Identification of a rudimentary neural crest in a non-vertebrate chordate

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Neural crest arises at the neural plate border, expresses a core set of regulatory genes and produces a diverse array of cell types, including ectomesenchyme derivatives that elaborate the vertebrate head4,5. The evolution of neural crest has been proposed to be a key event leading to the appearance of new cell types that fostered the transition from filter feeding to active predation in ancestral vertebrates6. However, the origin of neural crest remains controversial, as homologous cell types have not been unambiguously identified in non-vertebrate chordates4,7. Here we show that the tunicate Ciona intestinalis possesses a cephalic melanocyte lineage (a9.49) similar to neural crest that can be reprogrammed into migrating ‘ectomesenchyme’ by the targeted misexpression of Twist (also known as twist-like 2). Our results suggest that the neural crest melanocyte regulatory network pre-dated the divergence of tunicates and vertebrates. We propose that the co-option of mesenchyme determinants, such as Twist, into the neural plate border and expresses several neural plate border genes, as Ciona intestinalis possesses a cephalic melanocyte as a positional cue to trigger differentiation of the posterior a10.97 melanocyte.

Wnt signalling was selectively perturbed in the a9.49 lineage by the targeted misexpression of Wnt7, stable β-catenin and dominant-negative Tcf (ΔTcf, which lacks the β-catenin amino-terminal binding domain) using the Mitf enhancer (Fig. 1c–f). A β-cry-crystallin reporter was used to distinguish the melanocytes, as it is expressed in the otolith but not the ocellus (Fig. 1c). Both pigmented precursors were converted to the presumptive ocellus (Fig. 1b), suggesting that it might serve as a positional cue to trigger differentiation of the posterior a10.97 melanocyte.

Whole-genome phylogenetic analyses place the tunicates as the true sister clade to vertebrates8, and consequently they are well suited for investigating the evolutionary origins of the neural crest. In a previous report on the mangrove tunicate, a migratory cell population originating from the vicinity of the neural tube was likened to the neural crest9. However, subsequent studies of eleven additional tunicates provided unequivocal evidence that these cells arise from the mesoderm flanking the neural tube7. It was then suggested that a mesoderm-derived mesenchyme lineage (A7.6) in Ciona possessed some of the properties of the neural crest8, although these cells do not arise from the neural plate border and lack expression of key neural crest regulatory genes.

We present evidence that the bilateral a9.49 pigment cell lineage of Ciona embryos represents a rudimentary neural crest. It arises at the neural plate border and expresses several neural plate border genes, as well as a number of neural crest specification genes, including Id, Snail, Ets and FoxD4–13 (Fig. 1a and Supplementary Fig. 1). In vertebrates, MITF (microphthalmia-associated transcription factor) directly activates several target genes required for melanogenesis of neural-crest-derived melanocytes, including TYR and TYRP1 (ref. 14). In tunicates, Mitf is expressed in the a9.49 lineage15, which can be labelled by electroporation of a reporter, Mitf>lacZ (Mitf regulatory sequence driving the expression of lacZ) (Fig. 1b). The posterior daughters of the lineage (a10.97) intercalate at the dorsal midline and form the gravity-sensing otolith and melanocyte of the light-detecting ocellus (Fig. 1c)16. We sought to understand the basis for the differential specification of these pigmented cells.

Wnt signalling has a conserved role in neural crest induction, and promotes melanocyte formation from cephalic neural crest in zebrafish17. Both a10.97 cells express Tcf/Lef, the transcriptional effector of Wnt signalling18, thus Wnt may also have a role in Ciona melanogenesis. We found that Wnt7 is expressed along the dorsal midline just posterior to the presumptive ocellus (Fig. 1b), suggesting that it might serve as a positional cue to trigger differentiation of the posterior a10.97 melanocyte.

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to ocelli after misexpression of Wnt7 in the a9.49 lineage (Wnt7 is not normally expressed in this lineage) (Fig. 1d). A similar transformation was observed after targeted expression of a stabilized form of β-catenin, the coactivator of Tcf (Fig. 1e). In contrast, misexpression of a dominant-negative form of Tcf (Mitf→ΔTcf) produced the reciprocal transformation: both a10.97 melanocytes differentiated into otoliths and expressed the β-crystallin reporter (Fig. 1f).

Supernumerary otoliths were induced by the expression of a constitutively active form of the transcription factor for MAPK signalling, Ets1/2 (also known as Ets/pointed2) (Fig. 1g and Supplementary Fig. 2). Co-electroporation of Mitf→Wnt7 transformed these extra otoliths into ocelli (Fig. 1h). These results suggest that Wnt7 signalling is crucial in transforming otoliths to ocelli; it specifies the ocellus and suppresses the development of the otolith. To determine the underlying mechanism we sought to identify neural crest specification genes that are selectively activated in the presumptive ocellus in response to Wnt7 signalling.

In vertebrates, FoxD3 has been shown to repress melanogenesis of neural crest cells through downregulation of FoxD expression18. We found that FoxD is selectively expressed in the presumptive ocellus, (Fig. 2a, b) adjacent to the site of Wnt7 expression in the dorsal midline (Fig. 1b). A FoxD enhancer recapitulates this expression in the presumptive ocellus (Fig. 2c), and is dependent on Wnt signalling, as expression is lost in the presence of ΔTcf (Fig. 2d).

To investigate the role of FoxD in melanogenesis, we expressed variants of FoxD in the midline of the CNS, including the a9.49 lineage, using 5′ regulatory sequences from the Msx gene (Fig. 1a and Supplementary Fig. 3). Targeted expression of either full-length FoxD or the N-terminal third of FoxD (non-DNA binding) abolished expression of the Mitf→GFP reporter gene (Fig. 2e, f and Supplementary Fig. 3). However, misexpression of a constitutive repressor form of FoxD (DNA-binding domain fused to a WRPW repressor motif) had little effect on Mitf expression (Supplementary Fig. 3). These results suggest that FoxD represses Mitf independent of its DNA-binding domain, which is consistent with its mode of regulation in avian embryos, in which FoxD-mediated repression of Mitf is thought to occur through the sequestration of the transcriptional activator Pax3 (ref. 18).

Our results suggest a simple gene regulatory network (Wnt7 signalling activates FoxD, which inhibits Mitf expression) for the differential specification of the otolith and ocellus in the Ciona tadpole (Supplementary Fig. 4). Both a10.97 cells express Mitf before neurulation and during the convergence of the two cells along the dorsal midline of the anterior neural tube. Subsequently, the posterior a10.97 cell receives a localized Wnt7 signal and activates FoxD, which attenuates Mitf leading to diminished pigmentation in the ocellus. Mitf expression is sustained in the anterior a10.97 cell, which forms the densely pigmented otolith.

Notably, zebrafish uses a very similar mechanism to specify neural crest-derived pigmented melanophores and iridophores, which derive from a separate population of neural crest cells.
from a common bipotent Mitf+ progenitor\(^\text{14}\). The conservation of this network strengthens the argument that the a9.49 lineage of *Ciona* represents a rudimentary neural crest. However, the a9.49 lineage lacks some of the defining properties of cephalic neural crest, such as long-range migration and the potential to form ectomesenchyme derivatives.

We therefore sought to identify vertebrate neural crest determinants that are not expressed in the *Ciona* a9.49 lineage. In vertebrates, the craniofacial mesenchyme is derived from primary mesoderm and the ectomesenchyme arising from cephalic neural crest\(^\text{2,20}\). Both sources of cranial mesenchyme express the conserved mesodermal determinant *Twist*\(^\text{21}\) and produce diverse cranial tissues including muscle, cartilage and bone. In tetrapods, it seems that only the cephalic neural crest expresses *Twist* and produces ectomesenchyme\(^\text{21-23}\), whereas trunk neural crest lacks *Twist* expression and generates non-ectomesenchymal derivatives (for example, neurons, glia and melanocytes)\(^\text{2}\). Disruption of *Twist* activity causes severe cephalic neural crest phenotypes, including defects in cell migration and survival, as well as morphological defects of the skull vault and heart\(^\text{21,22,24}\).

There are three *Twist*-related genes in *Ciona*. In this study we focused on the gene most similar to *Twist1* in vertebrates (Supplementary Fig. 5). In *Ciona*, *Twist* is expressed solely in mesoderm-derived mesenchyme (Fig. 3a). It is not expressed in any region of the neural plate, including the a9.49 lineage. *Twist*-expressing mesoderm undergoes long-range migration (Fig. 3b) and produces a number of diverse tissues in juveniles and adults, including body-wall muscles, tunic cells (which populate the protective covering of the adult) and blood cells\(^\text{25}\). The migration and differentiation of these mesoderm tissues are inhibited when *Twist* expression is reduced\(^\text{25}\).

To determine whether ectomesenchyme could be formed in *Ciona*, we misexpressed *Twist* in the a9.49 lineage using the *Mitf* enhancer (Fig. 3c, d). The manipulated cells exhibit a mesenchymal phenotype, including protrusive activity, proliferation and long-range migration, which was not observed by the misexpression of other related genes (Supplementary Video 1 and Supplementary Fig. 6). Moreover, misexpression of *Twist* in the notochord and motor ganglion causes some disruptions in terminal differentiation, but does not transform these tissues into mesenchyme (Supplementary Fig. 7). The reprogrammed a9.49 cells exhibit expression of mesenchyme genes, including *Erg* (Supplementary Fig. 8), which is expressed in the ectomesenchyme of mouse embryos\(^\text{26}\). The affected lineage was visualized in juveniles using reporters for *Tyr*, a gene that is activated by *Mitf* in melanocytes\(^\text{14}\) (Fig. 3e, f). Normally the a9.49 derivatives are located solely in an anterior region of the CNS (Fig. 3e). In contrast, embryos expressing

![Figure 4](image-url)
5. Delsuc, F., Brinkmann, H., Chourrout, D. & Philippe, H. Tunicates and not vertebrates were cloned into a pCESA vector using the primer pairs shown in Supplementary Table 1. The Zick, Mss, FoxD, Tbr, Ttp, Brachyury and Dmef enhancers have been described previously.29,30,31,32,33,34,35. Conversion of Kaede was achieved by treating tailbud embryos with ultraviolet light using a fluorescence stereomicroscope. All images were generated on a Zeiss Axio Imager A2 or a Zeiss LSM 700 microscope.

METHODS SUMMARY

Ciona intestinalis transgenesis, and protein–RNA double-labelling assays, were performed as described previously.29 All enhancer and misexpression sequences were cloned into a pCESA vector using the primer pairs shown in Supplementary Table 1. The Zick, Mss, FoxD, Tbr, Ttp, Brachyury and Dmef enhancers have been described previously.29,30,31,32,33,34,35. Photoconversion of Kaede was achieved by treating tailbud embryos with ultraviolet light using a fluorescence stereomicroscope. All images were generated on a Zeiss Axio Imager A2 or a Zeiss LSM 700 microscope.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions P.B.A designed and performed most experiments in consultation with M.L. E.W. isolated the cis-regulatory element for the β-crystallin reporter and made the stable β-catenin transgene. A.H.N. examined Mech2 and Erg expression in wild-type and reprogrammed tailbud embryos. P.B.A., M.L. and E.W. wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.L. (mievine@berkeley.edu).
METHODS

Embryo preparation and imaging. *Ciona intestinalis* adults were obtained, *in vitro* fertilized and electroporated for transient transgenesis as described29. For each electroporation, typically 70 μg of DNA was resuspended in 100 μl buffer. Embryos were fixed at the appropriate developmental stage for 15 min in 4% formaldehyde. The tissue was then cleared in a series of washes of 0.01% Triton-X in PBS. Actin was stained overnight with Alexa-647-conjugated phalloidin at a dilution of 1:500. Samples were mounted in 50% glycerol in PBS with 2% DABCO compound for microscopy. Differential interference-contrast microscopy was used to obtain transmitted light micrographs with a Zeiss Axio Imager A2 using the 340 EC Plan Neofluar objective. Confocal images were acquired on a Zeiss LSM 700 microscope using a plan-apochromat 320 or 340 objective. Confocal stacks contained approximately 50 optical slices at a thickness of 1 to 2 μm each. Images were rendered in three dimensions using Velocity 6 with the three-dimension opacity visualization tool. For time-lapse microscopy, larvae and juveniles were anaesthetized in artificial sea water supplemented with 0.04% tricaine mesylate in a glass-bottom dish. Time-lapse images were taken on a Zeiss LSM 700 microscope at intervals of 3 to 4 min.

Molecular cloning. The University of California Santa Cruz Genome Browser Gateway facilitated the identification of conserved non-coding sequences between *Ciona intestinalis* and *Ciona savignyi*. Primers (Supplementary Table 1) were used to PCR (polymerase chain reaction) amplify these putative enhancer sequences which were cloned into a pCESA vector containing an HA-NLS peptide and a WRPW repressor motif using NheI or SpeI sites. Additional coding sequences for Ets–VP16, Ets–WRPW and ATF were subcloned from existing expression vectors13.

In situ hybridization and immunohistochemistry. The double-fluorescent *in situ* hybridizations and immunohistochemistry were performed as described30 using linearized complementary DNA clones for Wnt7 (cilv33g04), FoxD (citb8o13), Erg (cilv04i11), Mech2 (cilc04m09), and Twist (cilc20p07) from the cDNA library of N. Satoh.

Kaede lineage tracing. Embryos electroporated with Tyr–Kaede and co-electroporated with Mitf–lacZ or Mitf–Twist were developed in the dark until the late tailbud stage. Tailbuds expressing Kaede were then photoconverted with UV light using the DAPI filter on a Zeiss Stereo Lumar.V12 for 3 min. Embryos were continuously reared in the dark from tailbud stages and prepared for imaging.