Genetic lineage labeling in zebrafish uncovers novel neural crest contributions to the head, including gill pillar cells

Alessandro Mongera1,* Ajeet P. Singh1 Mitchell P. Levesque1‡ Yi-Yen Chen1,5 Peter Konstantinidis2 and Christiane Nüsslein-Volhard1,*

SUMMARY
At the protostome-vertebrate transition, a new predatory lifestyle and increased body size coincided with the appearance of a true head. Characteristic innovations of this head are a skull protecting and accommodating a centralized nervous system, a jaw for prey capture and gills as respiratory organs. The neural crest (NC) is a major ontogenetic source for the ‘new head’ of vertebrates and its contribution to the cranial skeleton has been intensively studied in different model organisms. However, the role of NC in the expansion of the respiratory surface of the gills has been neglected. Here, we use genetic lineage labeling to address the contribution of NC to specific head structures, in particular to the gills of adult zebrafish. We generated a sox10:ER<sup>+</sup>-Cre line and labeled NC cells by inducing Cre/loxP recombination with tamoxifen at embryonic stages. In juvenile and adult fish, we identified numerous established NC derivatives and, in the cranium, we precisely defined the crest/mesoderm interface of the skull roof. We show the NC origin of the opercular bones and of multiple cell types contributing to the barbels, chemosensory organs located in the mouth region. In the gills, we observed labeled primary and secondary lamellae. Clonal analysis reveals that pillar cells, a craniate innovation that mechanically supports the filaments and forms gill-specific capillaries, have a NC origin. Our data point to a crucial role for the NC in enabling more efficient gas exchange, thus uncovering a novel, direct involvement of this embryonic tissue in the evolution of respiratory systems at the protostome-vertebrate transition.

KEY WORDS: Neural crest, Pillar cells, Cre/loxP, Zebrafish, Cranial neural crest, Gill

INTRODUCTION
The evolution of a true head has been proposed as a major step in the protostome-vertebrate transition, enabling the shift from filter feeding to active predation (Northcutt and Gans, 1983). The vertebrate head that has been assembled stepwise over evolutionary time displays functional characteristics that serve prey detection and capture but also enable a more efficient respiratory metabolism (Gans and Northcutt, 1983). Indeed, a predatory lifestyle requires a higher metabolic rate, which in turn establishes a positive selective pressure for enhanced gas exchange and distribution. To increase gas exchange capacity, respiration shifted during evolution from ciliated epithelia along the walls of the pharynx to complex gill organs attached to the branchial arches. Craniates, which include vertebrates and hagfishes, developed gills with highly organized filamentous structures that enable a massive expansion of the surface area involved in respiration (Evans et al., 2005).

The ‘new head’ theory of Northcutt and Gans proposes that many morphological and functional innovations of vertebrates develop from the neural crest (NC), the epidermal placodes or the lateral plate mesoderm (Northcutt and Gans, 1983). Although the original claims of this theory regarding the origin of NC and placodes have been subsequently refuted, a huge body of work has confirmed the important role of NC in accelerating vertebrate evolution (Northcutt, 2005; Yu et al., 2008; Abitua et al., 2012).

NC is a pluripotent embryonic tissue that differentiates into numerous cell types, such as osteoblasts and chondroblasts in the skull, neurons of the peripheral nervous system, Schwann cells and pigment cells (Hörstadius, 1950; Le Douarin, 1986; Le Douarin and Dupin, 2012). Further, NC is suggested to have played a crucial role in vertebrate brain development by promoting forebrain viability, a prerequisite for brain expansion (Etchevers et al., 1999).

Although the NC origin of the cartilaginous gill arch endoskeleton (Landacre, 1921) and of the walls of pharyngeal blood vessels (Le Lièvre and Le Douarin, 1975) is long established, the embryonic origin of gill pillar cells, which represent the functional and structural core of the gill lamellae, is unclear (Hughes and Morgan, 1973). It has been suggested that this important component of the gill filaments might derive from the lateral plate mesoderm (Northcutt and Gans, 1983), from endothelial cells (Bietrix, 1895) or from smooth muscles (Datta Munshi and Singh, 1968).

Extensive fate mapping of NC has been performed in many organisms representative of different vertebrate taxa (Le Douarin and Kalcheim, 1999), notably by transplantation experiments creating chick-quail chimeras (Le Douarin, 1986), vital dye injection in the premigratory NC of chick and frog (Bronner-Fraser and Fraser, 1988; Collazo et al., 1993), tissue extirpation in medaka and lampreys (Langille and Hall, 1988a; Langille and Hall, 1988b), and genetic lineage labeling using Cre/loxP-mediated recombination in mice (Chai et al., 2000; Jiang et al., 2002). However, a more detailed map for adult structures in hagfishes, lampreys and ray-finned fishes (actinopterygians) would be extremely valuable, as important structures of the ‘new head’, such as the gills, were lost very early in the evolution of tetrapods with the acquisition of a terrestrial lifestyle. In zebrafish and medaka, vital dye injection, expression of reporter genes and transplantation experiments have been employed to follow the migration of NC-
derived cells in the larva (Langille and Hall, 1988a; Schilling and Kimmel, 1994; Li et al., 2003; Wada et al., 2005). However, these methods have severe limitations as far as adult structures are concerned, as the label becomes diluted or transgene expression does not persist through adulthood.

To overcome these limitations and to permanently and specifically label NC cells and their derivatives in zebrafish, we generated a transgenic line expressing tamoxifen-inducible Cre recombinase under the control of the sox10 promoter (Carney et al., 2006). Sox10 is expressed specifically in the NC at early stages of development (Dutton et al., 2001). We induced Cre/loxP-mediated recombination in various reporter lines during embryonic development and identified in larval, juvenile and adult fish numerous established NC derivatives, including multiple elements of the cranial skeleton, peripheral nervous system components, pigment cells and glia, thus confirming the specific expression of the sox10 promoter in NC cells during the time of induction.

Moreover, we were able to define the NC/mesoderm interface in the zebrafish frontoparietal bones, which are dermoskeletal elements whose origin across vertebrate taxa is still debated (Coulby et al., 1993; Gross and Hanken, 2008a; Noden and Trainor, 2005), and to demonstrate a NC contribution to other head structures of uncertain origin.

Strikingly, we observed labeled Gill filaments, including primary and secondary lamellae. In particular, we discovered labeled pillar cells, which represent a craniate innovation that provides the basis for the surface expansion of the gills (Evans et al., 2005). Pillar cells form capillary beds for blood perfusion and have mechanosensory properties (Smith and Chamley-Campbell, 1981; Evans et al., 2005). We propose that the development of this atypical NC-derived cell type at the protochordate-vertebrate transition led to the appearance of a more complex gill organ, supporting the relevance of the ‘new head’ theory to the evolution of the respiratory system.

**MATERIALS AND METHODS**

**Transgenic lines**

To generate Tg(cmlc:GFP-sox10:ERT2-Cre) (abbreviated to sox10:ERT2-Cre), the (~4.9kb)sox10 promoter (Carney et al., 2006) was subcloned into a pTol2-cmlc:GFP plasmid and the ER72-Cre coding sequence (Metzger et al., 1995) was PCR amplified and inserted by in-fusion reaction (Clontech) downstream of the promoter region. For Tgrps9:loxP-DsRed2-loxP-EGFP (abbreviated to rps9:switch), the EF1α promoter in the pTol-EF1α:loxP-DSRed2-loxP-EGFP plasmid (Hans et al., 2009) was replaced with a 3 kb promoter region of the Fugu rps9 gene (ENSTRUG00000015896.1). Primers used: 5'-AGAACTCACCTGTTAGAGAG-3' and 5'-GCGGCTTTAATGTCGCCGCA-3'. In addition, the following reporter lines were used: Tg(β-actin:loxP-STOP-loxP-DsRed-express) (abbreviated to β-actin:switch) (Bertrand et al., 2010); Tg(uibl:loxP-EGFP-loxP-mCherry) (abbreviated to uibl:switch) (Mosimann et al., 2011); Tg(EF1α:loxP-DsRed2-EGFP) (abbreviated to EF1α:switch) (Hans et al., 2009); Tg(hsp70l:loxP-DsRed2-loxP-nsEGFP) (abbreviated to hs:R to nG) (Knopf et al., 2011); Tg(crestin:Gal4-UBAS-GFP) (Y.Y. Chen, PhD thesis, Eberhard Karls Universität Tübingen, 2011); and Tg(~4725sox10:Cre) (Rodrigues et al., 2012).

For early NC tracing (within the first 3 days), the best line is EF1α:switch, as the expression level is high at early stages. For metamorphic and adult fish, the best ‘ubiquitous’ line in our experience is uibl:switch, followed by β-actin:switch, which generally leads to a more patchy labeling.

**Cre induction**

Fish carrying the sox10:ER72-Cre transgene were crossed to the different reporter lines and 16-hpf embryos were dechorionated and treated with 5 µM 4-hydroxytamoxifen (4-OHT; Sigma, H7904) for 8 hours unless otherwise specified. Control embryos were incubated in a corresponding dilution of ethanol.

**UAS:Cre injection**

Tg(crestin:Gal4-UAS-GFP;β-actin:switch) embryos at the 1-cell stage were injected with different concentrations (1-10 ng/µl) of a pTol2 vector containing the Cre coding sequence downstream of a 4xUAS site.

**Image acquisition and processing**

Images were taken using a Zeiss LSM 5 Live confocal microscope and a Leica M205 FA stereomicroscope and processed using Imaris (Bitplane), ImageJ (NIH) and Adobe Photoshop. For confocal imaging of adult gills, fish were fixed for 1 hour in 4% paraformaldehyde. The head was mounted in 0.4% agarose for imaging after the removal of the operculum. Adult maxillary barbels were surgically removed and mounted in 1% agarose for imaging.

**Cryosectioning and immunohistochemistry**

After fixation, juvenile fish (1 mm) were washed with PBS and incubated in 10%, 20% and 30% sucrose solutions. The samples were then incubated in 1:1 30% sucrose:OCT Compound (Tissue-Tek) for 30 minutes and then in OCT overnight. Fish were frozen in cryomolds and 20 µm cryosections were obtained with a CH3050 cryostat (Leica). Cryosections were treated with 100% methanol for 10 minutes to improve adherence to the slides.

Sections were rehydrated in PBST (PBS with 0.1% Tween 20), and blocked in 10% sheep serum in PBST for at least 1 hour at room temperature. The primary antibody [rabbit anti-DsRed (Clontech) at 1:200] was incubated overnight at 4°C in 10% sheep serum in PBST. Sections were then washed with PBST and incubated with the secondary antibody [anti-rabbit-Cy3 (Dianova) at 1:400] for 2 hours at room temperature. Sections were washed several times with PBST and nuclei visualized by adding DAPI (Sigma) to PBST in the last washing step.

**RESULTS**

**sox10:ER72-Cre allows inducible labeling of NC in zebrafish**

We generated a sox10:ER72-Cre transgenic line by fusing the NC-specific (~4.9kb)sox10 promoter to the tamoxifen-inducible Cre recombinase coding sequence [Fig. 1A (1)]. To test the system prior to NC differentiation, we created a reporter line that ubiquitously drives a red-to-green switchable cassette under the control of the Fugu (~3)bps9 promoter [Fig. 1A, before (2) and after (3) recombination]. The recombination was induced in double-transgenic embryos carrying the Cre driver construct and the reporter cassette by adding 4-hydroxytamoxifen to the water for 8 hours [16-24 hours postfertilization (hpf)], when NC delamination and early migration occur (Fig. 1B). At this time period, the sox10 promoter is reported to be specific for NC cells and oligodendrocytes (Simon et al., 2012; Carney et al., 2006). To assess the controllability and the potential leakiness of the system, we compared induced and uninjected embryos at 36 hpf. After injection we found many GFP+ cells resulting from Cre-induced recombination in the branchial arch region (Fig. 1C), in the dorsal neural tube, around the otic vesicles (Fig. 1E) and along the walls of the dorsal aorta and the ventral notochord (Fig. 1G). Uninduced embryos lacked GFP+ cells in these areas (Fig. 1D,F,H).

We then induced clones in the well-established β-actin:switch reporter line, which has already been shown to effectively trace embryonic cell populations to later stages of development and to adulthood (Bertrand et al., 2010). We analyzed the developing viscerocranium in larvae at 4 days postfertilization (dpf). In this...
region, chondroblasts were labeled in induced larvae (n=10; Fig. 1I), whereas no DsRed+ cells were detected in uninduced controls (n=10; Fig. 1J). Next, we analyzed the specificity of our system for NC derivatives using the EF1α:switch line and observed specific marking of pharyngeal arches 1-7 (Fig. 1K,K′) and of pigment precursors, peripheral nervous system glia, dorsal root ganglia (DRG) progenitors, lateral line Schwann cells and dorsal melanoblasts (Fig. 1L,L′, a-e, respectively). Induction of Cre at later time points (4 dpf) and for shorter periods (1-2 hours) results in isolated clones restricted to one or a few cell types, such as pigment cells (supplementary material Fig. S1A,D; n=50). These clones become smaller when the induction is performed at juvenile stages (i.e. 20 dpf; n=46; supplementary material Fig. S1B). Lack of labeling in adults of uninduced fish carrying both transgenes (supplementary material Fig. S1C) suggests that reporter expression was under tight control of the externally supplied tamoxifen.

Taken together, these results show that the sox10:ERT2-Cre line allows for rigorous control of the onset of tamoxifen-dependent recombination events in NC cells. In line with the previously characterized (−4.9)sox10:GFP reporter (Carney et al., 2006), our sox10:ERT2-Cre is occasionally expressed in a few random skeletal muscle progenitors in the trunk, which appear through adulthood as labeled clones (data not shown). These rare clones (on average one per flank), along with their clonally related recombined tissues, were not considered further in this analysis.

NC-derived structures in the trunk
To assess the NC contribution to postembryonic structures, we first analyzed labeled tissues in established postcranial NC derivatives of metamorphic juvenile fish (20 dpf) in which recombination had been induced at embryonic stages as described above (Fig. 2). We detected sympathetic ganglia (Fig. 2A), neurons of the enteric nervous system (Fig. 2B) and DRG (Fig. 2C). Moreover, we found sensory neurons innervating the body periphery, such as the pectoral and the caudal fins (Fig. 2D,D′, respectively). Glia providing myelination to axons in the peripheral nervous system were also marked (Fig. 2E-F′). We then searched for labeled chromatophores and found clones of iridophores, melanophores and xanthophores (Fig. 2G-I′). In several cases, we found labeled melanophores in proximity to recombined glial cells that surround the DRG (Fig. 2J, arrowheads). In the heart of metamorphic switched fish we found many labeled cells, of which at least some show overlap with cmlec:GFP+ cells, indicating that they are contractile cardiomyocytes (Kwan et al., 2007) (Fig. 2K,K′).

In the heart of metamorphic switched fish we found many labeled cells, of which at least some show overlap with cmlec:GFP+ cells, indicating that they are contractile cardiomyocytes (Kwan et al., 2007) (Fig. 2K,K′). In zebrafish, by means of cell
transplantation and different labeling techniques, a NC contribution to cardiomycocytes has already been demonstrated (Sato and Yost, 2003; Li et al., 2003). We also observed clones in the adrenomedullar region (Fig. 2L) and in the epithelial walls of the pronephros (Fig. 2M,M′), confirming the NC contribution to these internal organs (Collazo et al., 1993).

Taken together, these data show that our inducible Cre/loxP-based genetic labeling system enables the detection of a large set of trunk NC derivatives in juvenile and adult fish. With the rare exception of spurious muscle clones, we did not detect any tissue with an established non-NC origin. The sox10:ERT2-Cre-induced recombination may thus be used as a potent tool to uncover the NC origin of other postembryonic structures.

Analysis of labeled head structures reveals a NC contribution to frontal but not parietal bones

Next, we surveyed labeled chondrocranial and cartilaginous viscerocranial elements of the developing skull in 5-dpf larvae (Schilling et al., 1996; Piotrowski et al., 1996) and in 15- to 20-dpf fish (Fig. 3; Table 1). In the neurocranium, we detected recombined tissues in the ethmoid plate (Fig. 3A, e), trabeculae (Fig. 3A, t), taenia marginalis anterior (Fig. 3B, tma) and posterior (Fig. 3B, tmp), epiphyseal bar (Fig. 3B, eb) and in the otic capsule (Fig. 3C), whereas the basilar plate (parachordalia) and the anterior basicranial commissure were devoid of labeled cells. In the viscerocranium, we found a NC contribution to all its components (Fig. 3D,E).
In the head of metamorphic and adult fish we identified, by in vivo confocal imaging, neurocranial, viscerocranial and dermatocranial skeletal elements (Fig. 4; supplementary material Fig. S2). We decided to focus our attention on those structures that are still debated in the literature among different vertebrate taxa. Identifying their embryonic origin in zebrafish will be of great importance in deciphering key steps of vertebrate evolution.

First, we looked at the otic capsule and at the viscerocranium to confirm at later stages what we found in the larvae. A NC origin for the otic capsule cartilage has been shown previously in

*Xenopus*, chicken and mice (Gross and Hanken, 2008b; Le Lièvre, 1978; Noden, 1983; Cubbage and Mabee, 1996; O’Gorman, 2005), whereas extirpation/vital dye labeling experiments did not demonstrate a NC contribution to this structure in lamprey, medaka or toads (Langille and Hall, 1988a; Langille and Hall, 1988b; Olsson and Hanken, 1996). Notably, in the neurocranium of juvenile zebrafish, we detected labeled chondroblasts and osteoblasts in the otic capsule and in the structure that contributes to the formation of the inner ear (Fig. 4A-A″).

In the viscerocranium, Meckel’s cartilage, along with other important viscerocranial elements, is labeled, as expected (Fig. 4B). Remarkably, we detected labeled cells in the basihyal and the basibranchial (Fig. 4B′), two cartilages that form the hyobranchial skeleton. Unlike in zebrafish, NC does not seem to contribute to these bones in lissamphibians (Olsson and Hanken, 1996).

In the dermatocranium, we show that NC contributes to the entire opercular series, consisting of the opercle, inter-, sub- and preopercle (Fig. 4C,D). The vault series of dermal bones forming the roof of the skull consists of paired frontals and parietals. Both NC and mesoderm are known to contribute to frontoparietal bones. Intriguingly, we find that in zebrafish the frontals are labeled only in the anterior center of ossification (Fig. 4E, arrowhead; Fig. 4F) and the parietals (n=100, using three different reporter lines) are devoid of recombined osteoblasts (Fig. 4E, outlined by the dashed frame). The extent of their contribution defines a NC/mesoderm boundary in the skull, for which empirical data are inconsistent across vertebrae taxa (Gross and Hanken, 2008a). Our data point to a NC/mesoderm boundary in the frontal bones, at the border between the anterior and the posterior ossification centers.

### NC contributes to the barbels

Barbels are whisker-like tactile organs that protrude from the integument of the mouth region. The embryonic origin of the fibroblast-like cells that synthesize the extracellular rod matrix is unknown. As barbels have been recently used as a model for adult organ regeneration (LeClair and Topczewski, 2010), we sought to determine whether NC contributes to barbel development.

We detected labeled cells at the core of the maxillary barbels, surrounded by specialized epithelial cells (Fig. 4G). Specifically, we identified labeled nerve fibers (Fig. 4H, asterisks, red channel) in the region of the lymphatic (Fig. 4H, arrow, green channel) and blood vessels (Fig. 4H, arrowhead, green channel). Notably, we

---

**Table 1. List of chondrocranial and viscerocranial elements that form the developing skull with detected/undetected contribution from the NC**

<table>
<thead>
<tr>
<th>Division</th>
<th>Region</th>
<th>Element</th>
<th>NC contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurocranium</td>
<td>Base</td>
<td>Parachordal (pc)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anterior basicranial commissure (abc)</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trabecula (t)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethmoidal plate (e)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Orbito-temporal</td>
<td>Epiphyseal bar (eb)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Taenia marginalis anterior (tma)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Taenia marginalis posterior (tmp)</td>
<td>Yes</td>
</tr>
<tr>
<td>Viscerocranium</td>
<td>Mandibular</td>
<td>Meckel’s cartilage (m)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Palatoquadrate (pq)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basihyal (bh)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ceratohyal (ch)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyosymplectic (hs)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Branchial</td>
<td>Basibranchial (bb)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypobranchial (hb)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ceratobranchial (cb)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Abbreviations as in Fig. 3.
found labeled fibroblast-like cells embedded in the matrix of the central rod (Fig. 4H, arrowheads, red channel) and labeled flattened cells along the dorsal aspect of the rod (Fig. 4H, arrow, red channel). Interestingly, similar mesenchymal, fibroblast-like cells have been proposed to play a crucial role in blastema formation during fin regeneration (Knopf et al., 2011).

Pillar cells are NC derived

Finally, we focused on the gill organ and observed labeled gill filaments, including primary and secondary lamellae (Fig. 5A,B). In the primary lamellae, smooth muscle cells of the blood vessel tunica media are labeled (Fig. 5C, arrows) and are located in close contact with the endothelium, which is labeled by the flk:GFP transgene (Lawson and Weinstein, 2002) (Fig. 5C'). In the secondary lamellae, we found labeled pillar cells (Fig. 5D,E). Pillar cells are highly specialized, with autoregulatory contractile properties that offer mechanical support to the respiratory epithelium (Fig. 5D, green), and enable an adaptation of the lamellar surface to the oxygen content of water (Bettex-Galland and Hughes, 1973; Hughes and Morgan, 1973). Pillar cells are arranged in parallel columns and, with their cytoplasmic processes (Fig. 5D, arrowhead) supporting the respiratory epithelium (Fig. 5D, green), form lumina (Fig. 5E, arrowheads) that give rise to capillary networks through which blood perfuses and gas exchange occurs (Fig. 5F) (Hughes and Morgan, 1973).

In our experimental set-up, the definition of NC origin rests entirely on the specificity of the sox10 promoter. Although we did identify most of the previously known NC derivatives in the floxed clones, the discovery of new structures requires independent confirmation. We therefore induced recombination with an alternative NC promoter from the crestin locus (Luo et al., 2001) (Y.-Y. Chen, PhD thesis, Eberhard Karls Universität Tübingen, 2011) by injecting UAS:Cre DNA into Tg(crestin:gal4-UAS:GFP;β-actin:switch) embryos at the 1-cell stage. We detected, among previously identified NC derivatives (data not shown), labeled barbels (supplementary material Fig. S3), gill pillar cells and smooth muscles (Fig. 5G). Moreover, we identified these structures using a non-inducible sox10:Cre line with a longer (7.2 kb) promoter fragment and a different genomic insertion site (Rodrigues et al., 2012) (data not shown).

**DISCUSSION**

The appearance of NC was fundamental to subsequent vertebrate evolutionary history, tremendously accelerating the emergence...
of a predatory lifestyle and the exploitation of unexplored ecological niches. Although NC contribution to multiple cell types is well documented in different model organisms (Gross and Hanken, 2008a), the lack of a comprehensive analysis of NC-derived adult structures in actinopterygians leaves a gap in our understanding of important evolutionary transitions. Key innovations that endowed vertebrates with predatory capacity were the acquisition of muscular ventilation coupled with respiratory surface expansion, the emergence of a skull vault and a dorsoventrally articulated jaw. We have developed and employed a genetic long-term labeling method to uncover a potential NC origin of evolutionarily important cell types and tissues of previously unrecognized or debated origin in zebrafish, a model teleost. In particular, in the present study we focused on the gill respiratory system and showed that, by giving rise to gill pillar cells, NC might have expedited respiratory surface expansion and muscular ventilation.

**NC-driven expansion of the respiratory surface at the protochordate-vertebrate transition**

At the protochordate-vertebrate transition, the acquisition of a predatory lifestyle was facilitated by a massive expansion of the respiratory surface in the gills and the switch from ciliary to muscular ventilation (Northcutt and Gans, 1983). Recent paleontological analysis of crinoid fossils such as *Haikouella lanceolata* and *Yunnanozoon luidum* suggests a precraniate history of ‘crest animals’. These fossils are characterized by paired gill rays and jointed, widely spaced branchial arches, but do not possess a skull (Chen, 2008; Chen et al., 1999; Holland and Chen, 2001; Mallatt and Chen, 2003). Although many structures directly involved in prey capture have been shown to derive from NC, the embryonic origin of the new, expanded respiratory organ, which according to the recent paleontological interpretation seems to predate the appearance of the skull, has not been investigated. Our demonstration of a NC origin for gill pillar cells reinforces the importance of this embryonic tissue in the evolution of respiratory systems and, possibly, in the radiation of precraniate crest animals at the protochordate-vertebrate transition.

Pillar cells are found in the gills of hagfishes (Mallatt and Paulsen, 1986; Elger, 1987), lampreys (Youson and Freeman, 1976), chondrichthyans and osteognathostomes (Evans et al., 2005). However, cephalochordates lack specialized gill cells that increase the respiratory surface area. Instead, this group has rather simple collagen bars located in the pharynx atrium, and gas exchange occurs through ciliated epithelial cells (Welsch, 1975; Baskin and Detmers, 1976). Thus, pillar cells are a craniate synapomorphy and, as they provide the structural basis for the new expanded gill organ, represent a key innovation in vertebrate evolution. Pillar cells form small channels to conduct blood through the gills and possess the ability to expand or contract their cytoplasmic processes to control blood flow (Bettex-Galland and Hughes, 1973). This contraction/expansion activity also regulates the distance between neighboring secondary lamellae, thereby modulating water flow through the gills. The development of this atypical vertebrate-specific cell type is still poorly understood. Observations of cellular microanatomy and tissue ontogeny (Datta Munshi and Singh, 1968), supported by the expression of smooth muscle myosin (Smith and Chamley-Campbell, 1981), suggest a derivation of pillar cells from the smooth muscles forming the blood vessels of the primary lamellae. Cephalic NC is known to give rise to avian and mammalian smooth muscle cells of the blood vessels of the face and forebrain but not to the endothelium (Etchevers et al., 2001; Le Lièvre and Le Douarin, 1975; Yoshida et al., 2008). Our finding that, in addition to the smooth muscles of the pharyngeal arches, those forming the primary lamellae also have a NC origin supports the hypothesis that pillar cells evolved from this tissue rather than from endothelial cells, as originally proposed (Bietrix, 1895).

**NC contribution to cranial skeletal elements and barbels in zebrafish**

The contribution of cranial NC to the frontal and parietal bones is contentious and studies using different model systems and labeling techniques have led to inconsistent results (Coulby et al., 1993;
Noden and Trainor, 2005; Gross and Hanken, 2008a). For example, 
fate mapping in *Xenopus* using fluorescent dextran labeling 
suggested that cranial NC contributes to the entire length of 
the frontoparietal bones (Gross and Hanken, 2005), whereas studies in 
mouse using *Wnt1-Cre*-mediated genetic labeling suggested that 
NC does not contribute to the parietal bones (Jiang et al., 2002). 
Moreover, analysis of quail-chick chimeras has led to conflicting 
conclusions regarding a NC contribution to these dermatocranial 
elements in the avian skull, defining the NC-mesoderm boundary 
at the border between the anterior and posterior regions of the 
frontals (Noden, 1978; Evans and Noden, 2006) or extending NC 
contributions to both frontal and parietal bones (Couly et al., 1993).

The frontoparietal bones of the zebrafish are anatomically 
similar to their mammalian counterparts and start to ossify at 3-4 
weeks postfertilization (Cubbage and Mabee, 1996; Quarto and 
Longaker, 2005). We find that NC contributes to the anterior but 
not to the posterior ossification center of the frontals. Further, we 
find no contribution to the parietals, allowing us to suggest that the 
NC/mesoderm boundary in zebrafish lies in the frontal bones, 
between the anterior and the posterior ossification center, as has 
been shown in chicken (Noden, 1978; Evans and Noden, 2006) 
and mice (Jiang et al., 2002). Differences in the location of the 
NC/mesoderm boundary could also be attributed to species-specific 
factors, although we cannot rule out a possible contribution of 
experimental methodology to conflicting conclusions.

The embryonic origin of the otic capsule is debated. These paired 
bones accommodating the inner ear are considered to be a novelty of 
craniates and are absent in early crest animals (Chen, 2008; Gross 
and Hanken, 2008a; Mallatt and Chen, 2003). Studies in birds and 
mammals have shown a NC contribution to the otic capsule (Le 
Lièvre, 1978; O’Gorman, 2005; Noden, 1983). Previous studies using 
NC extirpation/vital dye labeling did not find a NC contribution to 
the otic capsules in lamprey (Langille and Hall, 1988b), medaka 
(Langille and Hall, 1988a) and oriental fire-bellied toad (Olsson 
and Hanken, 1996), although the otic capsules in *Xenopus* were shown to 
receive a contribution from the cranial NC (Gross and Hanken, 
2008b). These studies have led to the view that NC contribution to 
the otic capsule must predate the divergence of the mammalian and 
avian lineages (O’Gorman, 2005; Gross and Hanken, 2008a). We 
show that NC contributes to the otic capsule in zebrafish and could 
thus be a trait shared among vertebrates. In this regard, it is interesting 
to note that mutants with NC defects often have ear phenotypes in 
zebrafish (Kelsh et al., 1996; Whitfield et al., 1996).

Elegant fossil and molecular genetic analyses suggest that 
gnathostome jaw evolution occurred in a stepwise fashion, 
facilitated by skull reorganization and altered epithelial-
mesenchymal interactions among pre-existing molecular programs 
(Gai et al., 2011; Shigetani et al., 2002). We find that, as in other 
vertebrates, the complete lower jaw is NC derived. Contrary to 
observations in amphibians (Olsson and Hanken, 1996), the 
zebrafish hypobranchial skeleton is also NC derived.

The opercular flaps, which consists of multiple flat dermal bones, 
is also NC derived, as shown in the present study. The evolution of 
the opercular series has facilitated a novel mechanism for the 
depression of the mandible (Lauder, 1980a; Lauder, 1980b; Lauder, 
1982), allowing an improvement in suction feeding through better 
control over fluid movement (Lauder, 1980a). The NC origin of the 
opercular series, as conclusively shown using our long-term labeling 
approach, was previously assumed on the basis of its dermal bone 
composition and the expression of specific transgenes (Kimmel et 
al., 2010). Recent findings demonstrating the presence of an 
embryonic operculum in amniotes have rekindled interest in this 
structure, which was presumed to have been lost completely with the 
evolution of tetrapods and the emergence of a terrestrial lifestyle (Richardson et al., 2012).

We also find a NC contribution to zebrafish barbels. Barbels are 
tentacle-like chemosensory structures that arose independently 
many times during actinopterygian evolution (LeClair and 
Topczewski, 2010). Poor homology in terms of embryonic 
development and cellular composition across different vertebrate 
taxa complicates phylogenetic comparisons and makes it difficult 
to trace the evolutionary history of these sensory structures (Fox, 
1999). In the barbels of recombined fish we detected fibroblast-like 
cells in the matrix of the rod and flattened cells along its dorsal 
aspect. These two cell types are likely to be involved in the 
production of the connective tissue of the supporting central rod. 
Barbels are an attractive model for understanding tissue 
regeneration in adult organs (LeClair and Topczewski, 2010). That 
central components of these structures have a NC origin raises the 
intriguing possibility of studying the potential role of NC-derived 
adult tissues in organ repair.

**sox10:ER<sup>72-Cre</sup> as a tool to understand the evolution of developmental mechanisms underlying emergence of the vertebrate body plan**

Very little is known about the cellular and molecular events 
underlying NC-driven diversification of the vertebrate body plan. 
This is due, in part, to a dearth of tools to allow consistent and 
long-term labeling of NC derivatives. Inducible *sox10:ER<sup>72-Cre</sup>* recombination allows NC long-term labeling in a spatiotemporal 
manner: the spatial domain of Cre-mediated recombination is 
restricted by the *sox10* promoter and the temporal domain is under 
tamoxifen control. In the future, the *sox10:ER<sup>72-Cre</sup>* line will also 
enable the genetic manipulation of NC and its derivatives. For 
example, taking advantage of the new FlipTrap and FlEx 
technologies (Trinh et al., 2011; Ni et al., 2012), it will be possible 
to specifically manipulate particular genes in cell lineages that fall 
into the spatiotemporal domain of the *sox10:ER<sup>72-Cre</sup>* transgene. 
This will greatly improve our understanding of how these 
populations of migratory and proliferative cells give rise to diverse 
cell types and organs in the vertebrate body.

In summary, using an inducible *Cre/loxP* system for genetic 
lineage tracing, we demonstrate a NC contribution to various 
structures in metamorphic and adult zebrafish, an important model 
teleost. In particular, our analysis reveals a direct involvement of 
NC in the development of gill pillar cells, an atypical cell type 
found in vertebrates and hagfishes. A switch from ciliary 
ventilation to respiratory muscular ventilation with gills was an 
early event during the evolutionary history of modern vertebrates, 
followed by skull reorganization and the emergence of a jaw. A role 
for the NC in remodeling the respiratory system of early crest 
animals is underappreciated, in part owing to the lack of such 
analyses in non-tetrapod vertebrates. Respiratory systems 
underwent a profound transformation and pillar cells were lost 
along the tetrapod lineage. We propose that the evolution of the 
gills, with their highly expanded respiratory surface, was driven by 
the NC, and thus confirm the fundamental role played by this 
embryonic tissue in vertebrate radiation.

**Acknowledgements**

We thank F. Argenton, C. Baker, R. Kelsh and all our colleagues for insightful 
discussion and helpful comments on the manuscript; F. S. Rodrigues (R. Kelsh 
laboratory) for sharing unpublished data; and C. Dooley for valuable support in 
the early phases of this work.
Funding
This work was funded by the Max-Planck Society for the Advancement of Science and the European Molecular Biology Organization to A.P.S.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.091066/-/DC1

References


