22–23 onwards and becomes limited to its dorsal medial domain at HH29. phospho-Histone H3 and BrdU-labeling revealed that the *cScrt2* expression domain is located immediately external to the proliferative region. In contrast, *cScrt2* domain overlapped almost completely with that of the postmitotic neural transcription factor *NeuroM/Ath3/NEUROD4*. Together, these data define *cScrt2*-positive cells as a subset of immediately postmitotic neural progenitors. Previous data has shown that Scrt2 is a repressor of E-box-driven transcription whereas NeuroM is an E-box-transactivator. In light of these data, the co-localization detected here suggests that Scrt2 and NeuroM may have opposing roles during definition of neural subtypes.

The Scratch family is part of the Snail superfamily of zinc finger transcription factors. In vertebrates, these possess characteristic DNA-binding zinc finger motifs at the C-terminus and a basic amino acid-rich domain (SNAG domain) at the N-terminus. In addition, Scratch proteins also have a conserved Scratch domain that is not found in the other members of the Snail superfamily (Manzanares et al., 2001).

Accumulating evidence from expression patterns and loss and gain of function studies suggest that Scratch proteins have a conserved role in promoting neural fate across several phyla (Ellis and Horvitz, 1991; Roark et al., 1995; Nakakura et al., 2001b; Marín and Nieto, 2006; Rodríguez-Aznar and Nieto, 2011). In the fly embryo, ectopic expression of scratch (scrt) produces extra neurons and represses the expression of non-neural genes (Roark et al., 1995). However, deletion of *scrt* alone results in a very mild ocular phenotype. A significant effect was only seen when *scrt* was eliminated in conjunction with the pan-neural bHLH transcription factor *dpn* (*deadpan*). Similarly, in the nematode *Caenorharbditis elegans*, gain of function of the homologue of scrt (CES-1) was shown to prevent apoptosis of neuronal precursors but loss of function of CES-1 alone failed to generate an obvious phenotype (Ellis and Horvitz, 1991). Together, these data indicate that Scratch function in neural development is intertwined with other nuclear elements and that it can regulate bHLH transcription factor activity. In support of this, CES-1 represses the expression of pro-apoptotic genes through competition with bHLH heterodimers (Thellmann et al., 2003). Human SCRT1 also competes with bHLH transcription factors in binding to E-box motifs (Nakakura et al., 2001a; Paul et al., 2012). Finally, in the vertebrate embryo *Scrt1/2* have been consistently associated with postmitotic neural progenitors (Nakakura et al., 2001a; Marín and Nieto, 2006; Rodríguez-Aznar and Nieto, 2011).

In contrast to invertebrates, less is known in vertebrates about the expression domain of *Scratch* genes relative to other established neural differentiation transcription factors. Considering that neural differentiation occurs concomitantly with migration towards external layers of the developing nervous system (Leber and Sanes, 1995), the anatomical position of a particular gene's expression domain relative to others, whose function are known, contributes to refining its functional position in the neural differentiation hierarchy. The match between gene function and expression domain is clearer in the posterior neural tube, whose early anatomy is significantly simpler than the cortex (Diez del Corral and Storey, 2001).

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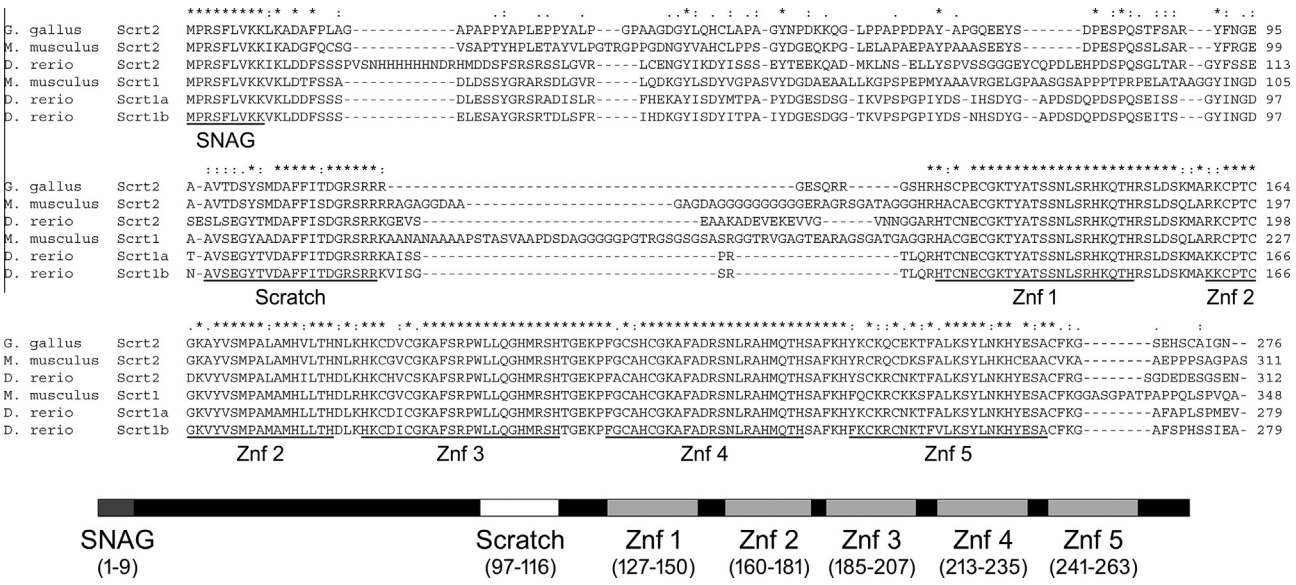

Fig. 1. Predicted protein sequence for chicken Scrt2 (*Gallus gallus*; AEW43643). Alignment with mouse Scrt1 (*Mus musculus*; NP_570963) and Scrt2 (NP_001153882) and zebrafish Scrt1a (*Danio rerio*; NP_001107073), Scrt1b (NP_001014369) and Scrt2 (NP_998802) and diagram showing positions of the previously described domains (Nieto, 2002) SNAG (aa 1–8), Scratch (aa 97–116) and 5 zinc fingers (Znf) (aa 127–150, aa 160–181, aa 185–207, aa 213–235 and 241–263) in the cScrt2 predicted amino acid sequence.

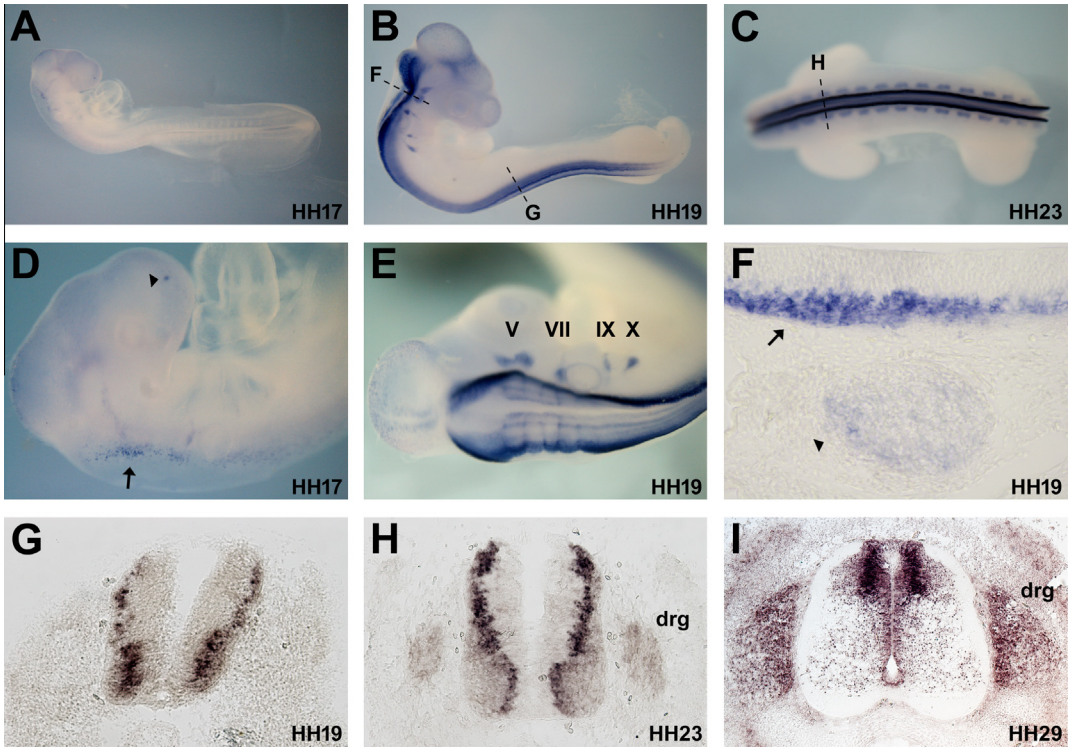

Fig. 2. Developmental expression of *cScrt2*. (A–E) Whole mount *in situ* hybridizations showing *cScrt2* expression in HH17 (A and D), HH19 (B and E) and HH23 (C). Dashed lines in (B) and (C) indicate sectioning plans and levels for (F–H). Arrow and arrowhead in (D) indicate positive cells in hindbrain and nasal placode, respectively. Roman numerals in (E) indicate cranial ganglia that express *cScrt2*. (F) Section of HH19 embryo subjected to whole mount *in situ* hybridization showing expression in the trigeminal ganglion (arrowhead); arrow indicates the hindbrain. (G–I) *In situ* hybridization in trunk spinal cord cross sections showing *cScrt2* expression in HH19 (G), HH23 (H) and HH29 (I). drg: dorsal root ganglia.

Thus, to better resolve Scratch's position in the neural differentiation transcriptional cascade in the vertebrate embryo, we cloned the full length coding sequence of the chicken *Scratch*2 homolog (*cScrt2*) and characterized in detail its expression pattern relative to other known markers in the trunk neural tube.

1. Results and discussion

The full-length chicken orthologue of *Scrt2* was cloned by RT-PCR using primers designed against a partial clone previously filed in the NCBI database (XM_426994) and clones obtained with

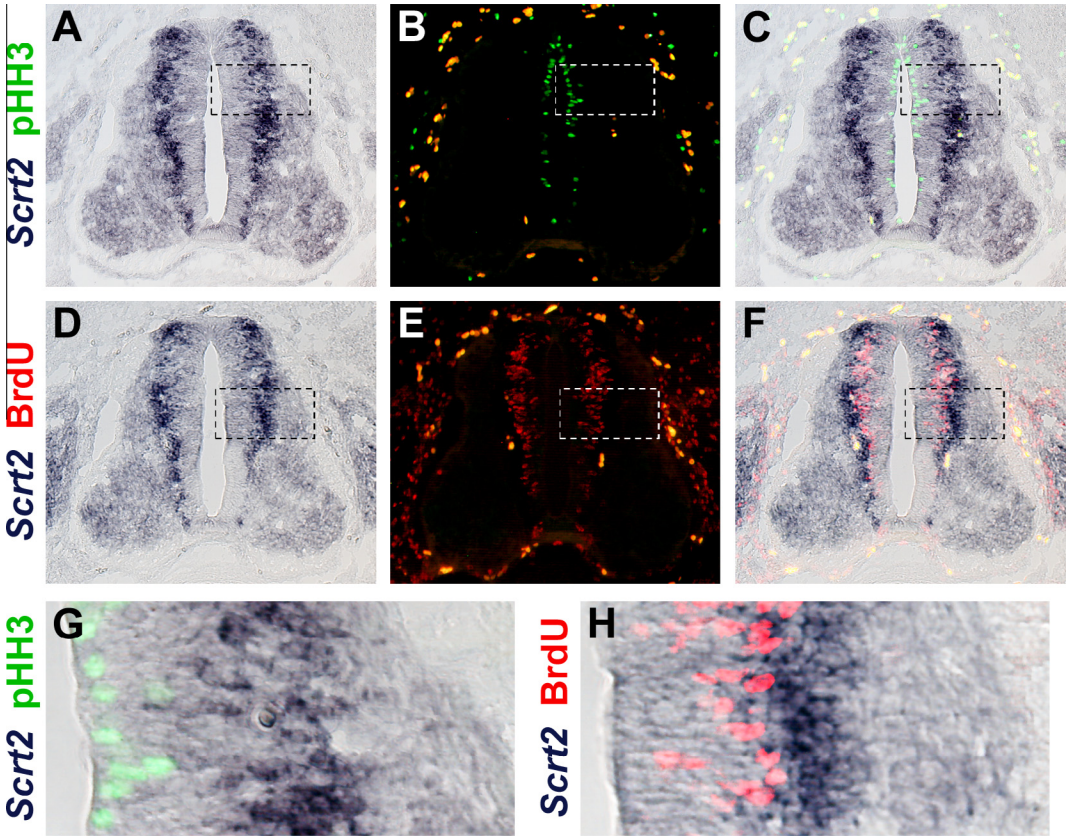

Fig. 3. *cScrt2* is not expressed in proliferating cells. (A–H) Double labeling for *cScrt2* by *in situ* hybridization (A and D) and immunofluorescence for pHH3 (B) or incorporated BrdU (E) showing expression of these markers in spinal cord cross sections of a HH25 embryo. (C and F) are merged images for each set. (G and H) are magnifications of delimited regions in (C and F), respectively. The yellow signal is generated from autofluorescent blood cells in the tissue.

RACE-PCR. The resulting full-length clone has 831 nucleotides (JN982016) and encodes for 276 amino acids. This complete coding sequence (CDS) has 99% identity (823/831) with a previously deposited cDNA sequence (FJ620691). The divergence between the two sequences is concentrated in the first 21 bases. Because the sequence of the primers we used for cloning the CDS were based in the sequencing of clones obtained with RACE-PCR that included both the 5′ UTR and the 5′ CDS in the same molecule, it is possible that the divergence with the previously deposited CDS results from alternative cloning procedures for the latter. To confirm the identity of our clone inside the Scratch family, we aligned the predicted translation product with other chordate Scratch orthologues. In the resulting phylogenetic tree, the clone grouped with the mammalian Scrt2 orthologues supported by the maximum bootstrap value (100%; data not shown), thus confirming the identity of the cloned gene as the chicken orthologue of *Scrt2* (*cScrt2*). The full sequence of the predicted protein shares identities of 59% with zebrafish Scrt2 and 68% with mouse Scrt2 (Fig. 1).

To determine the temporal and spatial pattern of expression of *cScrt2*, we performed *in situ* hybridization. We did not detect any hybridization or qPCR signal in embryos from HH5 to HH14 (data not shown). In whole mount embryos, *cScrt2* expression was first detected in few cells of the hindbrain and nasal placode of HH15 embryos (data not shown). This expression domain expanded significantly by HH17 and could be clearly detected in the mes-metencephalon and the nasal placode (Fig. 2A and D). In stage HH19 embryos the expression domain at the metencephalon, mesencephalon was broader and expanded posteriorly to the myelencephalon and posterior neural tube (Fig. 2B and E). In the latter region, *cScrt2* expression occurred as a continuous domain concentrated in the intermediate region of the trunk neural tube. This domain was thinner in the dorsal region of the neural tube and wider in ventral regions by HH19 (Fig. 2G), and this relative pattern is progressively inverted in later stages (Fig. 2H and I). In addition, we observed clear staining in the cranial ganglia (Fig. 2E). cScrt2 expression in the trigeminal ganglia was more widespread and weaker than in the neural tube (Fig. 2F). At HH23, subtle staining was detected in the prospective dorsal root ganglia (DRG) (Fig. 2C and H). *cScrt2* expression in the DRG was stronger in E6 (embryonic day 6; HH28–29) embryos and was clearly enriched in the dorsomedial domain (Fig. 2I).

Our *in situ* hybridization results differ slightly from the pattern reported for the mouse *Scrt2* orthologue. Two forms of Scratch have been identified in mouse: *mScrt1* and *mScrt2*. Here, we show that *cScrt2* expression is uninterrupted throughout the dorsoventral axis. In contrast, *mScrt2* is absent from a small region of the dorsoventral axis, corresponding to the V2 interneuron column, whereas *mScrt1* expression domain is continuous (Marín and Nieto, 2006). Thus, in this aspect, *cScrt2* expression pattern is more similar to *mScrt1* than to *mScrt2*, and may reflect a form of paralogue exchange across species.

On the other hand, *cScrt2* and both forms of mouse *Scrt* are excluded from the ventricular zone of the posterior neural tube suggesting that all are expressed in the early postmitotic progenitor cells. Furthermore, the ventral to dorsal wave of expression of *cScrt2* in the trunk neural tube recapitulates the reported overall cell pattern of proliferation in the neural tube, which stops ventrally on embryonic day 5 (E5) and continues dorsally until E8 (Langman and Haden, 1970). Indeed, in the mouse anterior neural tube labeling for PCNA (Proliferating Cell Nuclear Antigen) and *mScrt2* are mutually exclusive (Marín and Nieto, 2006).

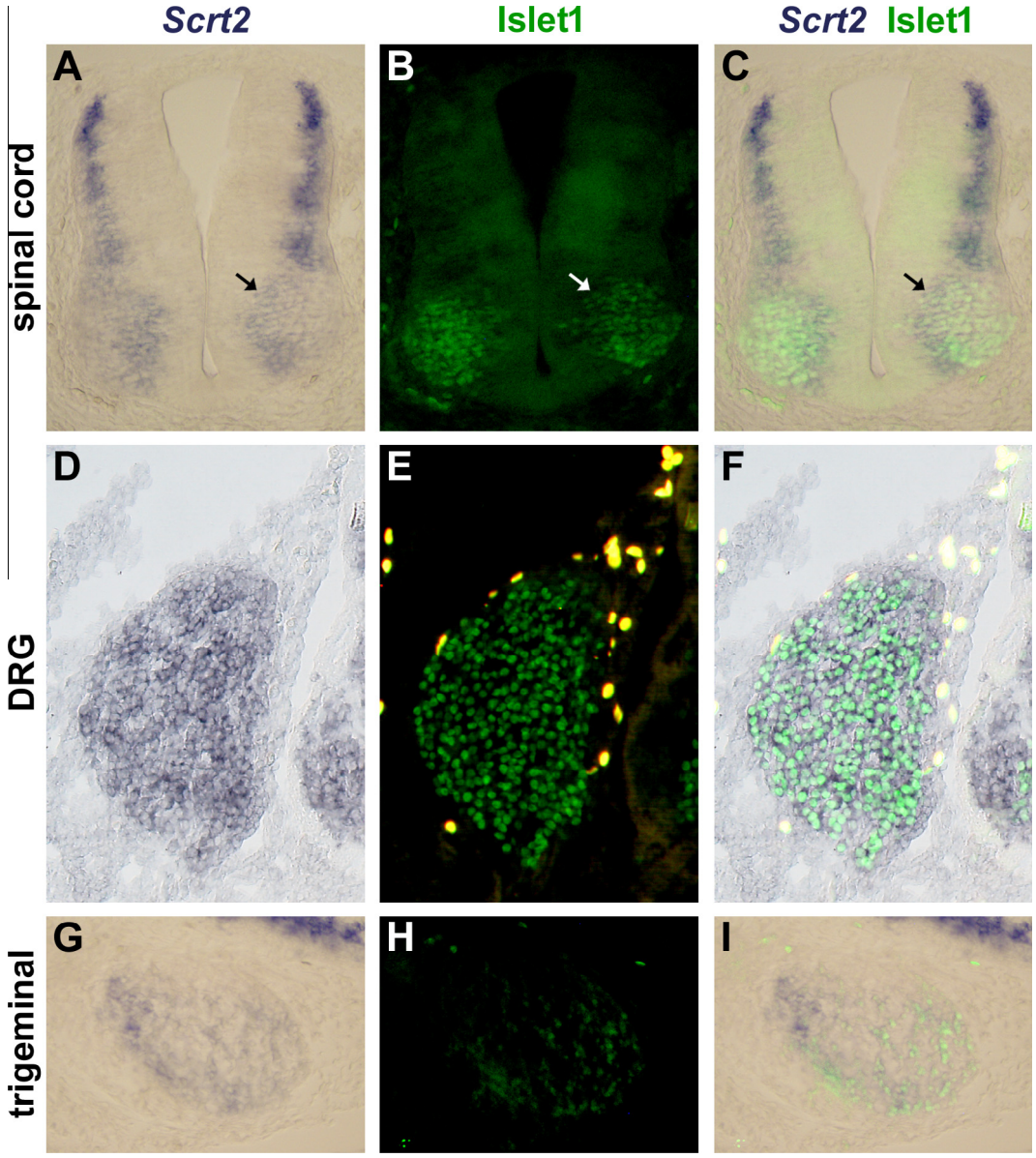

Fig. 4. *cScrt2* and Islet1 are coexpressed in the embryonic CNS and PNS. (A–I) Double labeling for *cScrt2* by *in situ* hybridization (A, D, G) and immunofluorescence for Islet1 (B, E, H) showing the relationship of their expression domains in the spinal cord (A–C) and trigeminal ganglion (G–I) in HH19 and in the DRG in HH25 (D–F). Arrows in (A–C) show a site in the motorneuron region where a stronger *cScrt2* signal overlaps with a weaker Islet signal. (C), (F) and (I) are merged images for each set. The yellow signal is generated from autofluorescent blood cells in the tissue.

To clarify the relationship between *cScrt2* and regions of cell proliferation in the chick, we investigated the expression domain of *cScrt2* relative to the ventricular proliferative zone by double-labeling for *cScrt2* expression and BrdU-incorporation or the presence of phospho-Histone H3 (pHH3) as markers for S and M phase, respectively. As expected, *cScrt2*expression domain in the chick embryo does not contain mitotic cells either in the posterior or anterior neural tube (Fig. 3 and data not shown). Rather, *cScrt2* is expressed in a region immediately external to the zone harboring S-phase cells in the proliferation zone. Therefore, as in other vertebrates, *cScrt2* is also associated with postmitotic neural progenitors.

In various developing neural tissues, the homeobox gene Islet1 is one of the earliest markers for post-mitotic neuroblasts (Ericson et al., 1992; Avivi and Goldstein, 1999). Thus, to determine if *cScrt2* is indeed expressed in early post-mitotic cells, we detected *cScrt2* transcripts and Islet1 protein through double-labeling. In the posterior neural tube, Islet1 is expressed in the ventral-most regions of the spinal cord where motor neuron progenitors differentiate (Ericson et al., 1992). The overlap between Islet1 and *cScrt2* is most obvious in the medial border of the motor neuron cluster, where newborn motor neurons reside (Fig. 4A-C; Hollyday and Hamburger, 1977). Interestingly, more mature cells, located ventro-laterally, display higher levels of Islet1 and lower levels of *cScrt2*. In the DRG, *cScrt2* expression coincides completely with that of Islet1 (Fig. 4D-F). In this tissue, Islet1 is also restricted to postmitotic cells and is one of the earliest markers for neural differentiation (Avivi and Goldstein, 1999; Cui and Goldstein, 2000). The DRG dorsal pole and perimeter are devoid of both Islet1 and *cScrt2* signals (Fig. 4D-F). Interestingly, these two sites harbor most of the proliferative cells at this stage (George et al., 2010). In the trigeminal ganglion, the *cScrt2* expression domain overlaps with Islet1 at the ventral-medial domain (Fig. 4G-I). However, unlike in the DRG, in this case the *cScrt2* domain is significantly larger than that of Islet1 and

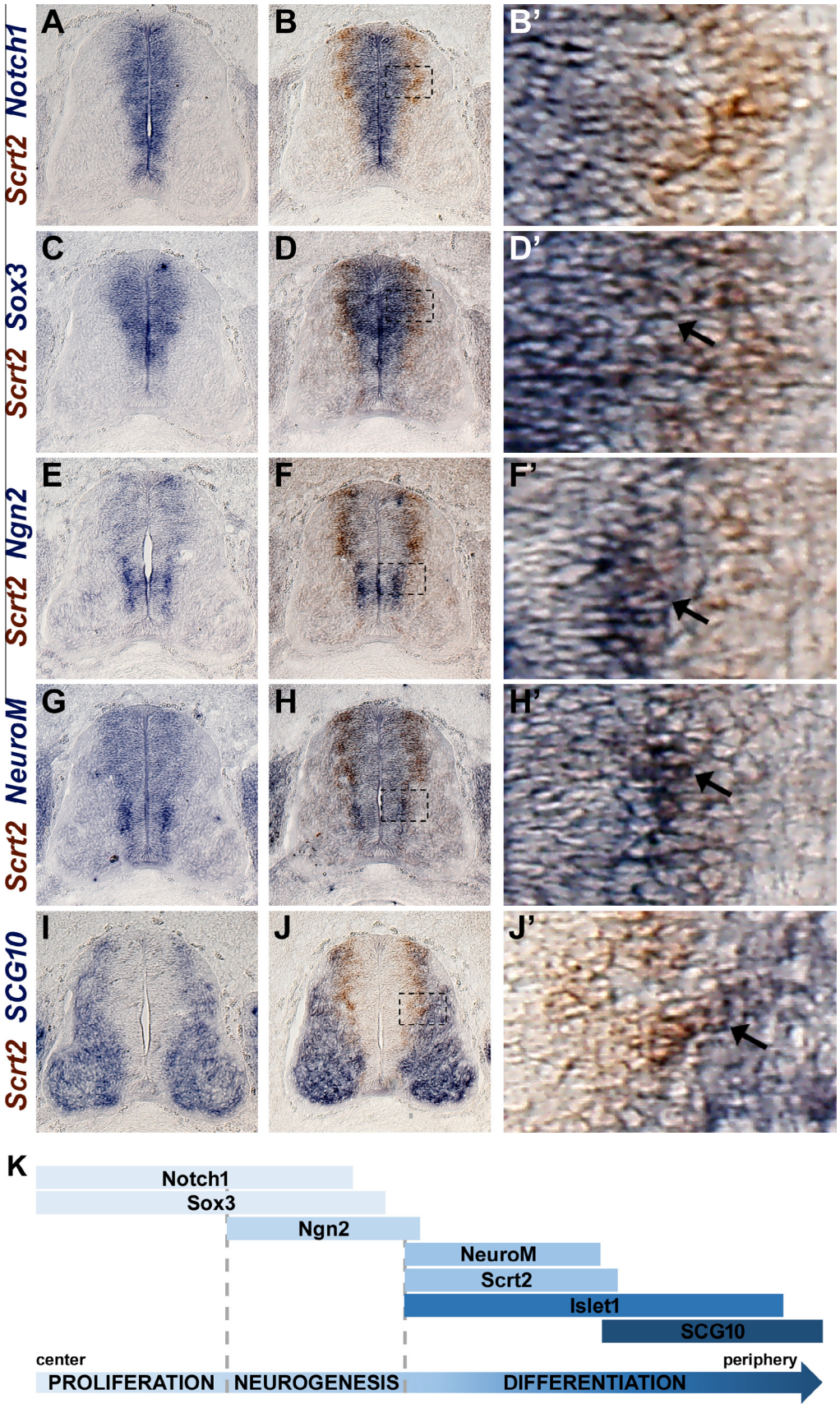

Fig. 5. Comparison between *cScrt2* expression and markers for different stages of neurogenesis and differentiation in the trunk spinal cord. (A–J) *In situ* hybridization in HH25 trunk spinal cord cross sections showing expression of *Notch1* (A), *Sox3* (C), *Ngn2* (E), *NeuroM* (G) and *SCG10* (I) or *cScrt2* combined with *Notch1* (B), *Sox3* (D), *Ngn2* (F), *NeuroM* (H) or *SCG10* (J). (B′, D′, F′, H′, J′) are magnifications of the delimited regions in (B, D, F, H, J), respectively. (K) Summary diagram representing a possible timeline of expression for Notch1, Sox3, Ngn2, NeuroM, Scrt2, Islet1 and SCG10 during neurogenesis and differentiation in the spinal cord. The left edge corresponds to the ventricular zone while the right edge corresponds to the marginal region of the neural tube.

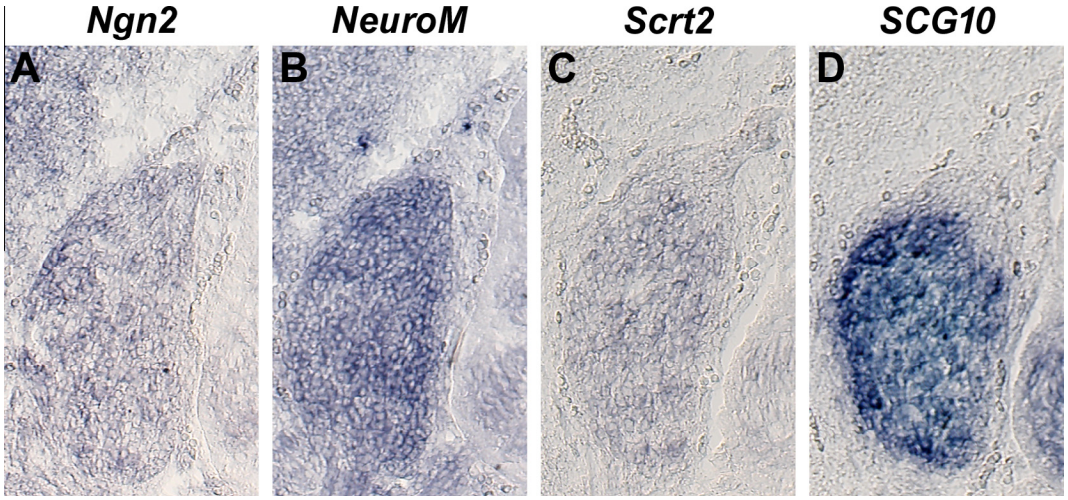

Fig. 6. Expression of neural differentiation factors in the DRG. (A–D) *In situ* hybridization showing expression of *Ngn2* (A), *NeuroM* (B), *cScrt2* (C) and *SCG10* (D) in HH25 DRG cross sections.

extends dorso-laterally encompassing the majority of the cells in this structure. At this stage, all the cells within the condensed ganglia have exited the cell cycle (McCabe et al., 2009; Blentic et al., 2011) and the presence of *cScrt2* throughout the trigeminal ganglia further supports the hypothesis that *Scrt2* is activated immediately after cell cycle arrest (Marín and Nieto, 2006; Paul et al., 2012).

If Scrt2 participates in the neural differentiation program of postmitotic cells, most likely it does so in conjunction with other genes involved in the early steps of differentiation. Scrt2 is a *bona fide* E-box-binding repressor (Nakakura et al., 2001a; Reece-Hoyes et al., 2009; Rodríguez-Aznar and Nieto, 2011; Paul et al., 2012). It is quite well established that a variety of E-box-binding proteins heterodimerize with transcription factors to regulate gene expression (Castro et al., 2006; Powell and Jarman, 2008). To further refine the localization of *cScrt2*-expressing cells in the developing neural tube relative to the neural differentiation transcription factor cascade and to identify potential binding partners, we performed double *in situ* hybridizations in HH25 embryos with transcription factors that are involved in cell cycle and in differentiation. As expected from our data above, the proliferative marker *Notch1* expression domain abutts but does not overlap with *cScrt2* (Fig. 5B). *cScrt2* expression domain is external to and overlaps partially with the external perimeter of the neural progenitor cell markers *Sox3* and *Ngn2/NEUROG2* (Fig. 5D and F and Supplementary Figure 1). However, *cScrt2* expression domain overlaps considerably with that of *NeuroM* (Fig. 5H and Supplementary Figure 1), which is expressed in early postmitotic neural progenitors (Roztocil et al., 1997). Similarly, in the DRG, *cScrt2* expression domain also coincides with that of *NeuroM* and *Ngn2* (Fig. 6A-C).

In the murine embryonic cortex Scrt2 expression also coincides with that of Ngn2 in the apical region of the ventricular zone (Paul et al., 2012). These expression data, together with data showing that Scrt2 overexpression decreases *Ngn2* expression in Xenopus neurulas (Paul et al., 2012) suggested that Scrt2 could downregulate *Ngn2* expression. In light of this, we performed overexpression of loss-of-function cScrt2 in chick neural tube using a truncated cScrt2 and a fusion protein with the activator domain of VP16. Neither of the two forms expanded *Ngn2* expression in chick neural tube (Supplementary Figure 2). A similar result was reported with Scrt2 gain-of-function in the mouse pallium, which also failed to alter local Ngn2 expression (Paul et al., 2012).

What then could be Scrt2 function? In the mouse, Scrt2 expression overlaps with that of neuronal differentiation marker beta-III tubulin in the pallium's intermediate zone. Consistent with a possible role in regulating neural differentiation, gain of Scrt2 function enhances mitotic exit and increases beta-III tubulin expression in the cortex (Paul et al., 2012). On the other hand, in the chick embryo the Scrt2 expression domain does not overlap with that of the other late differentiation marker *SCG10/STMN2* in the trunk neural tube (Fig. 5J and K). Furthermore, loss of cScrt2 function did not alter *SCG10* expression (Supplementary Figure 2). Finally, gain of cScrt2 function also did not alter the expression of the pan-neuronal markers HuC/D and Tuj1 (data not shown). Together, these data suggest that either cScrt2 is not directly involved in the later steps of neural differentiation in the chick spinal cord or that it is involved with neuronal subtype specification, the variations of which cannot be detected by variations in pan-neuronal markers expression.

Overexpression of NeuroM in the chick embryo also does not affect the expression of pan-neuronal markers. Instead, NeuroM indirectly induces expression of GDNF receptor alpha-1 (GFRalpha1), suggesting that NeuroM biases progenitor cells towards a specific subtype (Shimada et al., 2012). The expression pattern of *GFRalpha1* and that of *GFRalpha4* are very similar to that of both *NeuroM* and *cScrt2*. The original report on the dynamic pattern of *NeuroM* expression strongly suggests that its overlap with *cScrt2* occurs at other stages and tissues as well. Firstly, similar to *cScrt2*, *NeuroM* expression in the neural tube is first detected at HH17 in an intermediate domain between the ventricular zone and the outer mantle region and is excluded from BrdU-incorporating proliferating cells. Moreover, *NeuroM* expression follows a ventral to dorsal wave with the highest level of labeling around HH21 in the motor neuron domain, where it declines slowly and disappears in E6 embryos (Roztocil et al., 1997). In the developing DRG, *NeuroM* is also first detected around HH21, and becomes progressively restricted to the dorsomedial region (Roztocil et al., 1997). Finally, NeuroM is also present in the condensed trigeminal ganglia (Roztocil et al., 1997; Ohsawa et al., 2005).

Given their similarity in expression pattern, we investigated if cScrt2 regulated *NeuroM* expression. Reduction of cScrt2 activity did not affect *NeuroM* expression, suggesting that*NeuroM* is not under cScrt2 control (Supplementary Figure 2). Instead, it is very likely that both *cScrt2* and *NeuroM* are regulated by the homeodomain transcription factor Brn3a. Brn3a binds directly to *NeuroM* enhancer locus and represses *NeuroM* expression (Lanier et al., 2007; Dykes et al., 2011). Likewise, an *in silico* search for Brn-binding motifs in the mouse genome identified Scrt2 as a possible target gene (Castro et al., 2006).

In contrast to their similarity in expression pattern, Scrt2 and NeuroM have been ascribed with opposing molecular actions. While Scrt2 represses E-box-driven expression (Nakakura et al., 2001a; Reece-Hoyes et al., 2009; Rodríguez-Aznar and Nieto, 2011; Paul et al., 2012), NeuroM transactivates E-box-containing promoters (Roztocil et al., 1997). These data, taken together with the co-expression of *cScrt2* and *NeuroM* in a subset of neural progenitors shown here, raise the intriguing possibility that Scrt2 and NeuroM may act at similar levels but in an opposing manner in the neural differentiation hierarchy of neural subtype specification.

2. Experimental procedures

2.1. Cloning of chicken Scrt2

When we started this project, the chicken *Scrt2* homologue available in the NCBI database (XM_426994) was partial and lacked a portion of the 5′ coding sequence. Thus, we cloned the *cScrt2* 5′ UTR and coding terminal using nested RACE-PCR to amplify this sequence from RACE cDNA libraries from HH8, HH19 and HH24 (GeneRacer; Invitrogen). Gene-specific primers used in the nested RACE-PCR were cScrt2-Rv2: GAAGTAGCGGGCGGAGAAG GTGGA and cScrt2-Rv3: CCTGCTTCTTGTCGGGGTTGTAG.

The primers for full coding sequence were cScrt2-Fw1: CCCGCCATGCCCCGCTCCTT and cScrt2-Rv1: CTAGTTCCCTATTGCA-CAGCTGTGTTC. Expected amplicon was cloned in pGEM-T (Promega) and then subcloned in the expression vector pMES-GFP (Swartz et al., 2001). The final full-length clone was confirmed by sequencing (JN982016).

2.2. RNA in situ hybridization and immunohistochemistry

Embryos between developmental stages HH5 and HH28–29 were fixed in 4% paraformaldehyde in PBS overnight at 4 °C or 2 h at room temperature and submitted to hybridization as whole mounts (Figs. 2A–F and 4A-C and G–I), paraffin sections (10 μm, Fig. 2G-I) or cryosections (14-16 μm, Figs. 3, 4D–F, 5 and 6). *In situ* hybridization was performed at 70 °C using antisense RNA probes labeled with digoxigenin-11-UTP or fluorescein-12-UTP through *in vitro* transcription. For probe localization we used AP-conjugated antibodies and the signal was developed with NBT-BCIP, INT-BCIP or BM-Purple (Roche). The following probes were used: *SCG10/STMN2* (GenBank NM_205181, positions 54–885), *Notch1* (XM_415420, positions ~500–4502) (both kindly provided by P.K. Politis), *Ngn2/NEUROG2* (NM_204796, positions 101–646; kindly provided by E. Farley), *Sox3* (NM_204195, positions 572–1370), *NeuroM* (NM_205076, positions 654–1106) and *cScrt2* (JN982016, first 325 nucleotides or entire sequence). For double hybridization, after developing for the presence of the first probe the first antibody was inactivated in 0.1 M glycine, pH 2.2 for 10 min. For combined hybridization and immunofluorescence, the material was first hybridized and developed, washed and then subjected to standard immunolocalization protocols. Antibodies used were anti-BrdU (Accurate, 1:100), anti-pHH3 (Ser10, Millipore; 1:100) and anti-Islet1 (clone 39.4D5, DSHB; 1:50). The same sections were first captured in bright field for the hybridization signal and then in fluorescent microscopy. Afterwards, the two images were overlaid to facilitate visualization of the two domains. The contrast levels in the fluorescent images were heightened for better visualization and these were overlaid using the Blend tool in Adobe Photoshop.

2.3. BrdU labeling

Chicken eggs were incubated for 5 days and 20 μl of 2 mg/ml BrdU in Ringer's saline containing 0.05% Fast Green were injected in the space surrounding the embryo and delimited by the vitelline membrane. Eggs were closed and reincubated for 1 h. Thereafter, embryos were collected and fixed overnight in 4% PFA in PBS at 4 °C before being processed for cryosectioning. With this protocol, the BrdU-positive region was clearly distinct from the phospho-Histone H3-positive region.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gep.2013.03.004.

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