The vertebrate-specific VENTX/NANOG gene empowers neural crest with ectomesenchyme potential

Pierluigi Scerbo\textsuperscript{1,2} and Anne H. Monsoro-Burq\textsuperscript{1,2,3*}

INTRODUCTION

The neural crest (NC) is a multipotent cell population of ectodermal origin induced during gastrulation at the neural (plate) border (NB) of vertebrate embryos. Following a process of epithelial-to-mesenchymal transition (EMT) and extensive migration, NC cells colonize disparate embryonic locations, where they undergo terminal differentiation. NC cells form craniofacial bone, mesenchyme and cartilage, pigmented cells, and peripheral nervous system of the adult organism. The NC is a vertebrate-specific innovation proposed to be crucial for the elaboration of the vertebrate “new head” (vertebrates are thus referred to as “craniata”), for the development and evolution of sophisticated central and peripheral nervous system, favoring vertebrate predatory and active lifestyle as well as high adaptability to disparate ecological niches (1). Paleontological studies date the origin of vertebrates during the Cambrian period [541 to 485 million years (Ma) ago] (2). The presence of NC cells forming pigment, neurons, and cartilage in lamprey and hagfish embryos, two lineages that diverged from other vertebrates 505 Ma ago during the Cambrian ages (3), indicates that the last common ancestor of vertebrates likely had multipotent NC cells. Functional and molecular analyses in vertebrate embryos have demonstrated that Pax3/7, Zic, Msx, Tfap2, and Znf703 transcription factors are key players shaping the NB during gastrulation (4–6). Feedback interactions within NB circuitry ensure the activation of the early NC specification module (snai1/2, foxd3, and soxE genes, including genes important for stem cell self-renewal such as myc, and for EMT such as twist1) (7–11). Subsequently, in the cranial part of the embryo, the NB and early NC circuitry collaborate to activate in parallel the ectomesenchyme module, driven by the sox9 and twist1 genes (12–14), and a sensory neural progenitor regulatory module, driven by the runx1, pou4f1 (Brn3a), and tlc3 genes (table S3) (15). A molecular antagonism between foxd3 and sox9/twist1 promotes either the neural or the ectomesenchyme program, respectively, in cranial NC (9–11). In trunk NB/NC, foxd3 initially promotes several NC fates, including melanoblasts and neurons, while at later stages, a conserved circuitry comprising foxd3 and sox9/twist1 controls the commitment to a sensory neural identity (9, 13–15). Unexpectedly, multiple analyses propose that the global architecture of the gene regulatory network (GRN) controlling vertebrate NB/NC identity, multipotency, and fates is not a vertebrate-specific innovation but is a synapomorphic trait of bilaterian ectodermal cells fated to become sensory neurons (6, 16–20). Functional and scRNA-seq (single cell RNA sequencing) revealed that urochordates had putative proto-NC cells, the bipolar tail neurons (BTNs). BTNs share both expression of NB/NC specifiers (pax3/7, zic, msx, tfap2, znf703, snai, and foxd) and developmental equivalence with vertebrate NB/NC-derived sensory neurons [either intramedullary Rohon-Beard (RB) neurons or extramedullary dorsal root ganglia (DRG)]. Pou4 orthologs play a conserved function in controlling the sensory neural identity both in urochordates and vertebrates (12, 18, 21, 22). The discovery and molecular characterization of urochordate proto-NC cells has strengthened the hypothesis that a key step for the rise of vertebrate NC was the acquisition of a “multipotent progenitor regulatory state” in BTN-like cells, acting upstream of the commitment toward neuronal and ectomesenchyme lineages (18–23). However, the existence and the identity of molecular determinants that might confer such a regulatory state are still obscure. Therefore, how the vertebrate NB/NC territory is endowed with broader developmental potential than its invertebrate counterpart remains unanswered. Here, we present evidence suggesting that the ontogenetic and phylogenetic origin of vertebrate NC multipotency and ability to form cranial ectomesenchyme are intimately linked to the origin and evolution of the VENTX/NANOG gene family, a vertebrate-specific innovation important for the acquisition of NC identity and its functional multipotency in vivo.

RESULTS

Identification of a vertebrate-specific genetic innovation required for ectomesenchyme formation

The origin of key players controlling the broad developmental potential of embryonic cells, the POU5/OCT4, VENTX, and NANOG gene families, has so far been traced back to a stem gnathostome ancestor (24–26). We asked whether the phylogenetic origin of these
genes was linked to the vertebrate phylum and, thus, would represent a pan-vertebrate–specific transcriptional toolkit. By using public databases (National Center for Biotechnology Information (NCBI), Ensembl, JGI, SIMRBASE), we collected putative ventx orthologs from six cyclostome species (fig. S1A). Protein alignment (fig. S1A), three-dimensional (3D) protein reconstruction (fig. S1B), phylogenetic reconstruction (fig. S1C), and synteny analysis (fig. S1D) unambiguously demonstrated the existence of ventx orthologs in cyclostomes. In the bagfish genus, we retrieved two ventx-related sequences that colocalized in the same genetic locus. One of these sequences rooted with vertebrate nanog orthologs, suggesting that nanog is likely a ventx paralog issued by tandem duplication in the ancestor of living vertebrates (fig. S1E). Only conserved in marine gnathostomes, ved was also a ventx paralog that arose in a stem gnathostome ancestor and was lost in the last common ancestor of living tetrapods. Therefore, vertebrate ventx and nanog genes belong to the same family (VENTX/NANOG family). The most similar sequences to the VENTX/NANOG family that have been retrieved in urochordates and cephalochordates rooted with slowly evolving BSX/BARH family genes. Intron-exon structure (fig. S1F) and synteny analysis (fig. S1G) suggested that ventx and nanog genes are vertebrate-specific genetic innovations. Similarly, pou5f3 and pou5f3 genes have been retrieved in cyclostomes (fig. S1H and I). This led us to hypothesize that the integration of two copies of POU5F3/POU5F3 and Pou5f3 and two copies of VENTX/NANOG (ventx and nanog) in the genome of the last common ancestor of living vertebrates could have participated in the acquisition of vertebrate-specific traits in NC cells.

In amphibian embryos, the VENTX/NANOG ortholog ventx2 was expressed broadly in the NB/NC at the end of gastrulation, overlapping with the NB/NC markers pax3, myc, and snai2 (fig. 1B). Using our previous transcriptome-wide spatiotemporal mapping of the ectoderm germ layer in frog neurulas, we find ventx2 transcripts enriched in the NB ectoderm compared with the neural plate, and enriched relatively to the average levels in whole embryo (fig. S2A) (29). This distribution supports the hypothesis that Ventx2 could play cell-autonomous roles in the NB ectoderm. We, thus, analyzed the phenotype of embryos depleted for Ventx2 using a series of validated markers characterizing the different stages of NC development and the formation of specific lineages (details and references for each marker are listed in table S3). Unilateral depletion of Ventx2 by targeted injection of a validated translation-blocking morpholino (MO) (24, 30) affected neither the early expression nor the maintenance at later neurula stage of the NB markers pax3, zic1, and tfap2a, or of the pan-neurectoderm marker sox2 (fig. 1C, C and D, and fig. S2). In contrast, the induction of the early NC marker snai2 was defective at the late gastrula stage (fig. 1C and fig. S2D). At the mid and late neurula stages, Ventx2 depletion had no notable effect on the expression of a subset of early pan-NC markers such as myc, tfap2a, and foxd3, while it strongly affected other NC species, including snai2, sox9, sox10, and twist1. This suggested that the induction of the NC population had occurred on the injected side, with a partial specification for soxE genes and EMT regulators (snai2 and twist1).

In addition, the early expression of the RB markers pou4f1 (brn3a) and runx1 was not dependent on Ventx2 activity (fig. 1, D and E, and fig. S2, F and G). At tailbud stages, Ventx2 depletion reduced cranial NC migration toward the pharyngeal arches, as seen by the scarce green fluorescent protein (GFP)–positive cells, migrating ventrally and altered sox9 and twist1 expression in the reduced NC-derived frontal, mandibular, hyoid, and branchial stem (fig. 1F and fig. S3A). In contrast, at the same stages, the dorsal expression of cranial and trunk sensory neural progenitor markers remained unaltered in morphants (late foxd3 staining and pou4f1 expression in the prospective sensory ganglia and neurons; fig. 1G and fig. S3B). At differentiation stages, morphant tadpoles consistently showed strongly reduced craniofacial skeleton, while numerous cranial and trunk melanocytes formed from Ventx2-depleted NC (fig. 1H and fig. S3C). A subset of tadpoles showed reduced lateral melanocyte population in the trunk, but this effect was batch dependent and observed mainly for the strongest depletion conditions (fig. S3, C to E). In contrast to the major skeletal defects anteriorly, the morphant tadpoles responded normally to touch (on the injected side) by swimming away (fig. S3, E to H), suggesting that both peripheral sensory and motor nervous systems were functional. Last, we could trace MO-injected cells in late tadpoles, either forming melanocytes (cranial and trunk), mesenchyme above the neural tube and in the fin, Rohon-Beard intramedullary sensory neurons, and extramedullary DRG (fig. 1H). Together, these observations indicated that Ventx2 depletion, although altering early NC cells specification, did not prevent their differentiation toward the melanocyte and sensory lineages, but deeply and preferentially affected cranial ectomesenchyme NC derivatives. In sum, these results suggested that during neurulation, Ventx2 is needed to activate a subset of NC specifiers critical for the development of the ectomesenchymal lineage.

**Ventx2 is required for direct ectoderm programming toward NC**

To strengthen the results obtained in vivo above, and further test whether Ventx2 acted as a global regulator of NC identity or was required for the activation of selected NC genes acting in specific lineages, we used an independent assay using the direct induction of ectoderm into NC by the NB specifiers Pax3 and Zic1 (“induced NC” (iNC) assay) (2A) (5). This experimental “organoid” model allows NC induction and differentiation within the ectoderm-derived spheroid. The steps of EMT and migration can be bypassed if the cells are not put in presence of a fibronectin substrate. In this case, the differentiation into multiple derivatives occurs in situ (5). This assay, thus, allows us to evaluate iNC differentiation into multiple cell derivatives independently of potentially defective NC migration. Responsive ectoderm, injected with inducible forms of Pax3 and Zic1, was either cultured in control medium (not induced, –DEX, expressing k81a1 non-neural ectoderm marker) or induced at early gastrulation stage (forming iNC). Alternatively, the ectoderm was cojected with Pax3, Zic1, and Ventx2MO, followed by activation of Pax3 and Zic1 at early gastrulation stage. NC general specification and early lineage specification were first analyzed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) at a stage equivalent to late neurulation (st.18; Fig. 2, A and B, and fig. S4B). The expression of k81a1 was abolished in both wild-type (wt) and morphant iNC (fig. S4B). However, the induction of snai1, snai2, sox8, sox9, sox10, and twist1 expression by Pax3 and Zic1 was reduced upon Ventx2 depletion (Fig. 2B), similar to the observations in vivo (Fig. 1)
Fig. 1. Vertebrate-specific VENTX/NANOG is required for NC specification and ectomesenchyme development. (A) Schematic representation of VENTX/NANOG and POUS/OCT4 evolutionary history. (B) Expression profile of pou5f3.1, ventx2, pax3, and snai2 in early-neurula-stage embryos. (C to E) Eight-cell stage embryos injected in one dorsal-animal blastomere with 10 ng of ventx2-MO and 50 pg of GFP mRNA were processed for whole-mount in situ hybridization (WISH) at the late gastrula (st.13), early neurula (st.15), and late neurula (st.18) stages with the indicated probes. (F) GFP-labeled wild-type (wt) NC efficiently migrates and populates the branchial arches. In contrast, Ventx2 morphant NC exhibited defective migration, resulting in a reduced GFP-positive cranial area. Embryos were processed for WISH with sox9 and twist1 probes. (G) Embryos injected as in (C) were processed for WISH at the tailbud stage (st.25), with foxd3 and pou4f1 probes revealing normal sensory neuron development. (H) Craniofacial morphology in tadpoles (st.45): Morphant craniofacial morphology was strongly affected compared with the control side; cartilage dissection highlighted severe craniofacial defects, with reduced pharyngeal arch area in the Ventx2 morphant side. In contrast, morphant neural crest–derived melanocytes were detected in the dorsal cranial area above altered cartilages (live imaging). Moreover, mutant neural crest–derived cells (GFP-labeled) were detected in the mesenchyme above the neural tube and in the fin (a), in sensory Rohon-Beard neurons in the dorsal neural tube (labeled by kcnal1; b), and in the dorsal root ganglia (labeled by tlx3; c). Asterisks indicate injected side. Dotted lines indicate area of interest. A.U., arbitrary units. Asterisks indicate the injected side in control (red) or morphant (blue) embryos.
Fig. 2. Ventx2 is required for ectoderm direct programming into NC. (A and B) Two-cell-stage embryos were coinjected with 50 pg of inducible Pax3-GR and 50 pg of inducible Zic1-GR mRNA, with or without 10 ng of Ventx2-MO in each blastomere. Animal caps were explanted at blastula stage 9, cultured in the presence of ethanol (control, not induced) or dexamethasone (+DEX) starting at the gastrula stage (st.10.5), collected at the late neurula stage (st.18), and processed for reverse transcription quantitative polymerase chain reaction (RT-qPCR) to detect the indicated gene expressions. (C and D) Eight-cell-stage embryos injected in one dorsal-animal blastomere with 10 ng of Ventx2-MO and 50 pg of GFP mRNA were processed for WISH at the early neurula (st.15) and tailbud (st.25) stages with the indicated probes (see fig. S4 for late neurula stage). (E) The functional redundancy between frog Ventx2 and mouse Nanog was assessed by a rescue experiment: Ventx2 depletion was efficiently rescued by mNanog mRNA overexpression, restoring twist1 expression at the late neurula stage as well as global craniofacial morphology (see full fig. S7). Red asterisks indicate injected side. For qPCR graphs, error bars represent SEM values of five independent experiments with two technical duplicates. Student’s t test was used to determine statistical significance by unpaired Student’s t test. *P < 0.05, **P < 0.005, ***P < 0.0001.
and fig. S4A). In contrast, the induction of *znf703*, a known direct target of Pax3 and Zic1 at the NB, *foxd3, myc, ets1*, as well as *tfap2e*, a trunk NC early specifier, occurred whether Ventx2 was present or depleted in iNC (Fig. 2B and fig. S4B). This indicated that NC specification had occurred at least partially. In addition, expression of early sensory lineage markers *tlx3* and *runx1* was normally induced in morphant iNC, indicating that the sensory fate was activated in late-neurula-stage morphant iNC (Fig. 2B), matching the observations in vivo. In contrast, the earliest expression of *mitf* was poorly activated in morphant iNC (fig. S4B). Additional in situ analysis in neurulas and tadpoles further confirmed that expression of *tfap2e, tlx3,* and *znf703* did not depend on Ventx2 activity in vivo, while the sibling morphant embryos exhibited severely reduced *twist1* expression (Fig. 2, C and D, and fig. S4, C and D). Furthermore, in older iNC (analyzed at the equivalent of tailbud stage, st.35), we found that activation of ectomesenchyme markers (*runx2, twist1,* and *col2a1*) and melanoblast (*dct*) markers did not occur in the absence of Ventx2 (fig. S4E), whereas *foxd3,* labeling sensory neural progenitors at this stage, and the neurotransmitter *slc17a7 (vglut1),* marking differentiated sensory neurons, remained significantly activated morphant iNC (fig. S4E). In addition, the expression of *slc17a7* was normal on the injected side of tadpoles in vivo (fig. S4E). Globally, this analysis on NC directly induced from ectoderm showed that Ventx2-depleted ectoderm responded to induction by the NB specifiers Pax3 and Zic1, activated a subset of general NB/NC markers (*znf703, foxd3, myc,* and *ets1*), but failed to activate another subset of early NC specifiers (*snai2 and soxE* genes, *twist1*) and later ectomesenchyme lineage regulators and differentiation markers (*sox9, twist1, runx2,* and *col2a1*). In this assay, the early expression of *mitf* and expression of melanoblast gene (*dct*) were dependent on Ventx2, while the effect on melanocytes in vivo was not notable, suggesting that some compensatory mechanism may relay Ventx2 depletions in vivo. Furthermore, the sensory neuron fate was efficiently activated (*runx1, tlx3,* and *slc17a7*) both in iNC and in vivo (Figs. 1 and 2 and fig. S4, C to F). Last, to test whether Ventx2 and Nanog function was conserved, we rescued the iNC in vivo Ventx2 depletions by activating either Ventx2 (*Xenopus laevis*) or Nanog (*Mus musculus*). Both Ventx2 and Nanog showed efficient restoration of *twist1* and *sox9* expression in vivo, in early neurulas, as well as rescue of the craniofacial defects in tadpoles (Fig. 2E and fig. S7). Collectively, these results show the strong parallels between the function of Ventx2 in vivo and in iNC and demonstrate the evolutive conservation of Ventx2 and Nanog in the context of NC development.

Ventx2 promotes a multipotent-like NC state in NB ectoderm

To understand how Ventx2 may alter early NC specification and the emergence of specific lineage fates, we postulated that Ventx2 might be needed for the expression of multipotency-related genes (details in table S3). *Pou5f3/Oct4* genes label multipotent NC stem cells in zebrafish and chick embryos but not committed NC cells (13, 31). We, thus, analyzed the expression of *pou5f3.1* (oct91) in the iNC assay. At late neurula stage, *pou5f3.1* was similarly expressed in wt and morphant iNC, in both cases at lower levels than in the ectoderm (Fig. 2B). We further tested the induction of the telomerase reverse transcriptase gene *tert,* a readout of the activity of pluripotency factors such as Oct4, and of the ten-eleven translocation family dioxygenase *tet3,* involved in somatic reprogramming and lineage specification, including neural and NC in frogs (see table S3). Both were strongly activated in wt iNC, but significantly reduced in Ventx2 morphant iNC (Fig. 2B). In conclusion, depletion of Ventx2 in iNC altered the expression of *tert* and *tet3,* genes involved in the regulation of the broad developmental potential of cells in various models. Our observations thus highlight the previously unidentified and robust activation of these factors by the NB specifiers Pax3 and Zic1. In addition, we show that Pax3 and Zic1 do so in a Ventx2-dependent manner.

We then sought to understand the developmental timing and the biological relevance of Ventx2 activity during NC ontogeny in vivo. We injected an inducible form of Ventx2 (Ventx2-GR), targeting a dorsal-animal blastomere (31). We then activated Ventx2 either at the onset (st.10.5) or during the second half of gastrulation (st.11.5 to st.12) (Fig. 3 and fig. S5A). Focusing on embryos with normal gastrulation (fig. S5), we observed that the gain of Ventx2 activity in early gastrula ectoderm resulted in reduced expression of *snai2* and *twist1* (Fig. 3, A to C), indicating that NC induction was defective and that a smaller NC domain formed. In parallel, the expression of the early-expressed pluripotency gene *pou5f3.2* (oct25) was increased (Fig. 3C). In contrast, Ventx2 activation at the late gastrulation stage did not affect neural and NB markers (*sox2* and *znf703*), while it expanded expression of NC specifiers *snai2, foxd3, sox9,* and *twist1* (Fig. 3, A to D). The expansion was observed laterally, toward the non-neural ectoderm adjacent to the NC territory (Fig. 3, A to C) but not ectopically when the injection targeted farther lateral or ventral areas (fig. S5B), indicating that Ventx2 activation was not sufficient to elicit induction of these genes outside the dorsal ectoderm. Later expressed NC markers *sox10, tfap2e,* and sensory progenitor marker *tlx3* remained globally unaffected or slightly reduced (Fig. 3D). In the case of multipotency genes, Ventx2 gain of function at the late gastrula stage reduced *pou5f3* expression (Fig. 3C), but expanded the expression of the late-stage *oct4* ortholog *pou5f3.1* (oct91) and increased the level of expression for a series of stemness and multipotency-related factors: *tert, tet3, kdm4a,* *chd7,* and *smarcA4* (Fig. 3E and table S3). This suggested that Ventx2 cooperated with factors present close to the NB/NC domain to activate a subset of multipotency-related regulators and of early NC specifiers in late gastrulas. In contrast, the expression of later NC specifiers (*sox10,* sensory neuron markers (*tfap2e* and *tlx3*), or neural and NB markers (*sox2, znf703*) were not affected by Ventx2 gain of function in the second half of gastrulation (Fig. 3D). Thus, these results suggested that Ventx2 could promote/maintain a stem and multipotent-like state in early NC, in cooperation with other factors of the NB/NC-GRN.

**Ventx2 allows late Pax3/Zic1-dependent reprogramming of NB ectoderm into NC in vivo**

Since Ventx2 was necessary for the acquisition of a complete NC identity, for its multipotency in vivo, and could promote NC stem cell and multipotent state, we asked whether it was sufficient to confer an NC-inducing activity to the NB specifiers Pax3 and Zic1, at a stage when normally they had lost their capacity to reprogram embryonic ectoderm to NC identity in vitro (3). Here, in vivo unilateral activation of Pax3 and Zic1 at the late gastrula stage (st.12), targeted to ectodermal cells and observed in late neurulas, enhanced expression of the sensory neural progenitor marker *tlx3* (and moderately expanded *tfap2e*). It did not affect the patterns of epidermal (*sox9* and *sox10*) and neural (*sox2*) markers but globally altered early pan-NC identity (reduced *snai2, sox9,* *sox10, twist1,* and *foxd3* expression) (Fig. 4A). In sharp contrast, coinjection of *pax3/zic1* together with *ventx2,* all activated at the late gastrula stage (st.12), enhanced expression of the NC specifiers (*snai2, sox9, sox10, twist1,* and *foxd3*), strongly blocked expression of an epidermal marker (*sox8a1*), and reduced expression of sensory neural progenitor genes (*tfap2e* and *tlx3*) (Fig. 4B). This
suggested that ectopic expression of Ventx2 at the late gastrulation stage could reorient NB specifiers to activate a whole set of early NC genes. We hypothesized that these genes could be linked to the ectomesenchyme fate rather than sensory neural progenitor lineage. To check if their expanded activity ectopically around the NB would result in ectopic specification of various NC lineages, we analyzed later developmental stages. At tailbud stages, embryos injected with pax3/zic1 and ventx2 still displayed dorsally enlarged and/or ectopic expression of several NC markers: sox10, snai2, twist1, and foxd3 (Fig. 4C and Fig. S6A). We also observed increased expression of tfap2e in both conditions (pax3/zic1 with or without Ventx2; fig. S6A), indicating that the early lack of expression (Fig. 4A) was rather a delayed expression, perhaps due to increased expression of the multipotency-related genes described above. In addition, lineage-specification
Fig. 4. Ventx2 confers to neural plate border specifiers Pax3 and Zic1 the capacity to reprogram non-neural ectoderm to NC identity. (A) Two-cell-stage embryos coinjected in one blastomere with 50 pg of Pax3-GR, 50 pg of Zic1-GR, and 50 pg of GFP mRNAs were cultured in the presence of dexamethasone (+DEX) from the late gastrula stage (st.12) and processed for WISH analysis at the mid-neurula (st.18) with the indicated probes. At this late gastrula stage, the ectopic activation of Pax3 and Zic1 repressed early NC genes (snai2, sox9, sox10, twist1, and foxd3) and enhanced the sensory neural progenitor marker tlx3 (tfap2e remaining unchanged). (B) Embryos injected as in (A) together with 800 pg of Ventx2-GR mRNA were cultured in the presence of dexamethasone (+DEX) from the late gastrula stage (st.12) and processed for WISH at the late neurula stage (st.18), with the indicated probes. When coinjected with Ventx2, Pax3 and Zic1 expanded early NC genes (snai2, sox9, sox10, twist1, and foxd3) and repressed sensory neural progenitor genes (tfap2e and tlx3) and epidermal genes (xk81a1). (C) Embryos injected as in (A) and (B) were cultured in the presence of dexamethasone (+DEX) from the late gastrula stage (st.12) and processed for WISH at tailbud (st.25) with sox10 and snai2 probes. Only the Pax3/Zic1/Ventx2 combination induced ectopic expression of sox10 in the non-neural ectoderm (a strong phenotype is shown) and weaker ectopic snai2 expression. (D) Model of Ventx2 function in vertebrate NC development. Schematic illustration of the NB in invertebrates (left) and in vertebrates (right). We propose that the vertebrate NC (violet and red dots) evolved from an ancestral condition (violet dots) shared with invertebrates, by the introduction of Ventx/Nanog activity, which conferred multipotency and acquisition of ectomesenchyme fate.
markers $\text{dct}$ and $\text{scl7a7}$ were reduced, but we did not observe ectopic ectomesenchyme marker expression, suggesting that ectopic NC had not entered into a lineage-restricted state. Together, our results suggested that late-stage $\text{Ventx2}$ activity allows NB specification to reprogram embryonic ectoderm toward an early NC identity, which is further maintained in an immature and undifferentiated state.

**DISCUSSION**

The evolutionary origin and the molecular nature of vertebrate NC multipotency have been a matter of intensive studies, but so far, no vertebrate-specific gene innovation has been found to explain how the vertebrate NC may have acquired its multipotency. Here, using a phylogenetic analysis, we have demonstrated that the origin of the $\text{VENTX/NANOG}$ family was correlated with the emergence of vertebrates. Further maintenance and expansion of this family in vertebrates suggested that $\text{VENTX/NANOG}$ factors might confer a selective and heritable advantage to vertebrates by the means of survival, adaptation, and fitness manifested in the adult phenotype. This family is intimately related to the control of cell developmental potential and to the process of nuclear reprogramming (27, 30, 32–34).

To date, the architecture of the NB/NC circuity is a constrained and synapomorphic trait of bilaterians, whereas expression of $\text{ventx}$, $\text{nano}$, and $\text{vef}$ in NB/NC cells is a synapomorphic trait of vertebrates (13–30). To infer a causal link between $\text{VENTX/NANOG}$ emergence and their putative role in NC, we have modulated $\text{Ventx2}$ activity at several time points during vertebrate embryogenesis. Our results suggest that the proposed selective advantage conferred by the $\text{VENTX/NANOG}$ family could relate to vertebrate NC multipotency, as $\text{Ventx2}$ depletion led to the loss of NC selected lineages, affecting strongly ectomesenchyme and moderately pigment fates, resulting in a bias toward the neurogenic fate reminiscent of the unipotent ancestral bilaterian condition (Fig. 4D). Endogenous expression of the neurogenic markers ($\text{pou4f1}$, $\text{tlx3}$, and $\text{runtx1}$) was not dependent on $\text{Ventx2}$ activity. $\text{Ventx2}$-depleted embryos developed medullary and extramedullary sensory neurons, the vertebrate homologs of invertebrate proto-NC cells (e.g., the urochordate $\text{BTNs}$) (18, 21, 22). In addition, we showed that sustained $\text{Ventx2}$ activity during late gastrulation, but not before, enhanced NC identity, suggesting that the developmental function of $\text{Ventx2}$ in promoting NC identity was uncoupled from its role in pluripotent blastula cells (24–33). Activation of $\text{Ventx2}$ during late gastrulation led to the local expansion of the stem cell marker ($\text{pou5f3}$, $\text{oct4}$) and increased expression of other genes linked to broad developmental potential and reprogramming ($\text{tert}$, $\text{tet3}$, $\text{kdm4a}$, $\text{smarca4}$, and $\text{chd7}$). In parallel, all early NC specifiers were activated ($\text{snail1/2}$, $\text{soxE}$, $\text{twist1}$, and $\text{foxd3}$), indicating that $\text{Ventx2}$ promoted a multipotent NC stem cell progenitor state at the neural plate border. Furthermore, we found that later on, $\text{Ventx2}$ was sufficient to reprogram the NB circuity activity toward ectopic undifferentiated/immature NC fate in vivo. This paralleled the recent finding showing that $\text{Twist1}$ could reprogram chick NC cells biased to sensory neural identity, as well as urochordate $\text{BTNs}$, to ectomesenchyme-like identity, thus acting as an instructive lineage switch (12, 19). Together, our results, thus, suggest that $\text{Ventx2}$ action is twofold: first, $\text{Ventx2}$ participates in the acquisition of the early pan-NC regulatory program at the NB, thereby enhancing the coexpression of NC stem cell early markers ($\text{snail1/2}$, $\text{foxd3}$, and $\text{pou5f3}$), and second, $\text{Ventx2}$ is essential for the formation of the ectomesenchyme lineage. Thus, $\text{Ventx2}$ acts as a positive and permissive regulator at multiple steps of the NC-GRN. Our data support the view that a key step for innovation in vertebrate NC was the acquisition of a “multipotent progenitor regulatory state” in proto-NC cells of a vertebrate ancestor, upstream of both neural and ectomesenchyme potential (Fig. 4D) (1, 18, 23). This new multipotent and permissive regulatory state would have opened the possibility for co-opting new genes and promoting new fates into the NC-GRN. We propose that genetic innovation and neo-functionalization linked to $\text{VENTX/NANOG}$ (and $\text{POU5/OCT4}$) have helped the emergence of new competence in cells at the NB of a proto-vertebrate ancestor, allowing emergence of alternative cell fates. This implies that such innovation was not detrimental for preexisting cell fate programs (e.g., neurogenic) but permissive for a new one (ectomesenchyme). Heritable competence to form ectomesenchyme and/or neurogenic and melanogenic derivatives in NB progenitors would have arisen in the stem vertebrate ancestor. Selective and progressive stabilization of preexisting network(s), anchored in the evolution by the mean of genetic functional conservation predating vertebrate ancestor, would stabilize the competence for alternative cell fates in NC. In agreement with this hypothesis, recently, Martik and colleagues have proposed that the NC of basal vertebrates such as lamprey would be closer to the trunk NC in gnathostomes (35) and that the addition of new genes such as $\text{ets1}$ in the NC-GRN would have participated in the acquisition of the ectomesenchyme fate and its regionalization to cranial areas in jawed vertebrates. Our results show that in the absence of $\text{Ventx2}$, cranial NC, $\text{SoxE}$ activation (a pan-vertebrate NC feature), and ectomesenchyme formation are severely impaired, whereas trunk NC forms rather normally. We find that $\text{ets1}$, a pan-gnathostome feature (35), is normally induced in the iNC assay, independently of $\text{Ventx2}$ (Fig. S4), reinforcing the idea of a sequential and independent series of events promoting the vertebrate new head during evolution (1).

Last, we show that $\text{Ventx2}$ and $\text{Nanog}$ are functionally equivalent in NC formation and propose that the $\text{VENTX/NANOG}$ family would play a key evolutionary role in shaping such a multipotent regulatory state once added to the ancestral NB circuity ($\text{pax3/7}$, $\text{zic}$, $\text{mxe}$, $\text{tfap2}$, $\text{znf703}$, $\text{snai}$, $\text{foxd}$, and $\text{pou4}$) (6, 16–18, 21, 22). $\text{VENTX/NANOG}$ could have allowed the coexistence of multiple and alternative programs in proto-NC cells of a vertebrate ancestor, thus preserving the balance among new and ancestral fates of NB cells for further selection and evolution (Fig. 4D). Since $\text{Ventx2}$ also participates in reprogramming the late gastrula neurogenic NB to multipotent early NC fates, this raises the intriguing possibility that early NB cells may also undergo an endogenous reprogramming step to become multipotent NC during early embryogenesis. Our extensive phylogenetic analysis of the $\text{VENTX/NANOG}$ family in vertebrates (fig. S1) and the demonstration of their role and functional equivalence for NC development (Fig. 2 and fig. S7) bring important insights and will probably stimulate further studies on $\text{Ventx}$ function in amniotes. Last, whether a shared genetic circuity acts both in embryonic NC and adult multipotent NC stem cells retained postnatally (36) would be important in future investigations on tissue repair and regenerative medicine.

**MATERIALS AND METHODS**

* Xenopus embryo manipulation and explant culture: European and National Regulation for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes was strictly applied (license ¦ C91-471-108, Direction Départementale de Protection de la Population, Courcouronnes, France).
X. laevis embryos were obtained by in vitro fertilization, dejellied, injected, and cultured in Marc’s modified Ringer’s using standard procedure. Embryos were staged as in Nieuwkoop and Faber (37). Capped mRNAs for in vivo injection were synthesized with mMessage mMachine kits (Ambion, Austin, Texas). The pT7TSHA-GR-Vent2 plasmid was linearized with Xba I and mRNA was synthesized with T7 polymerase. The pT7TSHA-GR-Nanog was cloned by inserting mouse Nanog cDNA sequence instead of the X. laevis Ventx2 sequence. The pCS2 + Pax3-GR and pCS2 + Zic1-GR plasmids were used as described (5, 38). Inducible Pax3 and Zic1 (Pax3-GR and Zic1-GR), Nanog-GR, or Ventx2-GR was activated by dexamethasone at the indicated stages. Controls included injected embryos grown in 0.2% ethanol and un.injected embryos grown in dexamethasone. The Ventx2-MO was described in (24, 30). Embryos were injected unilaterally (in one dorsal-animal blastomere at the eight-cell stage) for whole-mount in situ hybridization (WISH)/morphology analyses. Lineage tracing was achieved by coinjection of Histone2B-GFP or membrane-bound GFP-CAAX. This injection in one dorsal-animal cell at the eight-cell stage targets the NB and NC predominantly but not exclusively [as detailed in fate maps by (39)]. For animal cap experiments, the two blastomeres of stage 2 embryos were injected at the animal pole. The blastocoeil roof ectoderm was dissected at stage 9 using Dumont #5 forceps and dishes coated with 3/4 normal amphibian medium (NAM)/2% agarose filled with 3/4 NAM. Small squares were cut to avoid taking ectoderm already subjected to the marginal zone inductive influence. Animal caps were grown at 15°C until the sibling controls reached stage 18 or 35. Throughout the study, each injection experiment was performed three or more times on different batches of embryos.

Phylogenetic analysis
Known VENTX, NANOG, BSX, BARH, and DBX orthologs were retrieved from public repositories (NCBI-GenBank, Ensembl, JGI Genome Browser, and SIMRBASE Genome Browser). BLAST (tblastn) searches were performed against available genomes and/or transcriptomes using the most conserved regions (encoded by the second and third exons, including the homeobox) of human, xenopus, coelacanth, zebrafish, and elephant shark VENTX and NANOG proteins as queries. The screened dataset was chosen so as to ensure the broadest taxonomic range among craniates (including cyclostomes and chondrichthyan) and invertebrate chordates (including urochordates, cephalochordates, and protostomes). Synteny has been performed using genome sequences available on public repositories (NCBI-GenBank, Ensembl, JGI Genome Browser, and SIMRBASE Genome Browser), and ID ENTRY is available in table S1. Translation start sites and exon–intron boundaries were assessed compiling automated predictions from the GENSCAN server (http://hollywood.mit.edu/GENSCAN.html) and manually refined on the basis of the protein sequence alignment using EXPASY Translate Tool. The sources for known or novel protein sequence models used in this study are listed in table S2. Known or predicted protein sequences were aligned using Jalview software. For phylogenetic tree reconstruction, we used the PHYML version (available online http://phylogeny.fr) and visualized using ETE Toolkit software using default parameters (http://etetoolkit.org/treeweek/). Phylogenetic tree in NEWICK format is available in table S3. 3D protein reconstruction has been performed using the I-TESSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) and visualized using DeepView–Swiss–PDB Viewer v4.1 software (https://spdbv.vital-it.ch/).

WISH, embryo sectioning, and cartilage staining
We used a fast WISH procedure optimized for NC staining (40). Plasmids used to make antisense riboprobes are available upon request. For sectioning, embryos were embedded in paraffin, and 10-μm-thick transverse sections were cut before immunostaining using commercial antibodies against GFP. For cartilage staining, tadpole embryos were stained with 0.05% Alcian Blue, destained in ethanol, rehydrated, and cleared with 4% KOH followed by graded glycerol solutions. Cartilage tissue was manually dissected out.

Reverse Transcription qPCR
Total RNA from animal cap explants was extracted and reverse transcribed. Ten animal cap explants or 10 whole embryos per biological replicate were used. The amplified material was detected using SsoAdvancedSYBR Green Supermix (#1725261; Bio-Rad) on a CFX96 Real-Time System (Bio-Rad). All results were analyzed with the Δ(ΔCq) method using ornithine decarboxylase (ode) as a housekeeping gene. Primer pairs are presented in table S2. Statistical analyses were done using GraphPad Prism.

Statistical analysis and imaging
All experiments were performed at least three times independently. The most frequent phenotypes are shown except for Fig. 4C, where a very strong phenotype is shown. For the analysis of NC surface area at migratory stage, we targeted the presumptive NC region by injecting gfp mRNA into a single blastomere at the 16-cell stage. We measured the area of gfp expression both in control and in ventx2 morphant embryos. Both NC surface area and pharyngeal cartilage area at the tadpole stage were measured using ImageJ software. The pigmented area was quantified by using ImageJ intensity quantification. The signal of pigmented cell (black in brightfield) was inverted, quantified, and compared to the same surface area among tadpoles. Statistical analyses were done using GraphPad Prism. For each phenotype, the error bars represent SEM between the experimental condition and the control condition. The number of embryos exemplified by the photographs over the total number of embryos analyzed is indicated.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/18/eaaz1469/DC1

REFERENCES AND NOTES
1. C. Gans, R. G. Northcutt, Neural crest and the origin of vertebrates: A new head. Science 220, 268–273 (1983).
2. S. C. Morris, J. B. Caron, A primitive fish from the Cambrian of North America. Nature 512, 419–422 (2014).
3. D. H. Erwin, M. Laflamme, S. M. Tweedt, E. A. Sperling, D. Pisani, K. J. Peterson, The Cambrian conundrum: Early divergence and later ecological success in the early history of animals. Science 334, 1091–1097 (2011).
4. A. H. Monsoro-Burq, E. Wang, R. Harland, Mxx1 and Pax3 cooperate to mediate FGF8 and WNT signals during Xenopus neural crest induction. Dev. Cell 8, 167–178 (2005).
5. C. Millet, F. Maczkowiak, D. D. Roche, A. H. Monsoro-Burq, Pax3 and Zic1 drive induction and differentiation of multipotent, migratory, and functional neural crest in Xenopus embryos. Proc. Natl. Acad. Sci. U.S.A. 110, 5528–5533 (2013).
6. Y. Li, D. Zhao, T. Horie, G. Chen, H. Bao, S. Chen, W. Liu, R. Horie, T. Liang, B. Dong, Q. Feng, Q. Tao, X. Liu, Conserved gene regulatory module specifies lateral neural borders across bilaterians. Proc. Natl. Acad. Sci. U.S.A. 114, E6352–E6360 (2017).
7. N. de Crozé, F. Maczkowiak, A. H. Monsoro-Burq, Reiterative AP2a activity controls sequential steps in the neural crest gene regulatory network. Proc. Natl. Acad. Sci. U.S.A. 108, 155–160 (2011).
8. T. Sauka-Spengler, D. Meulemans, M. Jones, M. Bronner-Frase, Ancient evolutionary origin of the neural crest gene regulatory network. Dev. Cell 13, 405–420 (2007).

9. N. A. Mundell, P. A. Labosky, Neural crest stem cell multipotency requires Foxd3 to maintain neural potential and repress mesenchymal fates. Development 138, 641–652 (2011).

10. N. Sasaki, K. Muzuski, Y. Sasaki, Requirement of FoxD3-class signaling for neural crest determination in Xenopus. Development 128, 2525–2536 (2001).

11. J. A. Lister, C. Cooper, K. Nguyen, M. Modrell, K. Grant, D. W. Ralife, ZebrafishFoxD3 is required for development of a subset of neural crest derivatives. Dev. Biol. 290, 92–104 (2006).

12. R. Soldatov, M. Kaucka, M. E. Kastri, J. Petersen, T. Chontorotzea, L. Engmaiera, N. Akkinatovab, Y. Yang, M. Häring, V. Dyachuk, C. Bock, M. Farlik, M. L. Piacentino, F. Boismoreau, M. M. Hilscher, C. Yokota, X. Qian, M. Nilsson, M. E. Bronner, L. Croci, W. Y. Hsiao, D. A. Guertina, J. F. Brunet, G. G. Consalez, P. Emfrs, K. Fried, P. V. Khrachenko, I. Adameyko, Spatiotemporal structure of cell fate decisions in murine neural crest. Science 364, eaaz9536 (2019).

13. M. Lukoseviciute, D. Gavriouchkina, R. M. Williams, T. Hochgreb-Hagele, U. Senanayake, V. Chong-Morrison, D. A. Guertin, J. F. Brunet, G. G. Consalez, P. Ernfors, K. Fried, P. V. Khrachenko, I. Adameyko, Spatiotemporal structure of cell fate decisions in murine neural crest. Science 364, eaaz9536 (2019).

14. N. A. Mundell, P. A. Labosky, Neural crest stem cell multipotency requires Foxd3 to maintain neural potential and repress mesenchymal fates. Development 138, 641–652 (2011).

15. J. A. Lister, C. Cooper, K. Nguyen, M. Modrell, K. Grant, D. W. Ralife, ZebrafishFoxD3 is required for development of a subset of neural crest derivatives. Dev. Biol. 290, 92–104 (2006).

16. R. Soldatov, M. Kaucka, M. E. Kastri, J. Petersen, T. Chontorotzea, L. Engmaiera, N. Akkinatovab, Y. Yang, M. Häring, V. Dyachuk, C. Bock, M. Farlik, M. L. Piacentino, F. Boismoreau, M. M. Hilscher, C. Yokota, X. Qian, M. Nilsson, M. E. Bronner, L. Croci, W. Y. Hsiao, D. A. Guertina, J. F. Brunet, G. G. Consalez, P. Emfrs, K. Fried, P. V. Khrachenko, I. Adameyko, Spatiotemporal structure of cell fate decisions in murine neural crest. Science 364, eaaz9536 (2019).

17. M. Lukoseviciute, D. Gavriouchkina, R. M. Williams, T. Hochgreb-Hagele, U. Senanayake, V. Chong-Morrison, D. A. Guertin, J. F. Brunet, G. G. Consalez, P. Ernfors, K. Fried, P. V. Khrachenko, I. Adameyko, Spatiotemporal structure of cell fate decisions in murine neural crest. Science 364, eaaz9536 (2019).

18. N. A. Mundell, P. A. Labosky, Neural crest stem cell multipotency requires Foxd3 to maintain neural potential and repress mesenchymal fates. Development 138, 641–652 (2011).

19. J. A. Lister, C. Cooper, K. Nguyen, M. Modrell, K. Grant, D. W. Ralife, ZebrafishFoxD3 is required for development of a subset of neural crest derivatives. Dev. Biol. 290, 92–104 (2006).

20. R. Soldatov, M. Kaucka, M. E. Kastri, J. Petersen, T. Chontorotzea, L. Engmaiera, N. Akkinatovab, Y. Yang, M. Häring, V. Dyachuk, C. Bock, M. Farlik, M. L. Piacentino, F. Boismoreau, M. M. Hilscher, C. Yokota, X. Qian, M. Nilsson, M. E. Bronner, L. Croci, W. Y. Hsiao, D. A. Guertina, J. F. Brunet, G. G. Consalez, P. Emfrs, K. Fried, P. V. Khrachenko, I. Adameyko, Spatiotemporal structure of cell fate decisions in murine neural crest. Science 364, eaaz9536 (2019).

21. M. Lukoseviciute, D. Gavriouchkina, R. M. Williams, T. Hochgreb-Hagele, U. Senanayake, V. Chong-Morrison, D. A. Guertin, J. F. Brunet, G. G. Consalez, P. Ernfors, K. Fried, P. V. Khrachenko, I. Adameyko, Spatiotemporal structure of cell fate decisions in murine neural crest. Science 364, eaaz9536 (2019).