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Reactivation of the pluripotency program precedes formation of the cranial neural crest

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Abstract

During development, cells progress from pluripotency to more restricted cell fates of a particular germ layer. However, cranial neural crest cells (CNCC), a transient cell population that begets most of the craniofacial skeleton, have much broader differentiation potential than their ectodermal lineage of origin. Here we identify a neuroepithelial precursor population characterized by expression of canonical pluripotency transcription factors, which gives rise to CNCC and is essential for craniofacial development. Pluripotency factor *Oct4* is transiently reactivated in CNCC and is required for subsequent formation of ectomesenchyme. Furthermore, open chromatin landscapes of Oct4⁺ CNCC precursors resemble those of epiblast stem cells, with additional features suggestive of priming for mesenchymal programs. We propose that CNCC expand their developmental potential via a transient re-acquisition of molecular signatures of pluripotency.

Cell differentiation progresses via a continuous lineage restriction process where cell potential is progressively reduced as the embryo develops. In the early embryo, pluripotent embryonic cells can differentiate into all somatic cell types, but this capacity is rapidly restricted during the formation of the three germ layers, each giving rise to specific and distinct cell types. However, in vertebrates, a stem cell-like population called the neural crest challenges this paradigm. Located at the border between the neural plate and the surface ectoderm, neural crest cells are induced as an epithelial cell type (1, 2) that subsequently

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undergoes an epithelial-to-mesenchymal transition (EMT), delaminates from the dorsal epithelium, and migrates through the embryo to populate ventral locations where they differentiate into diverse cell types (2, 3). Neural crest cells arising from the most rostral part of the embryo, called cranial neural crest cells (CNCC), generate not only derivatives typical of ectoderm, such as neurons and glia, but also give rise to cell types canonically associated with the mesoderm lineage such as bone, cartilage, and smooth muscle (4). Thus, mesenchymal CNCC derivatives, which make up most of the craniofacial skeleton, are often designated as ‘ectomesenchyme’ to differentiate them from classic mesoderm derivatives (5). The ability of CNCC to expand their differentiation potential beyond their germ layer of origin raises the question of whether this pluripotency is induced *de novo* in the ectoderm or, alternatively, retained from the early pluripotent embryo in a specific subset of neuroepithelial cells. Although the latter scenario has been suggested to be true in *Xenopus* (6), a single-cell transcriptome analysis of *Xenopus* embryogenesis did not find evidence for the maintenance of pluripotency program in developmental trajectories leading to the neural crest (7), leaving the question unresolved. Furthermore, the earliest steps of CNCC formation have not been characterized at the single-cell level in mammals, and it remains poorly understood how the expanded cell fate potential of CNCC arises during mammalian embryogenesis.

Transcriptional heterogeneity of early murine CNCC

We used single-cell RNA-seq (scRNA-seq) analysis to characterize the diversity of murine CNCC transcriptomes at four developmental stages from 4 to 10 somite stage embryos (corresponding to embryonic age (E) 8 to 8.75 days post-coitum [dpc]). In the developing head-fold this timespan captures CNCC specification in the dorsal neural folds, EMT, migration, and the earliest differentiation decisions. To label CNCC in the embryo, we took advantage of *Wnt1::Cre*, a well-established pre-migratory neural crest-specific driver (8). We generated *Wnt1::Cre; Rosa26^{TdTomato/+}* embryos at aforementioned developmental stages and used flow cytometry to isolate TdTomato⁺ (TdT⁺) cells from their *Hox*-negative cranial portions—dissected at rhombomere 1 level (Fig. 1A and S1A). Since TdT⁺ cells were first detected at the 3 to 4 somite stage, we used 4 somite stage embryos as the earliest developmental time point for our analysis. We examined single-cell transcriptomes using a modified Smart-seq2 protocol (9), which robustly detected 7000 genes per cell (Fig. S1B–C). Based on differential gene expression analysis using Seurat (10) we identified 10 cell clusters with distinct transcriptional profiles (Fig. 1B–C, S1D and Table S1), falling into two major subpopulations: neuroepithelial precursors (which encompass pre-migratory CNCC; clusters 1–4 and 10) and migratory mesenchymal CNCC (clusters 5–9) (Fig. 1B–C and S1D). Such distinct neuroepithelial and mesenchymal neural crest transcriptional programs have been previously detected in scRNA-seq studies from chick embryos (11, 12). The association of expression signatures with developmental stage from which each cell originated revealed that: (i) the majority of cells from the 4 somite stage embryos mapped to the neuroepithelial clusters, characterized by expression of previously recognized neural crest precursors with a primarily neural program (3, 11, 12), such as *Sox2*, *Zic3*, *Otx2*, *Gbx2*, *Pax2/8*, (ii) 6 somite stage was accompanied by an abrupt transcriptional identity switch, with emergence of delaminating CNCC cluster expressing canonical neural crest

specification and migration genes, such as *FoxD3*, *Sox10*, *Ets1* and *Twist1*, and (iii) by 8 somites, the majority of cells transitioned to migratory CNCC clusters and underwent first lineage commitment decisions separating ectomesenchyme from neural/glial progenitors (Fig. 1B–D, S1D and S2).

Neuroepithelial precursors could be further divided into several transcriptionally distinct subpopulations, characterized by high expression of either *Otx2* or *Gbx2*, but rarely of both (clusters 1–3 in Fig. 1B–C). *Otx2* and *Gbx2* are regionalization markers previously shown to define, respectively, anterior and posterior territories in the developing neural plate, including pre-migratory CNCC (13, 14). Given the expression of additional neural plate ‘positional’ genes such as *Rax*, *Hesx1*, and *Dkk1* in *Otx2*⁺ cluster 2, or *En1*, *Hes3*, and *Pax8* in *Gbx2*⁺ cluster 3 (Fig. 1C, S1D and S3–4), cells within these clusters correspond to anterior and posterior neuroepithelial precursors, respectively. These precursors encompass pre-migratory CNCC with transcriptional signatures reflecting positional information of the surrounding neuroepithelial cells, although they may also contain Wnt1⁺ neural progenitors that contribute to the brain (15, 16). However, this positional information is subsequently erased during delamination, as only a single delaminating CNCC cluster was identified (cluster 5 in Fig. 1B). Furthermore, developmental trajectory analysis showed that diverse neuroepithelial populations follow a single trajectory of delaminating CNCC, which do not express anterior-posterior (A-P) positional genes and are characterized by a fairly uniform transcriptional signature (Fig. 1C, S1D and S3–5). In agreement with previous studies (3), this suggests that although cells are transcriptionally heterogeneous prior to migration, delaminating CNCC acquire an equivalent transcriptional program, allowing them to subsequently adapt to environmental cues. Following this event, the CNCC population re-diversifies as cells undergo lineage decisions and generate their various derivatives.

Early Wnt1⁺ precursors dynamically express pluripotency factors

One neuroepithelial precursor population was composed mostly of cells isolated from 4 somite stage embryos, and devoid of 8 and 10 somite stage cells (cluster 1 in Fig. 1B, D and S2), suggesting this cluster may represent the earliest *Wnt1*-expressing CNCC precursors (Fig. S1D). Canonical pluripotency factors *Oct4*, *Sox2*, *Nanog* and *Klf4* were all specifically expressed in this cluster, *Oct4* being among the most highly enriched genes and *Nanog* and *Klf4* expression being almost exclusive to this cluster (Fig. 2A, S4 and S6A). We confirmed *Oct4* and *Nanog* expression in *Wnt1*⁺ cells arising in the dorsal neural fold using RNA fluorescent *in situ* hybridization (FISH) from *Wnt1::Cre; Rosa26^{TdTomato}* 4 somite stage embryos (Fig. 2B–C). We were intrigued by these observations because of the ability of canonical pluripotency factors to reprogram differentiated cells (17), and because these factors were shown to mark the stem cell niche from which neural crest arises in avian embryos (12, 18).

We noted that although *Oct4* was most highly expressed in early *Wnt1*⁺ precursors cluster comprised mainly of 4 somite stage *Otx2*⁺ cells, by 6 somite stage *Oct4* expression was downregulated in anterior precursors cells, whereas it increased in *Wnt1*⁺/*Gbx2*⁺ posterior precursors (Fig. S6B). This raised a possibility that *Oct4*⁺/*Wnt1*⁺ double positive cells first appear in the anteriormost embryo, and then *Oct4* expression shifts posteriorly as

development progresses. We used RNA FISH analysis of *Wnt1::Cre; Rosa26^{TdT/+}* embryos at 4 and 6 somite stages to perform a detailed mapping of *Oct4* and *Nanog* expression along the embryo A-P axis in relation to *Wnt1* expression (i.e. TdT⁺) and well-characterized positional markers, such as *Pax6*, *Otx2*, *En1* and *Gbx2* (Fig. 2B–C and S7–S11; (14)). We verified regional markers were enriched within distinct regions along the A-P axis, allowing us to define four domains (Fig. S7–11). Using this molecular map, we quantified *Oct4* and *Nanog* CNCC expression along the embryo A-P axis and observed strongly reduced expression in the two most anterior domains and increased expression in the most posterior *Gbx2* domain in 6 somite stage embryos as compared to 4 somite stages embryos (Fig. 2B–C and S7–11). These results support the anterior-to-posterior progression of *Oct4* and *Nanog* expression as the embryo develops.

We further confirmed this anterior-to-posterior shift in expression using *Oct4-GFP* mouse embryos (19), in which we monitored *Oct4* expression via GFP fluorescence in cranial regions of 4 to 8 somite stage embryos. We detected GFP expression in the developing neural folds where prospective CNCC form, and this expression was lost from the most anterior embryo by 6 somite stage (Fig. S12). Taken together, our data suggest a transient *Oct4⁺/Wnt1⁺* precursor population arises during early CNCC development in the most anterior neural plate and then shifts posteriorly.

Oct4 is re-expressed in prospective CNCC

To investigate whether Oct4 is re-expressed in prospective CNCC or retained from the early pluripotent embryo, we analyzed GFP fluorescence in whole *Oct4-GFP* embryos from E7.5 to the 2 somite stage. GFP was detected in the whole epiblast at the early neural plate stage (E7.5; Fig. 2D and (20, 21)). At the head-fold stage (late E7.5 and E7.75), GFP was not detected in developing head-folds (Fig. 2D, see zoom panel and arrowhead). It was then re-expressed in the most anterior embryo when first somites were forming (Fig. 2D, see arrow), and by the 2 somite stage, Oct4 expression extended posteriorly whereas the most anterior head-folds displayed decreased GFP fluorescence (Fig. 2D, see arrowhead).

To further substantiate that Oct4 is downregulated in the rostral neuroectoderm as the embryo transitions from early (E7.5) to late neurula stage (E7.75), we quantified Oct4 expression in Sox2⁺ cells along the embryo A-P axis (Fig. S13). In the early neurula embryo, the most anterior Sox2⁺ cells were also expressing Oct4. However, in the late neurula epiblast, we consistently found a strong decrease of Oct4 levels, with the first 10-15 anteriormost Sox2⁺ neuroepithelial cells not expressing Oct4 (Fig. S13). Altogether, these results show Oct4 is transiently re-expressed in prospective CNCC domain at the onset of somitogenesis.

Oct4⁺ precursors give rise to CNCC derivatives

To establish the contribution of the Oct4⁺ precursors to CNCC derivatives, we generated *Oct4-Cre^{ER/+}; Rosa26^{TdT/+}* embryos to enable tracking TdT⁺ cells at E9.5 following administration of tamoxifen at various earlier stages. Since *Oct4* is expressed throughout the pluripotent preimplantation epiblast (Fig 2D, S13 and (20, 21)), administering tamoxifen at

E6.5 resulted in fully labelled embryos (Fig. S14A). Therefore, we administered tamoxifen at E7.5. Since *Oct4* expression persists in the trunk through early somitogenesis (22), we inferred the actual onset of cell labeling based on which somites were TdT-negative 48 hours post tamoxifen administration (i.e. at E9.5; Fig. S14A). When labelling was initiated at 1 to 2 somites, TdT strongly labeled the fronto-nasal mass (FNM) and branchial arch 1 (BA1), confirming that *Oct4*⁺ cells descendants generate craniofacial structures. However, when labelling was initiated at later stages, such as 5 to 6 somites, TdT was absent from the embryo most anterior part, but detected in BA1 and BA2 as well as in streams of cells migrating to form cranial nerve ganglia IX and X (Fig. 3A–B and S14A–C). This anterior-to-posterior shift in TdT labelling dependent on the onset of *Oct4*⁺ cells was confirmed by quantifying the ratios of Sox10⁺/TdT⁺ double-positive cells to a total number of Sox10⁺ cells in craniofacial structures of E9.5 embryos (Fig. 3C). Finally, when labeling was induced at late E8.5, TdT was only detected in primordial germ cells (Fig. S15), the sole cell type maintaining *Oct4* expression after E9.0 (19, 20), but not in more posterior neural crest derivatives, suggesting *Oct4* reactivation is unique to CNCC.

If CNCC arise from transient *Oct4*⁺ precursors, ablation of *Oct4*⁺ cells at the onset of CNCC induction should result in the loss of CNCC derivatives. Hence, we genetically ablated *Oct4*⁺ cells upon tamoxifen treatment by using *Oct4-Cre*^{ER/+}; *Rosa26*^{DTA/+} embryos. We first administrated tamoxifen at E7.5 to induce diphtheria toxin (DTA)-mediated *Oct4*⁺ cell ablation between E7.5–8.0, corresponding to the onset of CNCC formation, but after *Oct4* requirement in post-implantation epiblast and germ layer specification (23, 24). Resulting mutant embryos analyzed at E9.5 displayed a complete absence of FNM (Fig. 3D–E). Neural folds were present, indicating the observed craniofacial phenotype was not a secondary effect of a massive failure in the neural plate/fold formation and confirming that cranial *Oct4*⁺ cell population is selectively required for CNCC development. This phenotype resembled that of *Wnt1::Cre*; *Rosa26*^{DTA/+} embryos (Fig. S16). Both phenotypes were similar to those previously reported in avian embryos that underwent cranial neural crest ablation (25). When we induced *Oct4*⁺ cell ablation at E8.5, mutant embryos presented with virtually normal fronto-nasal process and cephalic vesicles; however, nasal processes were absent (Fig. S17), consistent with the anterior-to-posterior shift in *Oct4* expression. Altogether, these results show *Oct4*⁺ cells define a transient CNCC precursor population, which is first induced anteriorly, then shifts posteriorly and gives rise to most, if not all, craniofacial structures.

Oct4 is required for proper ectomesenchyme specification, proliferation, and survival

To assess whether *Oct4* is necessary for CNCC formation we generated *Oct4*^{Flox/Flox}; *ActinCre*^{ER/+} mutant embryos to perturb *Oct4* function. About 24 hours are needed for *Oct4* mRNA levels to be significantly reduced following *Oct4* locus recombination (24). Thus, we administered tamoxifen at E6.5 to ablate *Oct4* expression after gastrulation but before *Wnt1* up-regulation (26) and neural crest induction. Resulting *Oct4* mutant embryos presented severely reduced facial prominences (Fig. 4A–B and S18A–B). However, RNA FISH against *Wnt1* revealed similar expression in controls and *Oct4* mutant embryos,

showing that Oct4 is not required for *Wnt1* induction in the neuroepithelium (Fig. 4C and S18C). Further, immuno-staining against neural crest markers AP2 α (Fig. 4D and F) and Alx4 (Fig. S18D–G) demonstrated that although facial prominences were reduced in mutant embryos compared to controls, similar proportions of the remaining cells expressed AP2 α and Alx4 (Fig. 4E, S18E and S18G), consistent with Oct4 marking early CNCC precursors, but being dispensable for CNCC induction and delamination.

In contrast, within CNCC derivatives, we observed a strong reduction in the proportions of Sox9⁺/Alx4⁺ and Sox10⁺/AP2 α ⁺ cells in facial prominences of E9.5 *Oct4* mutant embryos compared to controls. This was accompanied by an 80% reduction in the proportion of pH3⁺ cycling CNCC and a 10-fold increase levels of cleaved-Caspase3⁺ apoptotic CNCC, which together likely account for the observed decrease in size of facial prominences (Fig. 4D–E and S18D–G). However, development of neural/glial CNCC derivatives such as cranial ganglia, which also receive contribution from the Oct4⁺ precursors (Fig. S14B–C) appeared unaffected by Oct4 loss, as evidenced by lack of effects on Sox10 expression and proliferation in these derivatives (Fig. 4D–E). In aggregate, these data are consistent with the model whereby *Oct4* marks early CNCC precursor population but is not required for entry into the neural crest program. Instead, it is essential for ectomesenchyme specification and survival, directly linking this pluripotency factor to the expansion of developmental potency in the neural crest.

Ectomesenchyme priming of Oct4⁺ pre-migratory CNCC regulatory programs

To gain insights into open chromatin landscape and cis-regulatory features of Oct4⁺ CNCC, we performed ATAC-seq analysis of Oct4⁺ cells isolated from cranial region of *Oct4-GFP/+* 4–6 somite stages embryos (Fig. 5A). During early somitogenesis, in the posterior trunk of the embryo, *Oct4* expression is maintained from the post-implantation epiblast, through the posterior primitive streak to multipotent neuromesodermal progenitors (22, 24, 27). To enable stage-matched comparisons of hypersensitivity patterns, we sorted trunk Oct4⁺ cells from the same embryos. We compared ATAC-seq patterns of cranial and trunk Oct4⁺ cell populations focusing on promoter-distal peaks, most of which correspond to enhancers. We defined cranial-specific (blue) and trunk-specific (red) ATAC-seq peaks (Fig. 5A) and analyzed the underlying transcription factor (TF) sequence motifs. Cranial-specific ATAC-seq peaks were enriched in motifs for Otx2, Sox, Zic and Ap2 TF families, which are known to be expressed in the neuroepithelium during CNCC induction and specification (Table S2; (2)). In contrast, trunk-specific regions were almost exclusively enriched in various homeobox motifs, as might be expected given that at least a subset of trunk Oct4⁺ cells was undergoing axial specification when isolated (Table S3; (28, 29)).

We next performed gene ontology analysis using GREAT annotation tool (30). We found that although top enrichment categories at trunk-specific regions included pattern specification, regionalization, and limb development (Fig. S19A), top ontology enrichments for cranial-specific Oct4⁺ regions were all related to the neural crest and its derivatives, and included neural crest cell development and differentiation, regulation of glial cell

differentiation as well as cranial skeletal system development (Fig. 5B). Loci driving these associations included genes expressed not in Oct4⁺ CNCC, but later in delaminating and migrating CNCC, such as *Sox10*, *Mef2c*, *Pdgfra*, and *Twist1* (Fig. S19B–D). Examination of ATAC-seq signals at these loci confirmed the presence of cranial-specific accessible regions in their vicinity in the absence of detectable expression of the genes in early CNCC precursors (Fig. 5C, D and S19E). We detected a cranial-specific open chromatin region within the *Mef2c* gene corresponding to previously characterized Mef2c-F10N enhancer active in migrating CNCC (Fig. 5C; (31)). Similarly, at the *Sox10* locus, we identified three cranial-specific accessible regions (Fig. 5D), orthologous sequences for the two of which have already been characterized in migrating CNCC of avian embryos (12).

These observations suggest that the Oct4⁺ CNCC cis-regulatory landscape is primed for future activation of migration and differentiation programs. To systematically characterize the relationship between cranial Oct4⁺ cell-specific ATAC-seq peaks from the 4 to 6 somite stage embryos and gene expression, we linked these peaks to their closest genes and analyzed expression of the associated gene set in our scRNA-seq data at different developmental stages. This revealed the highest enrichments of the associated genes among those expressed at 10 somite stage (Fig. S19F), consistent with priming of distal cis-regulatory regions prior to expression of their target genes.

Similarity between cis-regulatory landscapes of cranial Oct4⁺ precursors and epiblast stem cells

We compared ATAC-seq patterns of cranial and trunk Oct4⁺ cells with a wide set of cell types at similar stage of development, E8.25 (32), and also included data from three well-characterized Oct4⁺ pluripotent cell states: mouse embryonic stem cells (ESC), epiblast-like cells (EpiLC) and epiblast stem cells (EpiSC) (33, 34). Clustering analysis revealed that: (i) trunk Oct4⁺ cells cluster with somitic mesoderm and neuromesodermal progenitors, (ii) cranial Oct4⁺ cells cluster with EpiSC and, consistent with their neuroepithelial origin, with mid/hindbrain, forebrain, and neural crest, and (iii) both cranial and trunk Oct4⁺ cells cluster away from other pluripotent cell types such as ESC and EpiLC. These results are consistent with predicted developmental relationships of cranial and trunk Oct4⁺ cells and uncover similarity of open chromatin landscapes of cranial Oct4⁺ and EpiSC (Fig. 5E). Indeed, 66% of distal ATAC-seq peaks from EpiSC are also hypersensitive in cranial Oct4⁺ cells (Fig. S20A). Furthermore, ATAC-seq peaks shared between EpiSC and cranial Oct4⁺ cells enrich for categories associated with transcriptional regulation and development, in contrast to ATAC-seq peaks unique to EpiSC (Fig. S20B–C). These observations suggest that mammalian CNCC transiently re-acquire developmental regulatory programs similar to those of pluripotent EpiSC.

Discussion

Through unbiased analysis of single-cell transcriptomes over 14 hours of early murine CNCC development we uncovered highly spatiotemporally dynamic and diverse molecular identities of this unique cell group. Our data show that upon formation, pre-migratory CNCC carry A-P information reflective of their neuroepithelial origin, as has been described

in central nervous system regionalization (35). However, in contrast to the latter system, positional identity is erased during delamination, as migratory CNCC lose expression of neuroepithelial positional genes and adopt a more uniform transcriptional signature. We speculate this erasure generates a functionally equivalent CNCC population, which can readily adapt to future migratory and post-migratory locations. Such a model would explain previously documented adaptation of pre-migratory CNCC to a novel position upon transplantation at a different axial level (36) and is consistent with widespread Polycomb-dependent bivalent chromatin marking at promoters of facial patterning genes in early CNCC, followed by the resolution of bivalency into transcriptionally active states in response to environmental cues (37).

Our work identified a transient precursor population which expresses both canonical pluripotency transcription factors and neuroepithelial markers, gives rise to CNCC, and is essential for the formation of craniofacial structures. Oct4 not only marks CNCC precursors, but is required for proper ectomesenchyme specification and survival, directly linking the function of this pluripotency factor with an expansion of CNCC developmental potential. Furthermore, recent work in *Xenopus* suggests the homeodomain protein Ventx, a frog homolog of Nanog, is also required for ectomesenchymal potential of CNCC (38). These observations raise a conundrum of how transcription factors expressed in neuroepithelial precursors affect downstream ectomesenchyme development—one possibility is through priming of distal regulatory regions. Such priming of developmental enhancers prior to activation of their target genes has now been observed in a variety of biological systems (11, 12, 37, 39, 40).

We also found that open chromatin patterns of Oct4⁺ CNCC precursors broadly resemble those of EpiSC. Although EpiSC are pluripotent, their transcriptional features are reminiscent of early primitive streak (41). Furthermore, we noted that both CNCC precursors and EpiSC express not only pluripotency factors, but also Zic3 and Otx2, two factors setting up EpiSC enhancer landscapes (42, 43). These similarities in transcription factor repertoire likely account, at least in part, for similarities in cis-regulatory programs. Thus, CNCC precursor molecular signatures are reflective of both transient re-activation of pluripotency, as well as priming towards future neural crest fates (Fig. 5F).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

All data needed to evaluate the conclusions in the paper are present in the paper, Supplementary Materials, and/or the Gene Expression Omnibus (accession number pending).

References and Notes:

1. Theveneau E, Mayor R, Neural crest migration: interplay between chemorepellents, chemoattractants, contact inhibition, epithelial-mesenchymal transition, and collective cell migration. *Wiley Interdiscip Rev Dev Biol.* 1, 435–445 (2012). [PubMed: 23801492]
2. Simões-Costa M, Bronner ME, Establishing neural crest identity: a gene regulatory recipe. *Development.* 142, 242–257 (2015). [PubMed: 25564621]
3. Soldatov R, Kaucka M, Kastri ME, Petersen J, Chontorotzea T, Englmaier L, Akkuratova N, Yang Y, Häring M, Dyachuk V, Bock C, Farlik M, Piacentino ML, Boismoreau F, Hilscher MM, Yokota C, Qian X, Nilsson M, Bronner ME, Croci L, Hsiao W-Y, Guertin DA, Brunet J-F, Consalez GG, Ernfor P, Fried K, Kharchenko PV, Adameyko I, Spatiotemporal structure of cell fate decisions in murine neural crest. *Science.* 364 (2019), doi:10.1126/Science.aas9536.
4. Le Douarin NM, Creuzet S, Couly G, Dupin E, Neural crest cell plasticity and its limits. *Development.* 131, 4637–4650 (2004). [PubMed: 15358668]
5. Weston JA, Yoshida H, Robinson V, Nishikawa S, Fraser ST, Nishikawa S, Neural crest and the origin of ectomesenchyme: neural fold heterogeneity suggests an alternative hypothesis. *Dev Dyn.* 229, 118–130 (2004). [PubMed: 14699583]
6. Buitrago-Delgado E, Nordin K, Rao A, Geary L, LaBonne C, NEURODEVELOPMENT. Shared regulatory programs suggest retention of blastula-stage potential in neural crest cells. *Science.* 348, 1332–1335 (2015). [PubMed: 25931449]
7. Briggs JA, Weinreb C, Wagner DE, Megason S, Peshkin L, Kirschner MW, Klein AM, The dynamics of gene expression in vertebrate embryogenesis at single-cell resolution. *Science.* 360 (2018), doi:10.1126/science.aar5780.
8. Debbache J, Parfejevs V, Sommer L, Cre-driver lines used for genetic fate mapping of neural crest cells in the mouse: An overview. *Genesis.* 56, e23105 (2018). [PubMed: 29673028]
9. Picelli S, Faridani OR, Björklund AK, Winberg G, Sagasser S, Sandberg R, Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc.* 9, 171–181 (2014). [PubMed: 24385147]
10. Butler A, Hoffman P, Smibert P, Papalexi E, Satija R, Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol.* 36, 411–420 (2018). [PubMed: 29608179]
11. Ling ITC, Sauka-Spengler T, Early chromatin shaping predetermines multipotent vagal neural crest into neural, neuronal and mesenchymal lineages. *Nat Cell Biol.* 21, 1504–1517 (2019). [PubMed: 31792380]
12. Williams RM, Candido-Ferreira I, Repapi E, Gavriouchkina D, Senanayake U, Ling ITC, Telenius J, Taylor S, Hughes J, Sauka-Spengler T, Reconstruction of the Global Neural Crest Gene Regulatory Network In Vivo. *Dev Cell.* 51, 255–276.e7 (2019). [PubMed: 31639368]
13. Wurst W, Bally-Cuif L, Neural plate patterning: upstream and downstream of the isthmic organizer. *Nat Rev Neurosci.* 2, 99–108 (2001). [PubMed: 11253000]
14. Kobayashi D, Kobayashi M, Matsumoto K, Ogura T, Nakafuku M, Shimamura K, Early subdivisions in the neural plate define distinct competence for inductive signals. *Development.* 129, 83–93 (2002). [PubMed: 11782403]
15. Zervas M, Millet S, Ahn S, Joyner AL, Cell behaviors and genetic lineages of the mesencephalon and rhombomere 1. *Neuron.* 43, 345–357 (2004). [PubMed: 15294143]
16. Hagan N, Zervas M, Wnt1 expression temporally allocates upper rhombic lip progenitors and defines their terminal cell fate in the cerebellum. *Mol Cell Neurosci.* 49, 217–229 (2012). [PubMed: 22173107]

17. Takahashi K, Yamanaka S, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 126, 663–676 (2006). [PubMed: 16904174]
18. Lignell A, Kerosuo L, Streichan SJ, Cai L, Bronner ME, Identification of a neural crest stem cell niche by Spatial Genomic Analysis. *Nat Commun*. 8, 1830 (2017). [PubMed: 29184067]
19. Kehler J, Tolkunova E, Koschorz B, Pesce M, Gentile L, Boiani M, Lomelí H, Nagy A, McLaughlin KJ, Schöler HR, Tomilin A, Oct4 is required for primordial germ cell survival. *EMBO Rep*. 5, 1078–1083 (2004). [PubMed: 15486564]
20. Schöler HR, Dressler GR, Balling R, Rohdewohld H, Gruss P, Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO J*. 9, 2185–2195 (1990). [PubMed: 2357966]
21. Downs KM, Systematic localization of Oct-3/4 to the gastrulating mouse conceptus suggests manifold roles in mammalian development. *Dev Dyn*. 237, 464–475 (2008). [PubMed: 18213575]
22. Aires R, Jurberg AD, Leal F, Nóvoa A, Cohn MJ, Mallo M, Oct4 Is a Key Regulator of Vertebrate Trunk Length Diversity. *Dev Cell*. 38, 262–274 (2016). [PubMed: 27453501]
23. Mulas C, Chia G, Jones KA, Hodgson AC, Stirparo GG, Nichols J, Oct4 regulates the embryonic axis and coordinates exit from pluripotency and germ layer specification in the mouse embryo. *Development*. 145 (2018), doi:10.1242/dev.159103.
24. DeVeale B, Brokhman I, Mohseni P, Babak T, Yoon C, Lin A, Onishi K, Tomilin A, Pevny L, Zandstra PW, Nagy A, van der Kooy D, Oct4 is required ~E7.5 for proliferation in the primitive streak. *PLoS Genet*. 9, e1003957 (2013). [PubMed: 24244203]
25. Creuzet SE, Regulation of pre-otic brain development by the cephalic neural crest. *Proc Natl Acad Sci U S A*. 106, 15774–15779 (2009). [PubMed: 19720987]
26. Rowitch DH, McMahon AP, Pax-2 expression in the murine neural plate precedes and encompasses the expression domains of Wnt-1 and En-1. *Mech Dev*. 52, 3–8 (1995). [PubMed: 7577673]
27. Edri S, Hayward P, Baillie-Johnson P, Steventon BJ, Martinez Arias A, An epiblast stem cell-derived multipotent progenitor population for axial extension. *Development*. 146 (2019), doi:10.1242/dev.168187.
28. Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, Rossant J, Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell*. 123, 917–929 (2005). [PubMed: 16325584]
29. Simandi Z, Horvath A, Wright LC, Cuaranta-Monroy I, De Luca I, Karolyi K, Sauer S, Deleuze J-F, Gudas LJ, Cowley SM, Nagy L, OCT4 Acts as an Integrator of Pluripotency and Signal-Induced Differentiation. *Mol Cell*. 63, 647–661 (2016). [PubMed: 27499297]
30. McLean CY, Bristor D, Hiller M, Clarke SL, Schaar BT, Lowe CB, Wenger AM, Bejerano G, GREAT improves functional interpretation of cis-regulatory regions. *Nat Biotechnol*. 28, 495–501 (2010). [PubMed: 20436461]
31. Aoto K, Sandell LL, Butler Tjaden NE, Yuen KC, Watt KEN, Black BL, Durnin M, Trainor PA, Mef2c-F10N enhancer driven β -galactosidase (LacZ) and Cre recombinase mice facilitate analyses of gene function and lineage fate in neural crest cells. *Dev Biol*. 402, 3–16 (2015). [PubMed: 25794678]
32. Pijuan-Sala B, Wilson NK, Xia J, Hou X, Hannah RL, Kinston S, Calero-Nieto FJ, Poirion O, Preissl S, Liu F, Göttgens B, Single-cell chromatin accessibility maps reveal regulatory programs driving early mouse organogenesis. *Nat Cell Biol*. 22, 487–497 (2020). [PubMed: 32231307]
33. Neijts R, Amin S, van Rooijen C, Tan S, Creighton MP, de Laat W, Deschamps J, Polarized regulatory landscape and Wnt responsiveness underlie Hox activation in embryos. *Genes Dev*. 30, 1937–1942 (2016). [PubMed: 27633012]
34. Dorigi KM, Swigut T, Henriques T, Bhanu NV, Scruggs BS, Nady N, Still CD, Garcia BA, Adelman K, Wysocka J, Mll3 and Mll4 Facilitate Enhancer RNA Synthesis and Transcription from Promoters Independently of H3K4 Monomethylation. *Mol Cell*. 66, 568–576.e4 (2017). [PubMed: 28483418]
35. Metzis V, Steinhauser S, Pakanavicius E, Gouti M, Stamatakis D, Ivanovitch K, Watson T, Rayon T, Mousavy Gharavy SN, Lovell-Badge R, Luscombe NM, Briscoe J, Nervous System

- Regionalization Entails Axial Allocation before Neural Differentiation. *Cell*. 175, 1105–1118.e17 (2018). [PubMed: 30343898]
36. Couly G, Creuzet S, Bennaceur S, Vincent C, Le Douarin NM, Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. *Development*. 129, 1061–1073 (2002). [PubMed: 11861488]
 37. Minoux M, Holwerda S, Vitobello A, Kitazawa T, Kohler H, Stadler MB, Rijli FM, Gene bivalency at Polycomb domains regulates cranial neural crest positional identity. *Science*. 355 (2017), doi:10.1126/science.aal2913.
 38. Scerbo P, Monsoro-Burq AH, The vertebrate-specific VENTX/NANOG gene empowers neural crest with ectomesenchyme potential. *Sci Adv*. 6, eaaz1469 (2020). [PubMed: 32494672]
 39. Rada-Iglesias A, Bajpai R, Swigut T, Brugmann SA, Flynn RA, Wysocka J, A unique chromatin signature uncovers early developmental enhancers in humans. *Nature*. 470, 279–283 (2011). [PubMed: 21160473]
 40. Wang A, Yue F, Li Y, Xie R, Harper T, Patel NA, Muth K, Palmer J, Qiu Y, Wang J, Lam DK, Raum JC, Stoffers DA, Ren B, Sander M, Epigenetic priming of enhancers predicts developmental competence of hESC-derived endodermal lineage intermediates. *Cell Stem Cell*. 16, 386–399 (2015). [PubMed: 25842977]
 41. Kojima Y, Kaufman-Francis K, Studdert JB, Steiner KA, Power MD, Loebel DAF, Jones V, Hor A, de Alencastro G, Logan GJ, Teber ET, Tam OH, Stutz MD, Alexander IE, Pickett HA, Tam PPL, The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. *Cell Stem Cell*. 14, 107–120 (2014). [PubMed: 24139757]
 42. Buecker C, Srinivasan R, Wu Z, Calo E, Acampora D, Faial T, Simeone A, Tan M, Swigut T, Wysocka J, Reorganization of enhancer patterns in transition from naive to primed pluripotency. *Cell Stem Cell*. 14, 838–853 (2014). [PubMed: 24905168]
 43. Yang S-H, Andrabi M, Biss R, Murtuza Baker S, Iqbal M, Sharrocks AD, ZIC3 Controls the Transition from Naive to Primed Pluripotency. *Cell Rep*. 27, 3215–3227.e6 (2019). [PubMed: 31189106]
 44. Corces MR, Buenrostro JD, Wu B, Greenside PG, Chan SM, Koenig JL, Snyder MP, Pritchard JK, Kundaje A, Greenleaf WJ, Majeti R, Chang HY, Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nat Genet*. 48, 1193–1203 (2016). [PubMed: 27526324]
 45. McQuin C, Goodman A, Chernyshev V, Kamensky L, Cimini BA, Karhohs KW, Doan M, Ding L, Rafelski SM, Thirstrup D, Wiegand W, Singh S, Becker T, Caicedo JC, Carpenter AE, CellProfiler 3.0: Next-generation image processing for biology. *PLoS Biol*. 16, e2005970 (2018). [PubMed: 29969450]
 46. Jiang H, Lei R, Ding S-W, Zhu S, Skewer: a fast and accurate adapter trimmer for next-generation sequencing paired-end reads. *BMC Bioinformatics*. 15, 182 (2014). [PubMed: 24925680]
 47. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR, STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 29, 15–21 (2013). [PubMed: 23104886]
 48. Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, Lennon NJ, Livak KJ, Mikkelsen TS, Rinn JL, The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol*. 32, 381–386 (2014). [PubMed: 24658644]
 49. Qiu X, Mao Q, Tang Y, Wang L, Chawla R, Pliner HA, Trapnell C, Reversed graph embedding resolves complex single-cell trajectories. *Nat Methods*. 14, 979–982 (2017). [PubMed: 28825705]
 50. Cao J, Spielmann M, Qiu X, Huang X, Ibrahim DM, Hill AJ, Zhang F, Mundlos S, Christiansen L, Steemers FJ, Trapnell C, Shendure J, The single-cell transcriptional landscape of mammalian organogenesis. *Nature*. 566, 496–502 (2019). [PubMed: 30787437]
 51. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ, Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods*. 10, 1213–1218 (2013). [PubMed: 24097267]
 52. Li H, Durbin R, Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 25, 1754–1760 (2009). [PubMed: 19451168]

53. Zhang Y, Liu T, Meyer CA, Eeckhoutte J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS, Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* 9, R137 (2008). [PubMed: 18798982]
54. Quinlan AR, Hall IM, BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics.* 26, 841–842 (2010). [PubMed: 20110278]
55. Love MI, Huber W, Anders S, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550 (2014). [PubMed: 25516281]

Materials and methods summary

For scRNA-seq, cells were isolated as single cells from dissected cranial portions of *Wnt1::Cre*, *Rosa26^{TdY/+}* embryos using flow cytometry. Developmental stages were inferred based on the number of somite pairs. Single sorted cells were processed using a modified Smart-Seq2 protocol (9) and data analyzed using Seurat. For ATAC-seq, cells were sorted as described above and processed following ATAC-seq protocol as described (44). Libraries were prepared following Illumina protocols and sequenced on NextSeq 500 (Illumina).

Embryos were imaged using a Leica M165 FC stereoscope coupled with fluorescence when needed. Immunostaining was done using classical procedures. FISH was performed following manufacturer guidelines (ViewRNA Cell Plus Assay, Cat# 88-19000-99, Thermo-Fisher). Probes were designed by Thermo-Fisher. Whole mount FISH was done following HCR v3.0 instructions for whole mount staining (Molecular Instruments). *Wnt1* probe was designed by Molecular Instruments. Samples were imaged using Zeiss LSM 800 confocal. Images were stitched together and processed with Adobe Photoshop software (Adobe Systems).

Image quantifications were performed using Cell Profiler 3.0 (45). Error bars were calculated as mean $\pm$ standard deviation (SD) using Mann-Whitney non-parametric statistical test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. At least 3 embryos were analyzed for each genotype and each developmental time point.

Fig. 1. Characterization of murine CNCC transcriptional heterogeneity with scRNA-seq
 (A) *Wnt1::Cre; Rosa26^{tdTomato/+}* embryos were dissected at the 4, 6, 8, and 10 somite stages of development at rhombomere 1 level. CNCC were enriched and single-cell sorted using flow cytometry. ScRNA-seq was performed using a modified Smart-Seq2 protocol.
 (B) tSNE plot representing all sequenced CNCC. Cell-clusters were obtained based on expressed transcriptomes similarities using Seurat. Clusters were annotated based on cluster-specific genes expression calculated by differential gene expression analysis and prior knowledge.

(C) Dot plot showing expression of select cluster-enriched genes in CNCC clusters. Dot size indicates percentage of cells expressing listed genes. Blue color intensity indicates average expression level. All genes are within the top 20 most significantly enriched genes for each cluster. Dashed rectangles indicate genes defining pre-migratory CNCC (in red) or migratory CNCC (in green).

(D) Original developmental stage of sequenced CNCC superimposed on tSNE plot showed in (B).

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Fig. 2. Pre-migratory CNCC transiently induce pluripotency factors

(A) *Oct4* (purple) and *Nanog* (green) expressions were superimposed on tSNE plot showed in Fig. 1B. *Oct4* and *Nanog* co-expressing cells are indicated in blue.

(B) RNA FISH analysis of *Oct4* and *Nanog* expression within transverse cross-sections of most anterior (*Pax6*) and most posterior (*Gbx2*) cranial domains at indicated stages of *Wnt1::Cre; Rosa26^{TdT/+}* embryos.

(C) Quantifications of *Oct4* (purple) and *Nanog* (green) expression changes between 4 and 6 somite stages along anterior-posterior (A-P) axis by RNA FISH in TdT⁺ cells in

dorsal epithelium, as defined by positional markers expression *Pax6*, *Otx2*, *En1* and *Gbx2* (see also Fig. S7–11). Error bars indicate mean $\pm$ standard deviation (SD). Mann-Whitney non-parametric statistical test; * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.001$.

(D) Side views of *Oct4-GFP/+* embryos cranial region at indicated stages. Top panels show GFP channel; middle panels represent merges between brightfield and GFP channels. Bottom panels are schematic representations of above images, A-P orientation indicated in right corner. For E7.75 embryos areas marked with red dashed squares are enlarged and shown in adjacent right panels, arrowheads show head-fold formation without detectable GFP. At 0 somite stage arrows mark GFP re-expression in anterior neural folds. At 2 somite stage, arrowhead indicates GFP downregulation in most anterior cranial region, arrows show GFP expression shift to more posterior region.

Fig. 3. Cranial Oct4⁺ cells are CNCC precursors essential for craniofacial development
 (A) Side views of E9.5 *Oct4-Cre^{ER/+}; Rosa26^{TdTomato/+}* embryos cranial region with time of labeling initiation (in number of somites) indicated. Top panels show TdTomato channel; bottom panels represent merges between brightfield and TdTomato channels. Asterisks mark TdTomato expression loss in anterior CNCC derivatives. Arrowheads show TdTomato expression gain in posterior CNCC derivatives when labeling initiates at later developmental stages. FNM: fronto-nasal mass, BA: branchial arch.

(B) Schematic summary of lineage tracking experiments shown in (A). Top panel shows schematic representations of embryos developmental stages at the time of labelling initiation with Oct4⁺ cells marked in green. Bottom panel shows schematic representations of resulting embryos at E9.5 with labelled cells in red. Asterisks mark craniofacial structures progressively losing TdT expression. Arrowheads indicate prominences gradually gaining TdT expression since labelling initiation is delayed in Oct4⁺ cells. Ov: Otic vesicle.

(C) Quantifications of Sox10⁺/TdT⁺ assessed by immunofluorescence within transverse cross-sections of indicated craniofacial prominences of E9.5 *Oct4-Cre/+; Rosa26^{TdT/+}* embryos with timing of labeling initiation (in number of somites) indicated. Error bars indicate mean $\pm$ SD. Mann-Whitney non-parametric statistical test; ns: non-significant, ** p < 0.01 and *** p < 0.001.

(D) Side and front views of E9.5 *Rosa26^{DTA/+}* and *Oct4-Cre/+; Rosa26^{DTA/+}* embryos treated with tamoxifen at E7.5. Asterisks indicate missing FNM in *Oct4-Cre/+; Rosa26^{DTA/+}* embryos, black and white arrowheads point to BA1 and neural fold (NF), respectively.

(E) Schematic representations of embryos shown in D. Asterisks indicate missing FNM in *Oct4-Cre/+; Rosa26^{DTA/+}* embryos, black and white arrowheads point to BA1 and NF, respectively.

Fig. 4: Oct4 is dispensable for CNCC induction but essential for ectomesenchyme specification
 (A) Side and front views of E9.5 *Oct4^{Flox/+}; ActinCre^{ER/+}* and *Oct4^{Flox/Flox}; ActinCre^{ER/+}* embryos treated with tamoxifen at E6.5. Asterisks indicate reduced FNM in *Oct4^{Flox/Flox}; ActinCre^{ER/+}* embryos, black and yellow arrowheads point to BA1 and cephalic vesicle, respectively.
 (B) Schematic representations of embryos shown in D. Asterisks indicate reduced FNM in *Oct4^{Flox/Flox}; ActinCre^{ER/+}* embryos, black and yellow arrowheads point to BA1 and cephalic vesicle, respectively.

(C) Side views of whole mount RNA FISH against *Wnt1* performed on E8.5 *Oct4^{Flox/+}*; *ActinCre^{ER/+}* and *Oct4^{Flox/Flox}*; *ActinCre^{ER/+}* embryos treated with tamoxifen at E6.5. Images are maximum projections. Dashed line indicates the NF limit.

(D) Immunofluorescence against AP2 α , pH3 and Sox10 within transverse cross-sections of E9.5 *Oct4^{Flox/+}*; *ActinCre^{ER/+}* and *Oct4^{Flox/Flox}*; *ActinCre^{ER/+}* embryos treated with tamoxifen at E6.5. Cranial ganglia (CG) are indicated by yellow dashed lines.

(E) Quantification of proportions of AP2 α ⁺ cells, Sox10⁺/AP2 α ⁺ cells and pH3⁺/Sox10⁺/AP2 α ⁺ cells in CG (top) and FNM (bottom). Error bars indicated mean $\pm$ SD. Mann-Whitney non-parametric statistical test; *** $p < 0.001$.

Fig. 5. Regulatory landscape of cranial Oct4⁺ cells shows similarity with EpiSC and epigenetic priming for migratory fates

(A) *Oct4-GFP/+* embryos were dissected at the 4 to 6 somite stage to assess chromatin accessibility in cranial (dissected at rhombomere 1 level) and trunk (dissected at first somite level) Oct4⁺ cells using ATAC-seq. ATAC-seq enrichments at distal regulatory regions are shown, with cranial-specific Oct4⁺ cells in blue, trunk-specific Oct4⁺ cells in red and shared regions in orange.

(B) Gene ontology using GREAT showing the five most enriched biological processes associated with cranial-specific promoter-distal hypersensitive regions.

(C and D) Genome browser tracks representing ATAC-seq signals in the vicinity of *Mef2c* (C) and *Sox10* (D) in mouse ESC, EpiSC, cranial and trunk Oct4⁺ cells. In (C), shaded box indicates cranial Oct4⁺ cell-specific accessible region overlapping with Mef2c-F10N enhancer active in migrating CNCC (31). In (D), shaded boxes indicate cranial Oct4⁺ cell-specific open regions, two out of three overlapping regions orthologous with previously characterized enhancers (87 and 99) active in migratory avian CNCC (12).

(E) Clustering analysis of ATAC-seq data from cranial and trunk Oct4⁺ cells together with ESC, EpiLC (34) and EpiSC (33) and all major cell types present in E8.25 mouse embryo from an atlas of single cell ATAC-seq (32) represented as a heatmap. Red square indicates tissue clustering with trunk Oct4⁺ cells. Blue square highlights cell types clustering with cranial Oct4⁺ cells.

(F) Model representing CNCC formation through a transient precursor state expressing canonical pluripotency factors and primed for acquisition of mesenchymal potential.