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Evolution of Developmental Control Mechanisms

Trunk lateral cells are neural crest-like cells in the ascidian *Ciona intestinalis*: Insights into the ancestry and evolution of the neural crest

William R. Jeffery [a,b,*], Takuto Chiba [a,1], Florian Razy Krajka [b], Carole Deyts [b], Nori Satoh [c], Jean-Stéphane Joly [b]

[a] Department of Biology, University of Maryland, College Park, MD 20742, USA
[b] INRA U1126 Group, Institut de Neurosciences A. Fessard, CNRS, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette, France
[c] Department of Zoology, University of Kyoto, Kyoto, Japan

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ABSTRACT

Neural crest-like cells (NCLC) that express the HNK-1 antigen and form body pigment cells were previously identified in diverse ascidian species. Here we investigate the embryonic origin, migratory activity, and neural crest related gene expression patterns of NCLC in the ascidian *Ciona intestinalis*. HNK-1 expression first appeared at about the time of larval hatching in dorsal cells of the posterior trunk. In swimming tadpoles, HNK-1 positive cells began to migrate, and after metamorphosis they were localized in the oral and atrial siphons, branchial gill slits, endostyle, and gut. Cleavage arrest experiments showed that NCLC are derived from the A7.6 cells, the precursors of trunk lateral cells (TLC), one of the three types of migratory mesenchymal cells in ascidian embryos. In cleavage arrested embryos, HNK-1 positive TLC were present on the lateral margins of the neural plate and later became localized adjacent to the posterior sensory vesicle, a staging zone for their migration after larval hatching. The *Ciona* orthologues of seven of sixteen genes that function in the vertebrate neural crest gene regulatory network are expressed in the A7.6/TLC lineage. The vertebrate counterparts of these genes function downstream of neural plate border specification in the regulatory network leading to neural crest development. The results suggest that NCLC and neural crest cells may be homologous cell types originating in the common ancestor of tunicates and vertebrates and support the possibility that a putative regulatory network governing NCLC development was co-opted to produce neural crest cells during vertebrate evolution.

Introduction

The neural crest is responsible for the formation of many different cell types during vertebrate development (Hall, 1999; Le Douarin and Kalcheim, 1999). Neural crest precursors arise between the neural and non-neural ectoderm, delaminate during neural tube formation, and migrate through prescribed pathways to their final sites of differentiation. Neural crest development occurs in several steps (Nieto, 2001; Sauka-Spengler et al., 2007). First, inductive signals, such as FGF, BMP, and Wnt, establish the neural plate border and activate the transcription factors Pax 3/7, Dlx3/5, Zic, and Msx1/2. The latter transcription factors in turn activate the expression of downstream transcription factors, such as Snail, FoxD3, and SoxE, which specify neural crest identity at the neural plate border and trigger the expression of effector genes responsible for migratory activity and pluri potency of neural crest cells.

Neural crest cells are ubiquitous in vertebrates, including the basal agnathan groups (McCauley and Bronner-Fraser, 2003; Ota et al., 2007). It has been argued that the appearance of the neural crest was a major step in the evolution of vertebrate complexity, particularly in the cranium (Gans and Northcutt, 1983; Shimeld and Holland, 2000; Northcutt, 2005). The presence of complex populations of neural crest cells in all vertebrates raised questions concerning their evolutionary origin and spearheaded searches for similar cell types in invertebrate chordates. In cephalochordates and ascidians, some of the early regulatory genes typical of vertebrate neural crest cells are expressed along the neural plate border (Ma et al., 1996; Corbo et al., 1997; Wada et al., 1997; Sharman et al., 1999; Holland and Holland, 2001; and others). However, neither the expression of downstream genes in the pathway leading to neural crest development nor delamining and migratory cells were observed in this region (Wada et al., 2001; Baker and Bronner-Fraser, 1997), which supported the hypothesis that neural crest cells first appeared during vertebrate evolution.

Recent phylogenomic evidence suggests that tunicates (including ascidians), rather than cephalochordates, may be the sister group of vertebrates (Bourlat et al., 2006; Delsuc et al., 2006; Vienne and Pontarotti, 2006). Accordingly, a cell type with features resembling

* Corresponding author. Department of Biology, University of Maryland, College Park, MD 20742, USA. Fax: +1 301 314 9358.
E-mail address: Jeffery@umd.edu (W.R. Jeffery).
[1] Present address: Department of Biological Sciences, Vanderbilt University, Nashville, TN 37232 USA.

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vertebrate neural crest cells (neural crest-like cells or NCLC) was discovered in the ascidian *Ecteinascidia turbinata* (Jeffery et al., 2004). *Ecteinascidia* NCLC resemble vertebrate neural crest cells in (1) showing long distance migration from the dorsal midline, (2) expression of HNK-1 antigen, a neural crest marker (Hall, 1999; Le Douarin and Kalcheim, 1999), and a gene homologous to *zic2*, which is crucial for neural crest development (Elms et al., 2003), and (3) differentiation into body pigment cells, one of the major fates of vertebrate neural crest cells. It was subsequently shown that these HNK-1 positive NCLC are found in diverse ascidian species (Jeffery, 2006). Thus, it was concluded that ascidian NCLC and neural crest cells have many similarities and share at least one common derivative: body pigment cells (Jeffery, 2007).

Here, we address two issues required to understand the extent to which ascidian NCLC are related to neural crest cells: their (1) embryonic origin and (2) expression of neural crest related genes. We have selected the solitary ascidian *Ciona intestinalis* for this analysis. First, embryonic cell lineages are well known in *Ciona*, and some of these have been traced to specific adult tissues (Tukuoka et al., 2005). Knowledge of cell lineage is critical in determining the origin of NCLC. Second, the existence of a large EST collection (Satou et al., 2002a) makes *Ciona* the species of choice for characterizing NCLC gene expression patterns.

Materials and methods

Animals and embryos

C. intestinalis were purchased from Station Biologique, Roscoff, France and maintained at Gif-sur-Yvette, France or collected and maintained at the Marine Biological Laboratory, Woods Hole MA, USA. Eggs and sperm were obtained from dissected gonoducts. Cross insemination was carried out, the chorion was removed from fertilized eggs with protease (Mita-Miyazawa et al. 1985), and dechorionated embryos were raised at 15–18 °C in Millipore filtered sea water (MFSW) in Petri dishes coated with 1% agarose.

Cleavage arrest experiments

In cleavage arrest experiments, embryos or hatched larvae were treated with 3 μg/ml cytochalasin B (Sigma, St. Louis, MO) in MFSW beginning at various stages of development and incubated in the inhibitor until untreated controls reached 6 h post-hatching (hph). The cleavage arrested embryos and controls were fixed in 4% paraformaldehyde (PF) and processed for HNK-1 immunoreactivity as described below.

HNK-1 immunoreactivity

Fixation and immunostaining with HNK-1 monoclonal antibody (BD Biosciences Pharingen, San Jose, CA) was carried out as described by Jeffery et al. (2004) and Jeffery (2006). Alternatively, specimens were fixed in 4% PF in MFSW-0.1% Tween (pH 8.2; overnight), stained with antibody diluted 1:2 in PBS, and antigen–antibody complexes were detected with biotinylated goat anti-mouse IgM secondary antibody (1:200 in PBS; Vector Laboratories, Burlingame, CA). Both procedures gave similar results. Antigen–antibody complexes were detected using the ABC Peroxidase Kit (Vector Laboratories). Controls treated with non-immune mouse serum (1:10 dilution in PBS; AbCam, Cambridge, MA) did not exhibit peroxidase labeled cells. The stained specimens were viewed by light microscopy and photographed.

Genes and in situ hybridization

The cDNA clones used for *in situ* hybridization were obtained from the *Ciona* Gene Collection release 1 (Satou et al., 2002a) and are listed in Table 1. To obtain the *Ciona TLC2* cDNA, the DNA sequences homologous to *Halocynthia roretzi* (*Hr-TLC2*; GenBank BAB20902) was identified in the ghost database by BLAST, and the corresponding cDNA was then selected from the gene collection.

Table 1
List of *Ciona intestinalis* genes tested for expression in the A7.6/TLC lineage by *in situ* hybridization

Gene name	JGI model	Clone ID	Vertebrate orthologue
Ci-ap2-like1		cie009n12	AP-2
Ci-ap2-like2		cilv008i03	AP-2
Ci-cadherin2a	ci0100146860	cinc012h04	Cadherin II
Ci-collagen2a		cilv002e01	Collagen II
Ci-Dll-B		cicl022f04	dlx3
Ci-emc		cicl010f24	Id
Ci-foxD-b		citb008o13	foxD3
Ci-macho1	ci0100150779	cieg016n12	Zic
Ci-msxb		cign067l18	msx1/2
Ci-myc	ci0100150934	cieg017l16	c-*myc*
Ci-NoTrlc	ci0100140298	citb018l16	–
Ci-pax3/7		cign078f09	pax3, pax7
Ci-rhoABC	ci0100142667	cilv044l14	rho A/B/C
Ci-snail		cibd020p17	Snail, slug
Ci-TLC-2	ci0100143931	cilv075a14	–
Ci-twist-like1		cicl029j13	Twist
Ci-twist-like2		cicl020p07	Twist
Ci-zicL		cicl002e04	Zic

Whole mount *in situ* hybridization was done on specimens fixed in 4% PF and processed as described above, using digoxigenin labeled RNA probes, and NBT-BICP staining according to the procedures of Wada et al. (1995) and Satou and Satoh (1997) or Christiaen et al. (2002). The stained specimens were viewed by light microscopy and photographed.

Results

HNK-1 expression during development

The pattern of HNK-1 expression was determined in embryos, larvae, and juveniles. No HNK-1 positive cells were detected in embryos from the early cleavage through the tailbud stages (see Fig. 1A for a gastrula stage). In a few mid to late tailbud embryos, weak HNK-1 staining was seen in clusters of cells on the dorsal side of the posterior trunk (Fig. 1B). In the majority of embryos, however, strong HNK-1 staining began at the time of larval hatching in scattered cells located lateral and posterior to the sensory vesicle (Fig. 1C). In swimming larvae, the labeled cells appeared to migrate anteriorally toward the developing oral siphon (Figs. 1E, G; and see below). HNK-1 staining initially appeared in the central region of each cell, presumably in perinuclear vesicles, and later spread to mark the cell boundaries (Figs. 1D, F, H). Serial sectioning showed HNK-1 stained cells concentrated on the dorsal side of the swimming larva (Figs. 1I–N). At 6 hpf, HNK-1 stained cells were present throughout the trunk but most prevalent in the oral siphon primordium, the atrial siphon primordia, and near the developing cerebral ganglion (see Fig. 2A).

In post-metamorphic juveniles, HNK-1 expressing cells were observed in the following locations: the developing siphon primordia and siphons (Figs. 1O, P, S, T), the first two branchial gills slits (Fig. 1S and data not shown), a region dorsal and lateral to the developing cerebral ganglion (Fig. 1V), and in some but not all animals in the gut (data not shown) and endostyle (Figs. 1S, U). Rarely, HNK-1 staining was also seen in cells closely associated with the ocellus, which are lens cells (Figs. 1Q, R). Most individuals, however, showed no HNK-1 staining within the sensory vesicle, suggesting that lens staining is transitory. The tunic cells, which stain with HNK-1 antibody in some ascidian species (Jeffery, 2006), were not HNK-1 positive in juvenile *Ciona* (Figs. 1S, T, U). By 10 dph HNK-1 staining could not be detected in juveniles (data not shown). The results show that HNK-1 positive

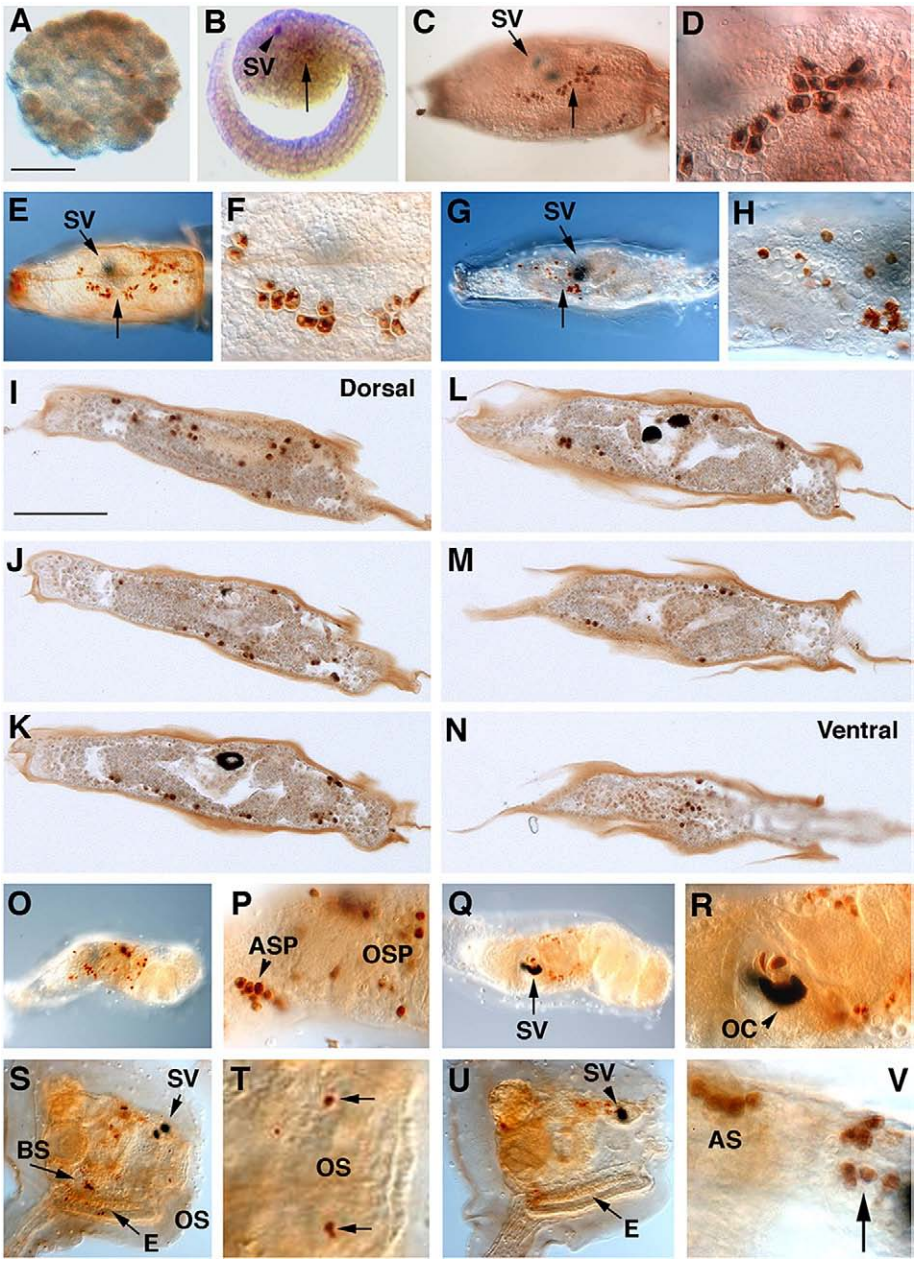

Fig. 1. Expression of HNK-1 antigen during embryonic and adult development. (A) HNK-1 antigen is not detected in a mid-gastrula stage embryo. Dorsal view. (B) HNK-1 antigen is expressed weakly (arrow) in a few cells located lateral and posterior to the sensory vesicle (SV) in a mid to late tailbud embryo. (C–H) HNK-1 antigen is strongly expressed (arrows) in cells anterior, lateral, and posterior to the sensory vesicle (SV) between hatching (C, D), the beginning of larval swimming (E, F), and elongation of the trunk (G, H). Panels D, F, and H represent higher magnifications of HNK-1 positive cells in panels C, E, and G respectively. Panels C–H are dorsal views with anterior on the left. (I–M) A serially sectioned swimming larva showing HNK-1 stained cells concentrated on the dorsal side of the trunk. Sections are shown in sequence from dorsal most (I) to ventral most (N) with anterior on the left. (O–V) HNK-1 expression continues during metamorphosis (2 dpf) (O–R) and in juveniles (5 dpf) (S–V). Panels P, R, T, and V represent higher magnifications of HNK-1 stained cells in panels O, Q, S, and W, respectively. Panel P is focused on HNK-1 stained cells near the atrial (ASP) and oral (OSP) siphon primordia. Panel Q is focused on HNK-1 stained cells near the ocellus (OC). Panel T is focused on HNK-stained cells in the oral siphon (OS). Panel V is focused on HNK-1 stained cells in the atrial siphon (AS) and posterior to the sensory vesicle (arrow). E: Endostyle. BS: Branchial gill slit. Scale bar: 50 μm: magnification is the same in panels A, B, C, E, G, O, Q, S, and U with approximately 4× increase in panel D, F, H, P, R, T, and V. Scale bar 30 μm in panel I; magnification is the same in panels I–N.

cells appear in the larval trunk about the time of hatching, then apparently disperse and become localized in different tissues and organs of the post-metamorphic juvenile, and finally lose HNK-1 reactivity, presumably as they differentiate.

The broad distribution of HNK-1 positive cells in the swimming larva can be interpreted in one of two ways. First, HNK-1 expression could be initiated at different times at various locations in the larva. Second, HNK-1 expression could be initiated in a defined region and the antigen expressing cells could later migrate into other regions of the larva. To test these alternatives, larvae were treated with the cell division/migration inhibitor cytochalasin B. Cytochalasin B treatment began shortly after hatching, and after 6 h of treatment the distribution of HNK-1 positive cells was compared to untreated control larvae (Fig. 2). As described above, the controls showed HNK-1 positive cells concentrated in the oral and atrial siphon primordia and near the sensory vesicle (Fig. 2A). In contrast, the cytochalasin-treated larvae showed HNK-positive cells posterior and lateral to the sensory vesicle (Fig. 2B), the same place HNK-1 labeling was detected in mid to late tailbud embryos and immediately after hatching (Figs. 1B–D; data not shown). In addition, HNK-1 positive cells were less numerous and larger in cytochalasin-treated larvae than in the untreated controls (Fig. 2), suggesting that they normally undergo division in swimming

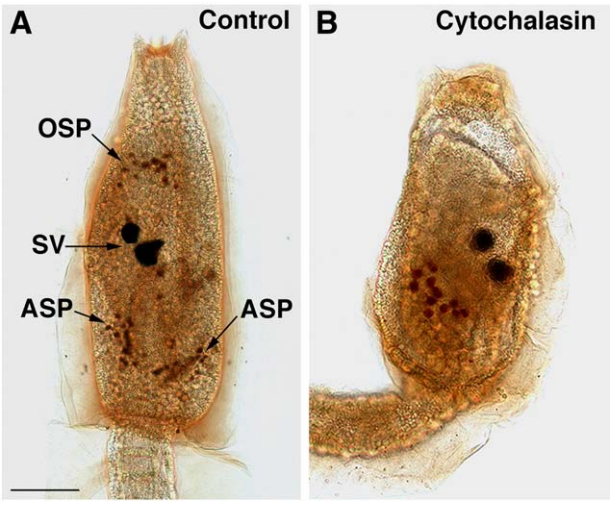

Fig. 2. Migration of HNK-1 positive cells determined by cytochalasin B inhibition. (A) Control untreated larva at 6 hph with focus on small HNK-1 positive cells in the oral (OSP) and atrial (ASP) siphon primordia anterior and posterior, respectively, to the sensory vesicle (SV). HNK-1 stained cells adjacent to the sensory vesicle are also present but out of focus. A larva from the same clutch as A that was treated with cytochalasin B from hatching to 6 hph showing large HNK-1 positive cells restricted to a position in the trunk posterior to the sensory vesicle. Scale bar: 30 μm: magnification is the same in panels A and B.

larvae. The results suggest that HNK-1 positive cells initially appear at the posterior margins of the sensory vesicle, then migrate through the larval trunk, and are eventually distributed in many different locations in the juvenile.

Embryonic origin of HNK-1 positive cells

Because HNK-1 expression did not appear until about the time of larval hatching, the embryonic origin of HNK-1 positive cells could not be determined by direct inspection. Instead, the cleavage arrest method (Whittaker, 1973), which permits blastomeres expressing a molecular marker to be positively identified in early embryos, was used to determine the lineage of HNK-positive cells. Cytochalasin B treatment was initiated after every cleavage up to the 64-cell stage, during gastrulation and neurulation, and at several times during larval tail development. Incubation with the drug was continued until untreated controls hatched and became swimming larvae, and the arrested embryos and controls were fixed and assayed for HNK-1 expression.

HNK-1 expression was first detected at the late gastrula stage in two cells located near the lateral lips of the blastopore (Fig. 3A). The location of the stained cells earmarked them as A7.6 blastomeres (Figs. 3A, B). The HNK-1 stained cells became internalized late during gastrulation and were positioned immediately under the lateral margin of the neural plate, which is marked by melanized precursors of the otolith and ocellus (Figs. 3C, D). Prior to neurulation, the neural plate extends posteriorly on the dorsal surface of the embryo to close the blastopore. Sections through cleavage arrested embryos at this stage showed HNK-1 stained cells (now represented by two cells in some section planes) located immediately below and lateral to the edge of the neural plate, as defined by the melanized otolith/ocellus precursors (Figs. 3E, F).

The A7.6 cells are progenitors of the trunk lateral cells (TLC), which flank the dorsal midline of the posterior trunk, adjacent to the developing sensory vesicle (Fig. 1I; Mita-Miyazawa et al., 1987). In *H. roretzi*, the A7.6 cells divide four times from the 110-cell stage to the mid-tailbud stage, producing a total of 32 TLC, and no further cell division occurs until after hatching (Nishida and Satoh, 1985). This pattern of cell division distinguishes the TLC from the other two types of mesenchyme cells located ventral to them in the larval trunk, which proliferate throughout the tailbud stages. Cleavage arrest at various times between the neurula and swimming larval stages showed that the number of HNK-1 stained cells resemble the cell division pattern of the TLC (although they do not reach the final number seen in *Halocynthia*) and not the more ventral clusters of mesenchyme cells (Table 2; Figs 1G, H, J). To further compare TLC and HNK-1 stained cells, we determined the expression pattern in tailbud embryos of the *Ciona TLC-2* gene, a specific TLC marker in *Halocynthia* (Takahashi and Satoh, 2001). *In situ* hybridization showed *TLC-2* expressing cells located in positions similar to HNK-stained cells in the dorsal posterior region of the developing trunk (compare Figs. 3G, H, J and K, L). In summary, the results show that HNK-1 stained cells and therefore NCLC are members of the A7.6/TLC lineage.

Expression of neural crest regulatory network genes in the A7.6/TLC lineage

The results suggest that A7.6/TLC lineage cells have features resembling neural crest cells: HNK-1 expression, localization at the edge of the neural plate/CNS, and migratory activity. The genes involved in vertebrate neural crest development have been ordered in a putative four-tiered hierarchy: the neural crest regulatory gene network (NC-RGN) (Meulemans and Bronner-Fraser, 2004, 2005). To determine whether NC-RGN genes are expressed in the A7.6/TLC lineage, and if so, which tiers they represent, we surveyed the expression of orthologous genes by *in situ* hybridization. The expression patterns of some these genes were already known from previous studies and *in situ* hybridizations in the *Ciona* ghost database (http://ghost.zool.kyoto.u.ac.jp/indexrl.html), although expression in the A7.6/TLC lineage was sometimes not completely resolved. The results are shown in Fig. 4 and summarized in Fig. 5.

The first and second tiers of the NC-RGN involve patterning signals (FGF, Notch, BMP, and Wnt) and transcription factors that refine and specify the neural plate border, respectively. The importance of the FGF, Notch, and Wnt/β-catenin signaling systems in A7.6/TLC induction has been previously demonstrated (Kawaminani and Nishida, 1997; Shimauchi et al., 2001; Imai et al., 2002a, 2003, 2006). Ascidian homologues of NC-RGN tier 2 genes (*msx1/2*, *pax7*, *dlx3*, and *zic*) are expressed along the margin of the neural plate (Ma et al., 1996; Wada et al., 1997; Caracciolo et al., 2000; Imai 2002b; Satou et al., 2002b; Gostling and Shimeld, 2003; Russo et al., 2004). We investigated the *Ciona* orthologues of *msx* (*Ci-msxb*), *pax3* and *7* (*Ci-pax3/7*), *dlx3* (*Ci-dll3*), and *zic* (*Ci-zicL* and *Ci-macho*) in the A7.6/TLC lineage. The results showed that none of these NC-RGN genes were expressed in the A7.6/TLC lineage (Fig. 5), although expression occurred in other regions of the embryo, including the neural plate border and developing CNS.

The third tier of the NC-RGN consists of transcriptional regulators, such as Snail/Slug, Id, FoxD3, Twist, AP2, and c-Myc, which are activated downstream of tier 2 genes to specify neural crest fate in vertebrate embryos. The *Ciona* orthologues of some of these genes had been examined previously, but others had not been studied. We investigated the expression patterns of the *Ciona* orthologues of these genes from the 64-cell to the swimming larval stage (Fig. 5). Previous studies (Fujiwara et al, 1998) showing *Ci-snail* gene expression in tail muscle cells but not in the A7.6/TLC lineage were confirmed. Similarly, the *id* orthologue *Ci-emc* was expressed in neural plate and CNS cells but not in the A7.6/TLC lineage (Figs. 4E, F). We confirmed that the *foxD3* orthologue *Ci-foxDb* is expressed in A6.3 cells (the immediate precursors of the A7.6 cells) at the 64-cell stage (Imai et al., 2002c) but not in the A7.6/TLC lineage later in development (Figs. 4G, H). *Ciona* has two *twist* orthologues, the paralogous *Ci-twist-like 1* and *Ci-twist-like 2* genes. Both *twist* genes are expressed in the A7.6/TLC lineage, as well as in the mesenchyme lineages derived from the B7.5 and B8.5 blastomeres (Imai et al., 2003; Tokuoka et al., 2004; Imai et al., 2006); this was also confirmed in this study (Figs. 4C, D, 5).

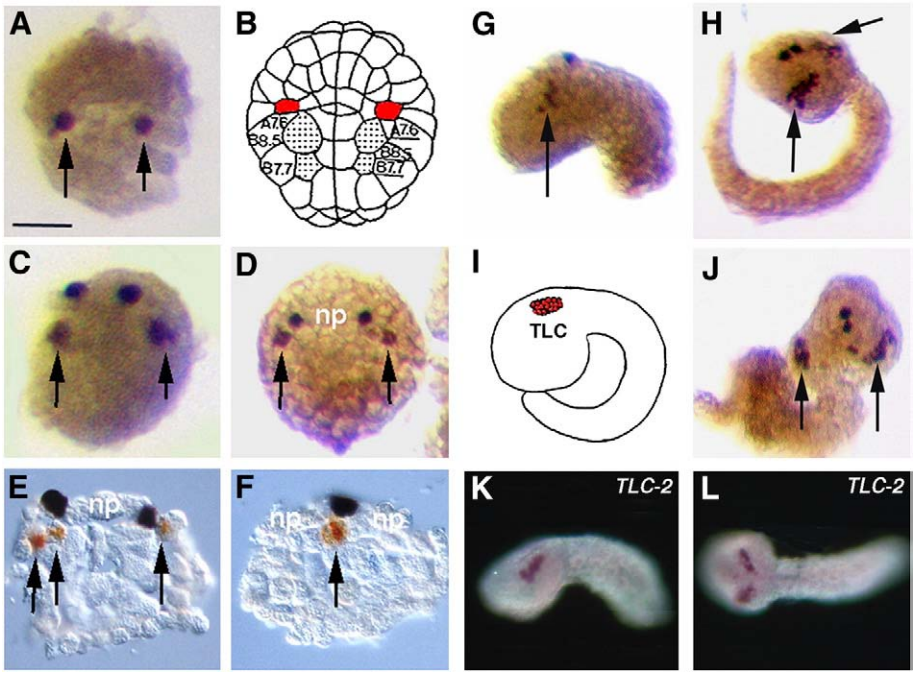

Fig. 3. Embryonic origin of HNK-1 positive cells determined by cleavage arrest. (A, C–H, J) Embryos subjected to cleavage arrest at the late gastrula (A), neural plate (C–F), and early (G), mid (H), and late (J) tailbud stages showing HNK-1 expression in a pair of A7.6 (A, C–F) cells and later in TLC (G, H, J) (arrows). Note the position of melanin pigmented cells, the otolith and ocellus precursors, marking the lateral margins of the neural plate (np) in panels C–F. (E, F). Cross (E) and sagittal (F) sections through the anterior–posterior axis in the plane of one or both melanin pigment cells and the neural plate. HNK-1 stained cells lie in the cell layer immediately below the margins of the neural plate (arrows). (B) Diagram of a gastrula embryo viewed dorsally showing the A7.6 cells in red and the B8.5 and B7.7 mesenchyme cells posterior to them. (I) Diagram of mid-tail bud stage embryo viewed laterally showing the position of trunk lateral cells (TLC) in red. (K, L) *In situ* hybridization showing *TLC-2* expression in TLC of mid-tailbud embryos viewed laterally (K) and dorsally (L). Scale bar in panel A is 40 μm; magnification is the same in all frames.

The expression patterns of *No trunk lateral cells* (*Notlc*) (Figs. 4A, B), a TLC marker (Imai et al., 2003), and *twist-like-2* (Figs. 4C, D) were compared to additional tier 3 and 4 genes in the A7.6/TLC lineage. Accordingly, we found that the A7.6/TLC lineage expressed one of the two AP2 genes, *Ci-ap2-like1* (Fig. 4I), but not *Ci-ap2-like2* (Fig. 5), and *Ci-myc* (Figs. 1K, L), the *c-myc* orthologue (Satou et al., 2003). *Ci-myc* was expressed in the neural plate at the neurula stage and the A7.6/TLC lineage from the late gastrula through the tailbud stages (Figs. 4G, H; 5). Furthermore, *Ci-ap2-like2* was expressed in A7.6 cells at the neurula stage, but expression was not detectible in TLC during the tailbud stages (Figs. 4I–J; 5). Comparison with *Ci-twist-like-2*, which is expressed in all three mesenchymal lineages, and *Notlc*, which is expressed in the A7.6/TLC lineage at the stage shown in Fig. 4, suggests that all mesenchyme lineages express *Ci-ap2-like1* and *Ci-myc*. The results indicate that orthologues of five NC-RGN tier 3 genes, *foxDb*, *twist-like1|2*, *ap2-like1*, and *myc*, are expressed in A7.6/TLC or its immediate precursors during *Ciona* development.

The fourth tier of the NC-RGN consists of the so-called neural crest effector genes, including *collagen 2*, *cadherin 2*, and *rhoA or B*, which control the delamination and migration of neural crest cells. We determined the expression patterns of the *Ci-collagen 2a*, *Ci-cadherin 2a*, and *Ci-rhoABC* (Sasakura et al., 2003) genes during development (Figs. 4M–P; 5). The *Ci-collagen 2a* gene was expressed in the developing tail but not in the A7.6/TLC lineage (Fig. 5). Although low levels of *Ci-cadherin2a* and *Ci-rhoA/B/C* were seen throughout the embryo, their expression levels were enhanced in the A7.6/TLC lineage and other mesenchyme cells (Figs. 5M–P).

In summary, *in situ* hybridization showed that the A7.6/TLC lineage expresses seven of the sixteen surveyed NC-RGN genes: none of the neural crest border genes, five of eight neural crest specifier genes, and two of three neural crest effector genes.

Table 2
Number of HNK-1 stained cells in cleavage arrested embryos and larvae

Beginning of cleavage	*n*	HNK-1 stained arrest cells/embryo +/− SD
Neural plate	16	2.8 +/− 1.0
Early TB	18	12.2 +/− 1.7
Mid TB	15	28.5 +/− 5.2
Late TB	19	28.7 +/− 3.7
Hatched larva	13	29.4 +/− 5.1

Discussion

The present study addressed two critical issues concerning NCLC development in *C. intestinalis*. First, what is the embryonic origin of NCLC? We demonstrate that NCLC are members of the A7.6/TLC lineage, the most dorsal of the three migratory mesenchymal lineages in ascidian embryos. Second, which if any of the NC-RGN genes are expressed in NCLC? We show that NCLC express some of the downstream neural crest specifier and effector genes of the NC-RGN, but none of the neural plate border specifying genes. Our results have implications regarding the chordate ancestry of neural crest cells and possible NC-RGN re-wiring during vertebrate evolution.

TLC are NCLC in Ciona

The restriction of HNK-1 expression to larvae and juveniles obscured the embryonic origin of NCLC in previously studied ascidian species, including *C. intestinalis* (Jeffery, 2006). In lieu of being able to directly trace the lineage(s) of HNK-1 positive cells, the cleavage arrest method was used to identify their progenitors in early *Ciona* embryos. *Ciona* is well suited for this analysis because lineage-founding cells are clearly visible on the surface of the embryo up to the gastrula stage. Accordingly, we traced HNK-1 expression to the paired A7.6 blastomeres, which are located on the lateral margins of the

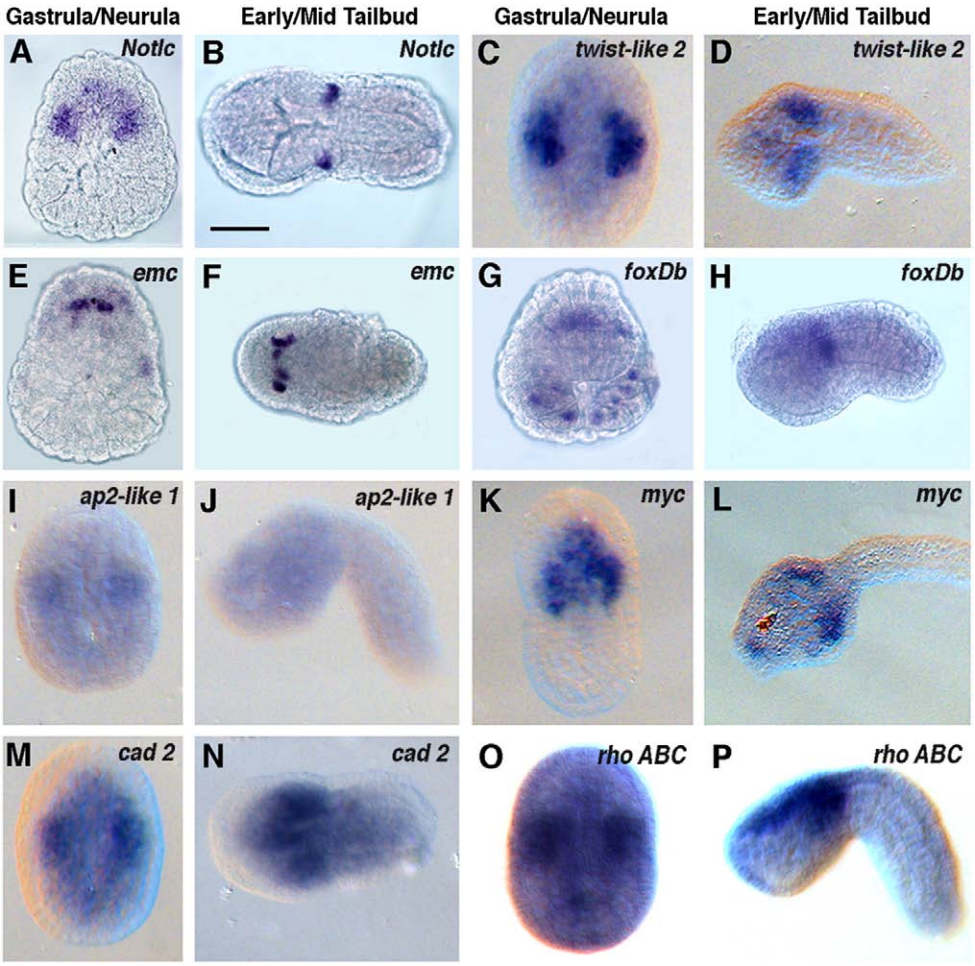

Fig. 4. Expression pattern of *Ciona* NC-RGN gene orthologues in the A7.6/TLC lineage determined by *in situ* hybridization. First and third column from the left: neurula stages with anterior at the top. Second and fourth column from the left: early or mid-tailbud stages with anterior on the left. (A, B) *Notlc* gene expression. (C, D) *Ci-twist-like 2* gene expression. (E, F) *Ci-emc* gene expression. (G, H) *Ci-foxD3* gene expression. (I, J) *Ci-ap2-like1* gene expression. (K, L) *Ci-myc* gene expression. (M, N) *Ci- cadherin* 2 (*cad 2*) gene expression. (O, P) *Ci-rhoABC* gene expression. Scale bar in panel B is 30 μm; magnification is the same in each frame.

blastopore from the 110-cell to the mid-gastrula stage. The A7.6 cells are progenitors of TLC, one of the three types of migratory mesenchymal cells in *Ciona* embryos (Tokuoka et al., 2004, 2005). The other two mesenchymal cell types, which arise from the B8.5 and B7.7 blastomeres and are positioned ventral to the TLC, do not express the HNK-1 antigen.

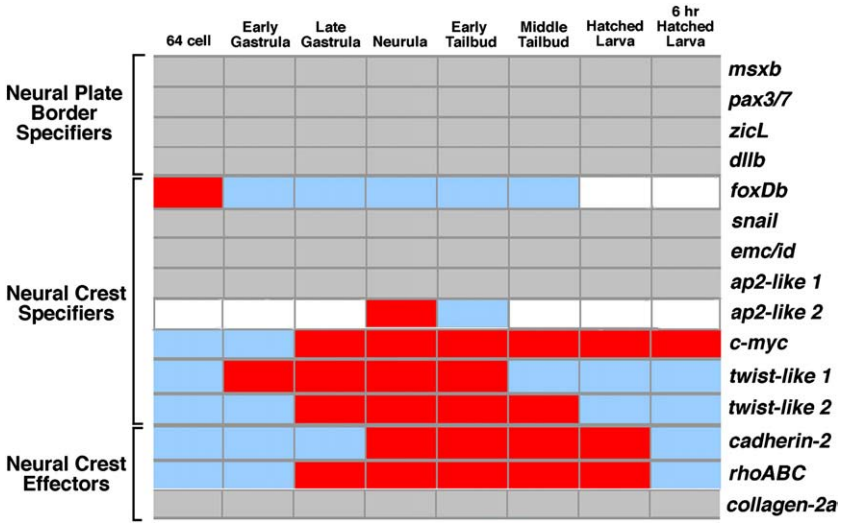

Fig. 5. Summary of the expression patterns of the *Ciona* orthologues of vertebrate neural crest related genes from the 64-cell through the 6 hr larva stage. Red and blue rectangles represent genes expressed in the 7.6/TLC lineage with red indicating a stage that the gene is expressed and blue a stage in which it is not expressed. Gray rectangles represent genes expressed at a given stage but not in the A7.6/TLV lineage. White rectangles indicate stages that were not investigated. Results are compiled from *in situ* hybridization data, some which is shown in Fig. 4.

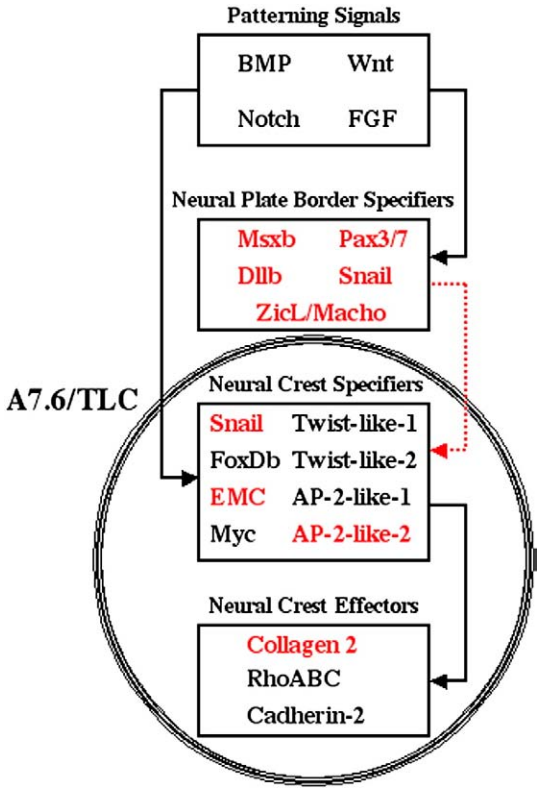

Fig. 6. Diagram illustrating the hierarchical relationship of the *Ciona* orthologues of vertebrate NC-RGN genes in the A7.6/TLC lineage. Boxes indicate hypothetical tiers of the hierarchy with the black arrow showing hypothetical interactions in both ascidians and vertebrates and the red dotted line arrow showing the interaction that is thought to be missing in ascidians and co-opted in the vertebrate lineage. Genes in black represent those expressed in the A7.6/TLC lineage or in cells that induce this lineage. Genes in red represent those not expressed in the A7.6/TLC lineage.

Several other findings support the identification of TLC as NCLC. First, in *Halocynthia* four cell divisions in the A7.6 lineage between gastrulation and the mid-tailbud stage produce two bilateral clusters of 16 cells, a total of 32 TLC (Nishida and Satoh, 1985). Cell proliferation then ceases until after hatching. In contrast, the two other types of mesenchyme cells continue to proliferate through the period of tail morphogenesis. We showed that *Ciona* HNK-1 positive cells resemble TLC in undergoing about four divisions to produce a total of 29 cells prior to the mid-tailbud stage, and they do not divide again until the swimming larval stage. Second, HNK-1 positive cells (in cleavage arrested embryos) are localized in the same positions as TLC in the posterior region of the trunk, bilateral to the developing cerebral vesicle precursor. This location was confirmed by following the expression of *TLC-2*, a specific TLC marker (Takahashi and Satoh, 2001). Third, the timing of migration and dispersed distribution of HNK-1 positive cells is very similar to TLC. Finally, some of the tissues and organs in which HNK-1 positive cells become localized in juveniles, namely the oral siphon and branchial gill slits, are the same as TLC derivatives (Hirano and Nishida, 1997; Tokuoka et al., 2005).

According to previous studies, the so-called blood cells are major TLC derivatives. Ascidian blood cells are a very heterogeneous population of five or more different cell types (Satoh, 1994). In the swimming tadpole larvae, during metamorphosis, and in adults, these cells move through extracellular spaces along with other migratory cells (Davidson et al., 2003). Indeed, some "circulating" blood cells, namely the body pigment cells, can be stationary and embedded in various juvenile and adult tissues and organs. More detailed cell tracing and histological studies will be necessary to determine whether *Ciona* NCLC are restricted to a single fate or are pluri potent, like vertebrate neural crest cells. Along a similar line, it is still questionable whether all *Ciona* NCLC have been discovered: if some NCLC do not express the HNK-1 antigen, express HNK-1 at very low levels, or are unable to express the HNK-1 antigen during cleavage arrest, they would have remained unidentified in the present analysis. Likewise, all HNK-1 positive cells may not be NCLC; although we believe that vast majority of HNK-1 positive migratory cells are NCLC. An exception, however, may be lens cells in the larval sensory vesicle.

The *Ciona* tadpole is relatively small and streamlined, consisting of only about 3000 cells (Satoh, 1994). The larvae of many other ascidian species, particularly *Ecteinascidia* and other colonial forms, are much larger and more complex. From existing phylogenetic evidence, it cannot be decided whether ascidian species with small simple larvae are basal to those with large complex larvae, or *vice versa* (Zeng et al., 2006). If ascidians with simple larvae are derived from a more complex ancestor, however, then the *Ciona* situation, in which TLC appear to be the exclusive cognates of NCLC, may not be a general ascidian (or tunicate) feature. In this regard, it is important to note that more HNK-1 positive cells (and body pigment cells) are usually observed in ascidians with large larvae, such as *Botryllus schlosseri*, than we have observed in *Ciona* (Jeffery, 2006). We envision several possibilities to account for the greater number of HNK-positive cells in ascidians with complex larvae. First, the final number of TLC may be larger due to additional rounds of cell proliferation, as appears to be the case for tail muscle cells (Jeffery and Swalla, 1992). Second, other lineages in addition to A7.6/TLC may contribute to the complement of HNK-1 positive cells. During larval simplification, ascidian species with small streamlined larvae, like *Ciona*, could have reduced the number of NCLC-forming lineages. It will be important to determine the embryonic origin(s) of NCLC in ascidians with large complex larvae, such as *Botryllus* and *Ecteinascidia*.

NCLC originate near the bilateral margins of the neural plate

Previous studies showed that migratory NCLC are generated near the developing neural tube in *Ecteinascidia* larvae (Jeffery et al., 2004). The present results expand our knowledge of ascidian NCLC with respect to their staging location prior to migration and permit further comparisons to be made with vertebrate neural crest cells.

TLC are derived solely from the A7.6 blastomeres of the 110-cell embryo (Nishida and Satoh, 1985; Tokuoka et al., 2004; 2005). The FGF, Notch, and Wnt/β-catenin signaling systems are important in TLC induction (Shimauchi et al., 2001; Imai et al., 2002a, 2003; Imai et al., 2006), just as they are in early specification of neural crest cells (Meulemans and Bronner-Fraser, 2004). Early during gastrulation, the A7.6 cells are positioned on presumptive dorsal surface of the embryo at the bilateral margins of the blastopore. The A7.6 cells leave the embryonic surface and move into the blastopore during the late gastrula stage. Their precise mode of internalization remains to be investigated but seems to be independent of surrounding cells and similar to the epithelial delamination of vertebrate neural crest cells. After internalization, the A7.6 cells and/or their progeny become located immediately below and slightly lateral to the edges of the neural plate, which extends posteriorly to close the blastopore. After neurulation, TLC are localized in bilateral clusters adjacent to the posterior sensory vesicle, which appears to be the staging zone for their subsequent migration.

In most vertebrate embryos, neural crest cells are derived from the neural folds during neural tube formation. Subsequently, the staging area for their departure from the dorsal midline is located bilateral to the neural tube, which is similar to the situation that we have described for NCLC in *Ciona*. Although *Ciona* NCLC resemble vertebrate neural crest cells in their location with respect to the neural plate, they differ in not being an integral part of the folding neural tube. Additionally, *Ciona* NCLC precursors flank a very small portion of the

neuroaxis. Thus, the evolution of neural crest cells in the vertebrate lineage could have involved an extension of the ability to form pre-migratory cells along a much larger portion of the neural plate margin, as well as the incorporation of primordial NCLC into the folding neural tube. Finally, TLC also differ from most vertebrate neural crest cells in that they do not begin to migrate promptly after the neural tube is formed. Instead, they pause and undergo several rounds of cell division in the staging zone next to the neural tube until about 12 h later, when the tadpole hatches and begins to swim. It is important to note, however, that *Xenopus* neural crest cells also remain adjacent to the neural tube for many hours before initiating migration (Sadaghiani and Thiebaud, 1987).

Ascidian NCLC gene expression patterns support NC-RGN co-option in vertebrates

The expression patterns of sixteen orthologues of vertebrate neural crest related genes were surveyed during *Ciona* development. We found seven of these genes (e. g. *Ci-twist-like 1*, *Ci-twist-like 2*, *Ci-foxDb, Ci-ap-2-like-1*, *Ci-myc*, *Ci-rhoABC*, and *Ci-cadherin-2*) expressed in the A7.6/TLC lineage or its immediate precursors between the 64-cell and the swimming tadpole stage. Of the latter, *Ci-myc*, *Ci-Ap2-like 1, Ci-rhoABC*, and *Ci-cadherin-1* expression were not previously described in the A7.6/TLC lineage. In addition, the likely *Ciona* homologues of two other vertebrate neural crest genes, *neurogenin* and *ets* (Sauka-Spengler et al., 2007), are also expressed in the A7.6/TLC lineage (Imai et al., 2006). All of these are tier 3 or 4 NC-RGN; no tier 2 genes were expressed in the A7.6/TLC lineage. Thus, the gene expression profile of NCLC resembles but is not identical to that of vertebrate neural crest cells.

It is important to note that the tier 3 and 4 NC-RGN genes are expressed in A7.6/TLC together around the neural stage (Fig. 5), facilitating potential interactions among their products. Accordingly, functional studies have demonstrated that some of these NC-RGN genes interact in a regulatory cascade in which FGF 9/16/20 and FoxDb (the latter via *Notlc*) induce *twist-like-1* expression, which in turn activates downstream *twist-like-2* during A7.6/TLC development (Imai et al., 2003).

The NC-RGN appears to be conserved in vertebrates (Meulemans and Bronner-Fraser, 2005; Sauka-Spengler et al., 2007) but its ancestry is uncertain in invertebrate chordates. In cephalochordates, tier 1 and tier 2 genes are activated along the neural plate border or adjacent non-neural ectoderm, but tier 3 and 4 genes do not appear to be co-expressed in this region, and migratory NCLC (as well as body pigment cells) are apparently absent (Yasui et al., 1998; Meulemans and Bronner-Fraser, 2002; 2003; Yu et al., 2002; Holland and Holland, 2001). These findings imply that the four-tiered NC-RCN was a vertebrate innovation with tier 3 and 4 genes co-opted into the pathway early during vertebrate evolution. After the latter studies were published, phylogenomic studies inferred that tunicates rather than cephalochordates are more closely related to vertebrates (Bourlat et al., 2006; Delsuc et al., 2006; Vienne and Pontarotti, 2006). If this reflects the true chordate phylogeny, then re-wiring of the NC-RGN could have happened either in the tunicate-vertebrate lineage, after it split from cephalochordates, or in the vertebrate lineage, after it diverged from tunicates.

Ascidians express the full range of border specifier genes along the lateral edge of the neural plate (Gostling and Schmeld, 2003; Ma et al., 1996; Mazet, et al., 2003; Satou et al., 2002; Wada et al, 1997). However, two critical pieces of information were previously missing that would enable a determination of the point during chordate evolution in which co-option may have occurred: (1) the identification of a tunicate neural crest homologue (e. g. NCLC; see below), and (2) the determination of NC-RGN gene expression patterns in tunicate NCLC. The present study has filled these gaps by showing that the A7.6/TLC lineage are NCLC that express some of the NC-RGN tier 3 and 4 genes but no tier 2 genes (Fig. 6). Thus, it can be concluded that downstream genes controlling neural crest cell identity were co-opted into the NC-RGN early during vertebrate evolution rather than prior to the divergence of tunicates and vertebrates. The co-option of these genes could have resulted in a neuroaxis flanked by a large population of neural crest cells with migratory potential and an increased repertoire of cell fates.

Chordate ancestry of the neural crest

We propose that NCLC and neural crest cells are homologous cell types that were derived from a primordial neural crest (Stone and Hall, 2004) in the common ancestor of tunicates and vertebrates. Furthermore, based on results of the present investigation, we suggest that the primordial neural crest had the following phenotype. They expressed some of the NC-RGN tier 3 and 4 genes but none of the tier 2 genes, and thus had migratory capacity but did not populate a large portion of the neural plate border. They were not an integral part of the neural tube but instead originated beside it, in a staging area for their subsequent migration. They may have had one or only a few cell fates, with a primary function to form body pigment cells. In the vertebrate lineage, primordial neural crest cells could have spread throughout the neural plate border and become incorporated into the folding neural tube after the gene network(s) responsible for their specification were co-opted. Moreover, they could have adopted a more diverse set of derivatives by including more neural crest specifier and/or effector genes into the network. In contrast, the primordial neural crest may have remained largely unchanged during tunicate evolution.

The scenario provided above is not the only possible interpretation of our results. Instead, NCLC with an incomplete NC-RGN and the other features described above may be a derived rather than an ancestral trait in *Ciona*. The presence of a larger number of HNK-1 positive cells and pigment cells in other ascidian species (Jeffery, 2006) and expression of a *zic* homologue (a neural plate border specifier in vertebrates) in *Ecteinascidia* NCLC (Jeffery et al., 2004) could be indicative of this ancestral complexity. However, *Ecteinascidia* situation is complicated by late *zic* expression in migratory cells, which is not observed in vertebrate neural crest cells (Nakata et al., 1998; Sauka-Spengler et al., 2007). Future investigations on the expression of neural crest related genes in ascidians with complex larvae should be able to resolve the problem of whether the *Ciona* NC-RGN is ancestral or derived.

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