**ALX1-related frontonasal dysplasia results from defective neural crest cell development and migration**

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**Abstract**

A pedigree of subjects presented with frontonasal dysplasia (FND). Genome sequencing and analysis identified a p.L165F missense variant in the transcription factor ALX1 which was imputed to be pathogenic. Induced pluripotent stem cells (iPSC) were derived from the subjects and differentiated to neural crest cells (NCC). NCC derived from ALX1¹⁶⁵F/iPSC were more sensitive to apoptosis, showed an elevated expression of several neural crest progenitor state markers, and exhibited impaired migration compared to wild-type controls. NCC migration was evaluated in vivo using lineage tracing in a zebrafish model, which revealed defective migration of the anterior NCC stream that contributes to the median portion of the anterior neurocranium, phenocopying the clinical presentation. Analysis of human NCC culture media revealed a change in the level of bone morphogenic proteins (BMP), with a low level of BMP2 and a high level of BMP9. Soluble BMP2 and BMP9 antagonist treatments were able to rescue the defective migration phenotype. Taken together, these results demonstrate a mechanistic requirement of ALX1 in NCC development and migration.

**Keywords** ALX1; frontonasal dysplasia; iPSC; neural crest cells; zebrafish

**Subject Categories** Development; Genetics, Gene Therapy & Genetic Disease; Stem Cells & Regenerative Medicine

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**Introduction**

The central part of the human face contains key anatomic features and sensory organs that enable us to interact with the environment and each other. The embryologic processes that form midface structures, including the eyes, nose, upper lip, and maxilla, are tightly regulated (Johnston, 1966; Minoux & Rijli, 2010; Rada-Iglesias et al, 2012). The midface structures form as the centrally located frontonasal prominence extends anteriorly, coalescing with elements derived from the paired maxillary prominences (Johnston, 1966, 1975; Le Lièvre & Le Douarin, 1975; Le Lièvre, 1978; Sadaghiani & Thiébaud, 1987). The embryonic facial prominences are derived from distinct migrating streams of cranial neural crest cells (NCC) that are conserved across vertebrates (Le Douarin et al, 1993; Schilling et al, 1996; Chai et al, 2000; Olsson, et al, 2002; Trainor et al, 2002; Barrallo-Gimeno et al, 2004; Wada, 2005; Dougherty et al, 2012). NCC migration and differentiation are highly coordinated and are associated with dynamic gene expression patterns (Simoes-Costa & Bronner, 2015). Key signaling pathways that regulate NCC development involve BMP, Wnt, FGF, or Notch which activate the expression of transcription factors such as PAX3, ZIC1, TFG2a, MSX1/2, and DLX5 (Meulemans & Bronner-Fraser, 2002; Khudyakov & Bronner-Fraser, 2009; Stuhlmillar & Garcia-Castro, 2012; Rada-Iglesias et al, 2013; Simoes-Costa & Bronner, 2015). Disruptions of NCC development contribute to a number of congenital malformations such as Waardenburg syndrome (WS), velocardiofacial syndrome/DiGeorge syndrome, Hirschsprung’s disease, congenital heart conditions, and craniofacial anomalies (Sedano et al, 1970; Fox et al, 1976; Pierpont et al, 2007; Uz et al, 2010).

Frontonasal dysplasia (FND) is considered a rare “orphan” disease (ORPHA250), with very few cases reported in the literature. The true prevalence of FND and its etiology remain unknown. To date, six genetic causes of subtypes of FND with varying patterns of...
inherited have been described in individual case reports: EFNB1 (MIM 300035) in X-linked craniofrontonasal syndrome (MIM 304110); ALX3 (MIM 606014) in FND type 1 (MIM 136760); ALX4 (MIM 605420) in FND type 2 (MIM 613451); ALX1 (MIM 601527) in FND type 3 (MIM 613456); ZSWIM6 (MIM 615951) in dominant acromelic frontonasal dysostosis (MIM 603671); and SPECC1L (MIM 614140) in Teebi syndrome (MIM 145420; Bhoj et al, 2015; Kayserili et al, 2009; Smith et al, 2014; Twigg et al, 2004, 2009; Ullah et al, 2016; Uz et al, 2010; Wieland et al, 2004). The heterogeneity of clinical phenotypes, including a wide range of possible ocular and craniofacial components, likely corresponds to different underlying genetic variants, genetic environments, and epigenetic modifications.

This study examined a pedigree of FND and identified a pathogenic variant in the homeodomain of transcription factor ALX1 resulting in a likely loss of function. A human stem cell model of FND was generated in order to investigate the effect of ALX1 mutations on NCC behavior. Cellular and molecular characterizations identified a number of differences between subject-derived and control NCC that shed light on the developmental processes that are disrupted in FND. In vivo characterization of alx1 in zebrafish revealed defective migration of the most anterior cranial NCC. This study underscores the utility of complementary human iPSC and zebrafish models to gain mechanistic insight into the molecular and cellular basis of ALX1-related FND.

Results

Clinical features of ALX1-related FND in a consanguineous pedigree

A family with four children born of consanguineous parents of Amish heritage presented with complex FND. The FND phenotype was inherited in a Mendelian recessive fashion. Both parents, one unaffected sibling and three affected children (one male and two females), were consented and enrolled in the study (Fig 1A, subject numbers indicated in red). The parents (subjects 1 and 2) and nine unaffected siblings (including subject 3) had normal facial structures without clinical stigmata suggestive of mild FND. All four affected children presented with bilateral oblique facial clefts, extending from either side of the nasal bone, involving both the primary and secondary palate. Among the affected children, there was some variability of the ocular phenotype, where the older affected girl (subject 4) presented with bilateral coloboma and asymmetric microphthalmia, whereas the three other affected children (including subjects 5 and 6) exhibited bilateral anophthalmia, with deficient upper and lower eyelids covering a shallow orbit. Subject 6 was the most severely affected and presented with bilateral oblique facial clefts and anophthalmia as well as no upper and lower eyelids, leaving the mucous membranes of both orbits exposed. Her nasal remnant also lacked the lateral alar subunits and is surrounded by several nodular skin tags.

Identification of pathogenic ALX1 variant

Whole-exome sequencing (WES) was performed on blood samples collected from subjects 1–5, which corresponded to both parents, one unaffected sibling, and two affected children. A missense p.L165F variant (c.493C>T) was identified in the homeodomain of ALX1, which was heterozygous in the parents (ALX1L/165F/165F), wild type in the unaffected sibling (ALX1L/165F/165L), and homozygous in both affected subjects (ALX1L/165F/165F). WES results were confirmed by Sanger sequencing of the entire ALX1 coding sequence. The ALX1 p.L165F missense variant has not been reported in connection with an ALX1-related instance of FND in the literature nor been recorded as a variant in the gnomAD database.

Figure 1. Clinical presentation of the FND pedigree and generation of control, father, and subject-derived iPSC.

A The pedigree family tree includes two unaffected parents, four unaffected male siblings, five unaffected female siblings, and two each female and male affected sibling. Subjects 1–5 are shown, with red, enrolled in the study. Subjects 4–6 show complex FND with ocular involvement. The eldest affected sibling (subject 4) presented with right coloboma, left microphthalmia, and bilateral Tessier 4 oblique facial clefts. Subject 5 presented with bilateral anophthalmia with fused eyelids and shallow orbits, with bilateral oblique facial clefts. Subject 6 presented with bilateral anophthalmia with open shallow orbits, absent upper and lower eyelids, exposed orbital mucosa, bilateral oblique facial clefts, and malformed nasal ala with nodular skin tags. iPSCs were generated using blood samples collected from subjects 1, 5, and 6.

B Whole-exome sequencing was carried out and analysis revealed a missense p.L165F variant (c.493C>T) in the ALX1 homeodomain, heterozygous in the parents (ALX1L165F/165F), wild type in the unaffected sibling (ALX1L165F/165L), and homozygous in both affected subjects (ALX1L165F/165F).

C Schematic of the ALX1 protein structure showing the position of the L165F substitution described here (red) and the locations of exon borders affected by two reported pathogenic variants (purple; Ullah et al, 2010; Uz et al, 2010).

D Schematic of the ALX1 genomic sequence, showing the locations of the three reported pathogenic variants. The purple bar at the bottom represents a FND-associated homozygous ALX1 deletion previously reported in the literature (Uz et al, 2010; Ullah et al, 2016).

E Immunofluorescence staining for pluripotent markers SSEA4, OCT4, SOX2, and TRA-1-60 and alkaline phosphatase staining of iPSC clones. One representative iPSC clone is shown for each genotype. Scale bar: 400 μm. Expression of pluripotent (OCT4, NANOG), endoderm (Endo., AFP, CATA, FOXA2), ectoderm (Ecto., NESTIN, GFAP, SOX2), and mesoderm (Meso., BRACHYURY, RUNX1, CD34) gene markers for ALX1L165F/165 (red), and ALX1L165F/165 (blue) iPSC relative to undifferentiated cells (UND). Data are represented as pooled mean ± SEM of three experiments on three clones for each genotype. Significance: P = 0.0167 for OCT4, P = 0.0005 for NANOG, P = 0.000004 for AFP, P = 0.0082 for CATA4, P = 0.0137 for FOXA2, P = 0.00002 for NESTIN, P = 0.0167 for GFAP, P = 0.0014 for SOX2, P = 0.0117 for BRACHYURY, P = 0.0008 for RUNX1, and P = 0.0068 for CD34 when comparing undifferentiated and differentiated ALX1L165F/165 F iPSC. P = 0.0013 for OCT4, P = 0.0011 for NANOG, P = 0.000003 for AFP, P = 0.0003 for CATA4, P = 0.0063 for FOXA2, P = 0.0001 for NESTIN, P = 0.027 for GFAP, P = 0.00002 for SOX2, P = 0.000009 for BRACHYURY, P = 3e-4 for RUNX1, and P = 0.000006 for CD34 when comparing undifferentiated and differentiated ALX1L165F/165 F iPSC. P = 0.0201 for OCT4, P = 0.0006 for NANOG, P = 1 × 10-12 for AFP, P = 5 × 10-15 for CATA4, P = 0.0031 for FOXA2, P = 0.0292 for NESTIN, P = 0.00001 for GFAP, P = 6 × 10-7 for SOX2, P = 0.0204 for BRACHYURY, P = 0.0009 for RUNX1, and P = 0.000003 for CD34 when comparing undifferentiated and differentiated ALX1L165F/165 F iPSC. Data from each clone were pooled, and the mathematical mean was calculated. SEM was used to determine the standard error. To test statistical significance, an ANOVA test was performed. A P-value < 0.05 was considered to be statistically significant.
Figure 1.
Generation of patient-derived iPSC model of ALX1-related FND

Induced pluripotent stem cell lines were generated using peripheral blood mononuclear cells (PBMC) obtained from whole blood samples that were collected from three unrelated wild-type individuals (ALX1<sup>165L/165L</sup>), the heterozygous father (ALX1<sup>165L/165F</sup>), and two of the four affected children (Subjects 5 and 6; ALX1<sup>165F/165F</sup>). PBMC were subsequently reprogrammed into iPSC (Fig EV1). Overall, 22 mutant ALX1<sup>165F/165F</sup> iPSC clones were successfully isolated and expanded from the two affected subjects, 13 ALX1<sup>165L/165F</sup> clones were isolated and expanded from the heterozygous father, and 35 ALX1<sup>165L/165L</sup> clones were isolated and expanded from healthy controls. Six ALX1<sup>165F/165F</sup> mutant clones (3 for each affected subject), 3 ALX1<sup>165L/165F</sup> clones from the heterozygous father, and 9 ALX1<sup>165L/165L</sup> clones from healthy controls (3 from each control) were fully characterized to confirm their pluripotency (Fig 1E) and ability to generate the three germ layers (Fig 1F). Sanger sequencing confirmed that the affected ALX1<sup>165F/165F</sup> and the heterozygous ALX1<sup>165L/165F</sup>-derived iPSC clones retained the ALX1 p.L165F variant through reprogramming. Copy number variant analysis did not show any amplifications or deletions.

Generation and characterization of iPSC-derived NCC

Given the primary role of neural crest cells in midface morphogenesis, the iPSC clones were differentiated into NCC using a protocol adapted from a previous study (Pini et al., 2018; Fig 2A). All NCC displayed similar morphological features and were indistinguishable at the colony level immediately following differentiation at passage 1 (Fig 2B).

Overexpression of neural plate border specifier genes in ALX1<sup>165F/165F</sup> NCC

A panel of marker genes at the center of the gene regulatory network required for NCC development and differentiation was selected to be examined in detail across the 14 days of the neural crest differentiation protocol (Fig 3; Barrallo-Gimeno et al., 2004; Sauka-Spengler & Bronner-Fraser, 2008; Sauka-Spengler et al., 2007; Simoes-Costa & Bronner, 2015). The NCC gene expression results can broadly be divided into three groups. The first group includes genes that did not significantly differ between affected, heterozygous, and unaffected controls. This group of genes comprises the
Figure 3.
neural crest specifiers FOXD3 and P75, as well as the lineage specifier HAND2. The second group includes genes where the affected cells exhibited expression patterns that differed significantly from the heterozygous and the unaffected control cells, with no difference between the heterozygote and the control. This group of genes includes the neural plate border specifier ZIC1, PAX7, PAX3, MSX1, and DLX5 and the neural crest specifiers SNAI2 and TWIST1 (P < 0.05 between days 2–8 when comparing subjects’, father, and control NCC for ZIC1, PAX7, DLX5; P < 0.05 between days 2–14 when comparing subjects’, father, and control NCC for PAX3, MSX1, SNAI2, and TWIST1). The final group includes genes that were significantly differentially expressed between the affected homozygous, heterozygous, and unaffected control cells. This group comprised the neural plate border specifier MSX2, DLX5, and the neural crest specifier TFAP2A (P < 0.05 between days 2–14 when comparing subjects’, father, and control NCC for MSX2, DLX5, and TFAP2A). Of note, all significantly differentially expressed genes in the affected cells were overexpressed above the levels observed in the heterozygous and unaffected control cells, consistent with a putative role of ALX1 as a transcriptional repressor.

ALX1 itself was found to be differentially expressed between affected cells when compared to the heterozygous and unaffected control cells at day 8 during NCC differentiation. The unaffected control and heterozygous cells exhibited similar ALX1 expression levels, with peak expression level reached at day 8 where unaffected cells exhibited a plateaued, lower level of expression. The greatest difference in gene expression levels was observed early in the NCC differentiation process, around days 2–8 (such as in the cases of ZIC1, PAX3, PAX7, DLX5, and TWIST1). This characterization suggests an early function for ALX1 in NCC differentiation and identifies the 2–8 day window for in-depth transcriptome analysis in future studies.

**Increased sensitivity to apoptosis in ALX1<sup>165F/165F</sup> NCC**

Since anomalies in cell cycle progression predispose cells to apoptosis and given the importance of apoptosis in regulating craniofacial development, the impact of the ALX1<sup>165F/165F</sup> gene variant on the sensitivity of the iPSC-derived NCC to apoptosis was analyzed. Basal apoptosis levels, determined by the percentage of Annexin V-positive cells, did not differ between control NCC (4 ± 0.2%) and ALX1<sup>165F/165F</sup> NCC (4.82 ± 0.65%); Fig 4A). After apoptosis induction via heat shock, the percentage of Annexin V-positive cells significantly increased specifically in ALX1<sup>165F/165F</sup> NCC (87.97 ± 2.44%) versus control NCC (24.15 ± 0.96%). These findings suggest that the affected subject’s ALX1<sup>165F/165F</sup> NCC are more sensitive to apoptosis.

These findings also indicate that ALX1 functions in proliferating NCC. To determine whether ALX1 function is required for cell cycle progression, we investigated expression of Cyclin D1 (CCND1), required for the cell cycle G1/S transition, and Cyclin A2 (CCNA2), required for the DNA synthesis during the S-phase. Both cyclins are expressed throughout the active cell cycle, from the G1/S transition to the G2/M transition (Pagano et al., 1992; Minarikova et al., 2016). Expression levels of CCND1 and CCNA2 were compared between control and ALX1<sup>165F/165F</sup> NCC at passages 2 and 3 post-differentiation. The ALX1<sup>165F/165F</sup> NCC were found to express significantly more CCNA2 and CCND1 at both passage 2 and passage 3 compared
to the control NCC, consistent with a greater degree of active cellular proliferation (Fig 4B).

**ALX1\textsuperscript{165F/165F} NCC do not undergo mesenchymal marker transition**

As NCC clones derived from the control \textit{ALX1}\textsuperscript{165L/165L}, heterozygous \textit{ALX1}\textsuperscript{165L/165F}, and homozygous \textit{ALX1}\textsuperscript{165F/165F} iPSC were maintained in culture, consistent qualitative morphologic differences were observed across cell passages. While control-derived NCC became progressively spindle-shaped and elongated, the mutant \textit{ALX1}\textsuperscript{165F/165F} NCC remained rounded (Fig 2B). In order to investigate these differences more fully, flow cytometry was performed across different cell passage cycles in order to investigate the effect of the in vitro maturation of the NCC via an examination of NCC marker expression. At passage cycles 1–4, a number of key

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**Figure 4.**

Control NCC

ALX1\textsuperscript{165F/165F} NCC
surface markers were examined. Expression of CD57 (synonym: HNK-1), indicative of NCC precursors before their commitment to downstream cell lineages (Minarcik & Golden, 2003), as well as markers of mesenchymal differentiation, CD105, CD73, and CD90, was assessed (Fig 4C).

Table 1 contains the precise percentage values of the FACS analysis of NCC at varying passage numbers. At passage 1 following differentiation, control and homozygous ALX1165F/165F NCC expressed similar levels of neural crest precursor marker CD57. The control and homozygous ALX1165F/165F NCC also expressed similar levels of mesenchymal markers CD90, CD105, and CD73. No significant differences were observed in marker expression between control and homozygous ALX1165F/165F NCC at this stage (P > 0.05). However, by passage 4, control NCC exhibited decreased CD57 expression and increased expression of CD90, CD105, and CD73, consistent with a progression to MSC differentiation. In contrast, homozygous ALX1165F/165F NCC displayed a similar expression of the aforementioned NCC and MSC markers at passage 4 as they did at passage 1 (Fig 4C).

The persistent CD57 expression in the homozygous ALX1165F/165F NCC, taken together with the elevated expression of neural crest specifier genes ZIC1, PAX7, PAX3, MSX1, MSX2, and DLX5, suggests that the mutant NCC may be unable to progress from the progenitor to the differentiating state. To understand whether the persistent CD57 expression had an effect on the ability of the homozygous ALX1165F/165F NCC to differentiate into downstream cell types, multilineage differentiation experiments were performed. Control and homozygous ALX1165F/165F NCC demonstrated equal ability to differentiate into Schwann cells (GFAP and S100B-positive expression), adipocytes (oil Red O. staining), chondrocytes (Alcian Blue, Safranin O. and Toluidine Blue staining), and osteoblasts (Alizarin Red S., Von Kossa staining and strong alkaline phosphatase activity; Fig EV2). The maintenance of CD57 and lack of elevation of CD90 / CD105 / CD73 and the same ability to differentiate into NCCs derivatives suggest that the homozygous ALX1165F/165F failed to progress through the process of NCCs differentiation despite multiple cell passages and are blocked into the progenitor state.

**Homozygous ALX1165F/165F NCC display a migration defect**

During embryonic development, NCC migrate to specific locations in order to form the prominences that coalesce to shape the face. To investigate the migratory properties of the iPSC-derived NCC in vitro, a wound healing assay with a central clearing was used. A significant migration defect was observed in the homozygous ALX1165F/165F NCC when compared with control NCC (Fig 5A, Movie EV1). Control NCC were able to migrate and fully cover the central clearing area of the wound healing assay after 24 h (recovery of 95.99 ± 3.22% of the surface area). In contrast, the homozygous ALX1165F/165F NCC covered less than half of the central clearing surface area (38.79 ± 3.22% for ALX1165F/165F NCC).

**ALX1165F/165F NCC show differences in BMP secretion**

The family of BMP family of growth factors plays a critical role in NCC migration (Tribulo et al., 2003; Sato et al., 2005). This, in combination with the increased expression of TWIST1 in ALX1165F/165F NCC, a known BMP inhibitor, led to us to hypothesize that ALX1165F/165F NCC might display abnormal levels of secreted BMP when compared to healthy control NCC (Hayashi et al., 2007). To test this hypothesis, the levels of secreted BMP in the culture medium of ALX1165F/165F and control NCC were measured via multiplex analysis. The concentration of BMP2 was found to be significantly reduced in control ALX1165F/165F NCC (11.9 ± 0.65 pg/ml) compared to control NCC (19.52 ± 0.9 pg/ml; P < 0.05; Fig 5B). In contrast, the BMP9 concentration was significantly increased in mutant ALX1165F/165F NCC (3.72 ± 0.85 pg/ml) compared to control NCC (0.25 ± 0.02 pg/ml). BMP4, BMP7, and BMP10 levels were undetectable.

To follow-up on the observed dysregulation of BMPs, we hypothesized that treatments to counteract BMP2 reduction or BMP9 elevation could result in an improved migration phenotype. The ALX1165F/165F NCC were treated with different concentrations of soluble BMP2, the BMP9 antagonist Crossveinless (CV2), or a combination of the two (Fig 5C, Fig EV3, Movies EV2 and EV4). Treatment with an increasing concentration of BMP2 from 10 to 50 ng/ml was able to restore the migration of homozygous ALX1165F/165F NCC in a dose-dependent manner. However, no difference was observed between 50 and 100 ng/ml of BMP2, suggesting a saturation effect (64.53 ± 3.17% for 50 ng/ml BMP2, and 67.31 ± 3.25% for 100 ng/ml BMP2).

Likewise, treatment with the BMP9 antagonist CV2 was able to partially rescue the migration defect of homozygous ALX1165F/165F NCC. The low dose of 10 ng/ml of CV2 did not show a significant effect on the migration defect of treated and untreated ALX1165F/165F NCC (37.5 ± 2.5% for 10 ng/ml CV2). As observed with BMP2, treatments with both 50 ng/ml and 100 ng/ml of CV2 were able to partially restore the ability of the homozygous ALX1165F/165F NCC to migrate, with no difference found between these two concentrations (57.6 ± 4.77% for 50 ng/ml of CV2, and 64.64 ± 3.36% for 100 ng/ml of CV2). Finally, we asked whether treatment with a combination of BMP2 and CV2 would exert an additive or synergistic effect to restore cell migration than single compound treatment.

### Table 1. Comparative FACS analysis of subject-derived ALX1165F/165F and control NCC at passages 1 and 4

|          | Passage 1 Control (ALX1165F/165F) | ALX1165F/165F | Passage 4 Control (ALX1165F/165F) | ALX1165F/165F |
|----------|----------------------------------|--------------|----------------------------------|--------------|
| CD57     | 45.6 ± 7.7%                      | 57.4 ± 7.3%  | 6.78 ± 2.36%                     | 49.55 ± 17.53% |
| CD90     | 55.05 ± 5.24%                     | 63.6 ± 6.9%  | 88.46 ± 2.05%                    | 67.8 ± 11.07%  |
| CD105    | 51.7 ± 7.8%                      | 47.6 ± 11.4% | 96.31 ± 0.95%                    | 60.02 ± 12.66% |
| CD73     | 97.4 ± 1.08%                      | 79.2 ± 9.6%  | 98.8 ± 0.44%                     | 83.95 ± 6.05%  |
**Figure 5.**

A) Bar graph showing the area recovered by migrating cells (%). The graph compares untreated ALX165F/165F NCC to those treated with BMP2 100 ng/ml and CV2 100 ng/ml at different times (0h, 6h, 12h, 18h, 24h).

B) Bar graph depicting the concentration (pg/mL) of BMP2 and BMP9. The graph compares control and subject samples.

C) Images of migrating cells at 0h and 24h under different treatments:
- Untreated ALX165F/165F NCC
- BMP2 10 ng/mL
- BMP2 50 ng/mL
- BMP2 100 ng/mL
- CV2 10 ng/mL
- CV2 50 ng/mL
- CV2 100 ng/mL
- BMP2 100 ng/mL
- CV2 100 ng/mL
No additive effect was identified, as BMP2 and CV2 cotreatment at 100 ng/ml was able to rescue the migration defect phenotype of mutant ALX1<sup>1165F/165F</sup> NCC at a similar level to what was observed with the individual treatments (73 ± 5.89% for BMP2 and CV2 cotreatment).

### Evaluation of alx1 function in the zebrafish

We and others previously showed that the zebrafish anterior neurocranium (ANC) forms from the convergence of the frontonasal and maxillary prominences (Wada, 2005; Eberhart, 2006; Dougherty et al., 2012). Since FND malformation is characterized by facial cleft affecting fusion of the frontonasal and maxillary structures, examination of the ANC morphology in zebrafish would be a sensitive assay for frontonasal development.

To generate an in vivo model of alx1, we employed CRISPR/Cas9-mediated targeted mutagenesis of the alx1 locus in zebrafish. This approach produced a frameshift mutation harboring a 16-base pair (bp) deletion in exon 2 of alx1 (NM_001045074), named alx1<sup>alu2016</sup> (Fig 6A, Fig EV4). The alx1<sup>alu2016</sup> mutation is likely to cause complete loss of function, since the encoded truncated protein lacks both the homeobox and transcription domains. While the majority of alx1<sup>alu2016</sup> homozygous zebrafish developed normally and were viable as adults, approximately 5% displayed specific craniofacial defects (Fig EV5). Alcian blue staining of alx1<sup>alu2016</sup> homozygous larvae at 5 days post-fertilization (dpf) revealed that the posterior neurocranium and ventral cartilages and Meckel’s cartilage were formed but smaller in size in a subset of zebrafish. In contrast, the ANC appeared dysmorphic, i.e., narrower in the transverse dimension and linear, rather than fan-shaped (Fig 6A). The chondrocytes of the ANC appeared cuboidal in mutant larvae, whereas wild-type ANC chondrocytes were lenticular and stacked in an intercalated pattern (Fig 6A).

The low penetrance of the ANC defect suggests the possibility of gene compensation by other alx family members (also see Discussion). To test this hypothesis, alx transcripts were quantified by qRT–PCR at several stages of embryogenesis in alx1<sup>alu2016</sup> homozygotes. We observed that alx1 mRNA level was significantly decreased in alx1<sup>alu2016</sup> mutants across several time points, likely because the mutation triggers nonsense-mediated decay (Fig 6B; El-Brollosy et al., 2019). Consistent with transcriptional gene compensation, alx3 and alx4a mRNA levels were significantly increased in the alx1<sup>alu2016</sup> mutant embryos compared to wild-type embryos (P < 0.05 for 10 ss, 24 hpf and 36 hpf embryos for alx3; P < 0.05 for 24 hpf and 36 hpf embryos for alx4a). These data suggest that alx3 and alx4a functions may compensate for the loss of alx1 during zebrafish development (Fig 6B).

Given the likely genetic compensation between different alx family members, we utilized a dominant-negative approach to interrogate the function of alx genes in embryonic development. It has
Figure 6.
been reported that ALX1 protein homodimerizes to be fully functional (Furukawa et al., 2002). In order to circumvent the genetic compensation by alx3 and alx4a, a truncated form of alx1 containing the N-terminal domain (Alx1DN) was generated. The Alx1DN truncation contains the DNA-binding homeodomain but is missing the transactivation domain (Herskowitz, 1987). Additionally, an alternative truncated protein that lacks the DNA-binding homeodomain and contains the transactivation domain, termed Alx1CT, was generated. We reasoned that, if a truncated Alx1 protein can occupy its binding sites but fail to dimerize or associate with its coactivators, it may function in a dominant-negative manner.

When Alx1DN was overexpressed by mRNA injection in wild-type zebrafish embryos, embryos displayed significant craniofacial defects (Fig 6C), while Alx1CT-expressing embryos developed normally. On closer inspection with Alcian blue staining, Alx1DN-expressing embryos showed complete abrogation of the frontonasal-derived median section of the ANC and Meckel’s cartilage, a similar phenotype. The anterior cranial NCCs were labeled at 10-somites stage and followed to 96 h post-fertilization (dpf), the NCCs were able to migrate into the frontonasal region of the palate (Fig 6D). In contrast, the anterior cranial NCC of Alx1DN injected embryos were unable to reach their final location of the median ANC. Instead, the NCC in the Alx1DN injected zebrafish were found in an ectopic anterior location outside of the frontonasal domain (Fig 6D, Movie EV3). While it is possible that increased cell death and altered cell division that were observed in the iPSC model are also operating here in the embryo, these cellular derangements are likely minor contributors to explain the ectopic localization of the cranial NCC, whereas altered cell migration is the dominant mechanism.

### Human genetics of ALX1

Genes of the ALX family encode homeodomain transcription factors and are associated with craniofacial malformations. Like other members of this family, the ALX1 protein is composed of two main functional domains: the N-terminal portion containing the DNA-binding homeodomain with two nuclear localization signals, and the C-terminal portion containing an OAR/aristaless domain required for transactivation and protein interaction (Furukawa et al., 2002). In this study’s pedigree, a novel missense variant p.L165F within the conserved homeodomain was identified. Leucine is an aliphatic, branched amino acid, while phenylalanine is an aromatic, neutral, and nonpolar amino acid. Due to properties of leucine, the substitution itself is likely disruptive to helix II in the DNA-binding element of the homeodomain within which it resides. Disruptive leucine to phenylalanine substitutions have been described in a number of published, genotyped disorders (Miller et al., 1992; Gomez & Gammack, 1995). Pathogenic missense variants within the homeodomains of both ALX3 and ALX4 have previously been identified as the causes of FND types 1 and 2, respectively (Wuyts et al., 2000; Kayserrill et al., 2009; Twigg et al., 2009).

Pathogenic ALX1 gene variants in FND have been reported in two case studies in the literature. The first study described two families (Uz et al., 2010). In the first, three siblings of consanguineous parents were described to suffer from a midline defect of the cranium, bilateral extreme microphthalmia, bilateral oblique cleft lip and palate, a caudal appendage in the sacral region, and agenesis of the corpus callosum. A homozygous 3.7 Mb deletion spanning ALX1 was identified in all affected subjects. In a second family, one affected subject was born with a midline defect of the cranium, bilateral microphthalmiamicrophthalmia, bilateral oblique cleft lip and palate, and a thin corpus callosum. A donor splice-site mutation c.531+1G>A of ALX1, homozygous in the child and heterozygous in the parents, was identified to be the likely cause of the child’s phenotype.

None of the affected subjects from the pedigree reported in this study demonstrated midline defects of the cranium or a cerebral phenotype. This is in spite of the fact that the missense mutation of the affected subjects in our study lies just proximal to that of family 2, within helix II, within the homeodomain.

A second case report described one family with FND (Ullah et al., 2016). It reported on four children born of consanguineous parents that presented with a broad nasal root, smooth philtrum, and mouth protrusion. An ALX1 gene variant c.661-1G>C was found to be heterozygous in the parents and homozygous in the affected children. The skipping of the exon via alternative splicing likely resulted in a mutant protein with some residual function, explaining the relatively mild phenotype.

### The ALX gene family: ALX1, ALX3, and ALX4

The ALX gene family consists of three members: ALX1, ALX3, and ALX4 (McConnell et al., 2011). In humans, mutations of each ALX gene have been associated with craniofacial malformations of the frontonasal-derived structures with variable phenotypic severity (Wu et al., 2000; Wuyts et al., 2000; Mavrogiannis et al., 2001; Kayserrill et al., 2009; Twigg et al., 2009; Uz et al., 2010). FND is a descriptive term that broadly describes a number of malformations...
of the midface. Previously classified based on appearance (see
Tessier, Sedano, De Myer classifications), FND related to variants
within the ALX gene family has recently been reordered on the basis
of genetics: Type I is caused by mutations of ALX3; type II is caused
by mutations of ALX4; and type III is caused by mutations of ALX1.
FND types 1 and 2 appear milder than type III, frequently presenting
with altered appearance of the nasal soft tissue (Twigg et al., 2009).

In mouse and zebrafish, Alx1, Alx3, and Alx4 have been shown to
be expressed in spatiotemporally restricted regions of the cranio-
facial mesenchyme (Zhao et al., 1994; Qu et al., 1997; ten Berge et al.,
1998; Beverdam & Meijlink, 2001; Dee et al., 2013; Lours-Calet et al.,
2014). Evidence of gene compensation has previously been reported
in animal studies (Beverdam et al., 2001; Dee et al., 2013). In studies
of sea urchins, Alx4 was shown to be directly regulated by Alx1
(Rafiq et al., 2012; Khor et al., 2019). The question remains how the
different ALX family members regulate craniofacial development,
through transcriptional activation or repression of shared and
unique target genes.

**IPSC-derived NCC for craniofacial disease modeling**

Most craniofacial structures are derived from a transient multipotent
embryonic population called NCC. The NCC progenitors give rise to
a wide variety of cell lineages including peripheral neurons, melanocytes,
and craniofacial mesenchyme (Retancur et al., 2010; Stuhl-
miller & Garcia-Castro, 2012). NCC exhibit a restricted expression of
ALX1 in the rostral domain during early developmental stages (Zhao
et al., 1996; Dee et al., 2013). Cellular and genetic mechanisms that
drive frontonasal NCC formation are poorly understood. In order to
gain insight into the functional consequences of the clinically patho-
genic ALX1 gene variant identified in this study’s pedigree, iPSC were
differentiated into NCC.

While a number of sophisticated protocols using chemically
deﬁned mediums and a combination of adherent and/or suspension
culturing approaches have been published in recent years, no
consensus has been established on a number of controversial issues
(Bajpai et al., 2010; Leung et al., 2016; Tchieu et al., 2017).

First, the deﬁnition of what a NCC in fact is remains based
entirely on the transcriptomic proﬁling performed in animal studies.
While we succeeded in identifying distinctive differences between the
ALX1<sup>165F/165F</sup> NCC and healthy controls, the results suffer from
an obvious limitation: a lack of understanding which stage of develop-
ment the NCC represent. The central challenge of the work with
iPSC models of human disease remains the lack of available human
transcriptomic cell data to allow for an understanding which stage
of development is modeled by the cellular lineages derived. NCC are
characterized *in vitro* by the expression of markers identiﬁed to be
speciﬁc to this cell population, namely P75, CD57, CD90, CD73, and
CD105 (Minarcik & Golden, 2003; Bilion et al., 2007; Kawano et al.,
2017) as well as their multilineage differentiation ability. NCC
formation is a stepwise process coordinated by a spatiotemporally
speciﬁc gene expression pattern. In this study, a putative loss of
ALX1 function did not impair NCC differentiation itself or the ability
of NCC to differentiate into multiple cell lineages. Rather, it
appeared that the clinically pathogenic ALX1<sup>165F/165F</sup> variant main-
tained the NCC in a precursor state. While control cells progressively
became craniofacial mesenchymal cells by CD57 downregulation and increases in MSC associated marker expression,

**ALX1<sup>165F/165F</sup> NCC did not undergo changes of their morphology or show a transition of progenitor to mesenchymal markers**. Second, a
lack of a 3D representation of NCC migration *in vitro* based studies
force scientists to either transplant human iPSC-derived NCC into
model organisms or retain a 2D system of representation (Bajpai
et al., 2010; Okuno et al., 2017). We focused on the validation of the
ﬁndings in human iPSC in zebrafish. Third, the development of
craniofacial mesenchyme is a product of the interactions of deriva-
tives of all three germ layers. This left the role of ALX1 in other
developmental derivatives unexplored in this study.

To allow for some insight into the expression proﬁle of key NCC
markers during the *in vitro* differentiation process, we surveyed
relative expression via qPCR every 2 days throughout our differenti-
ation protocol. We found the greatest differences between the unaf-
fected father and the affected children in the expression of PAX7,
PAX3, DLK5, SNAI2, and TWIST1. ALX1 has been described as a
transcription modulator capable of both activating and repressing
target gene expression, adapting its activity to different cell types
and environments (Gordon et al., 1996; Cai, 1998; Damle & David-
son, 2011). Its activity as a repressor, for example, has been docu-
mented with prolactin, as ALX1 binds directly to the prolactin
promoter (Gordon et al., 1996). In this study, all of the genes were
substantially upregulated in the affected children, with the greatest
changes found in the earlier time points of differentiation. ALX1
appears to play the role of a transcriptional repressor in NCC-based
craniofacial development.

Neural crest cells delamination and migration depend on signals
from the surrounding epidermis, including BMPs, which induce
expression of neural plate border specifier genes such as PAX3,
TFAP2a, MSX1/2 or ZIC1 (Tribulo et al., 2003; Sato et al., 2005;
Garnett et al., 2012; Dougherty et al., 2013; Simoes-Costa & Bronner,
2015). Fine temporospatial regulation of the level of these signaling
molecules is critical to allow for delamination and migration of NCC
craniofacial development. BMPs belong to the transforming growth
factor beta (TGFβ) superfamily and can be divided into several
subcategories based on molecular similarities. The two BMPs show-
ing dysregulation in this study, BMP2 and BMP9, belong to different
subcategories which exhibit different expression patterns and recep-
tors. BMP2 was identiﬁed as an important factor in migratory NCC
development, with a depletion of mobile NCC in knockout models
in transgenic mice resulting in hypomorphic branchial arches. (Kan-
zler et al., 2000). In a complementary mouse model, BMP2 increased
migration of NCC when added as a supplement to culture medium
(Anderson et al., 2006). BMP2 was also found to be required for the
migration of NCC in the enteric nervous system in the zebrafish and
to be signiﬁcantly decreased in the gut of patients affected by
Hirschsprung’s disease, a disease characterized by defective enteric
NCC migration (Huang et al., 2019a). BMP9 on the other hand was
shown to be required for tooth development in mice (Huang et al.,
2019b). It was previously identiﬁed as a potent inducing factor of
osteoogenesis, chondrogenesis, and adipogenesis during develop-
ment (Luther et al., 2011; Lamplot et al., 2013). Opposed to other
BMPs, including BMP2, BMP9 was found to be resistant to feedback
inhibition by BMP3 and noggin (Wang et al., 2013).

The relationship of BMP2 and BMP9 in NCC development, migra-
tion, and differentiation has yet to be examined. Why BMP2 and
BMP9 appeared to play antagonistic roles in the NCC modeling of
ALX1-related FND presented in this study remains unclear. On the
basis of the qPCR data and the multiplex assay revealing a decrease in BMP2 and an increase in BMP9 in NCC supernatant, we hypothesized that a lack of fully functional ALX1 may account for the overexpression of neural plate border specifiers, and the change of BMP signaling. In substituting or repressing BMP2 and BMP9, respectively, an almost complete rescue of the migration defect of the mutant ALX1<sup>165F/165F</sup> NCC was achieved. Pretreatment of subject-derived NCC could perhaps result in a complete rescue of migration.

Animal models of ALX1-related FND

Studies in sea urchins have contributed meaningful knowledge to the regulatory functions of Alx1 as a transcription factor. In the sea urchin Strongylocentrotus purpuratus, the alx1 gene was found to activate itself in a self-regulatory loop at lower levels. Once its level exceeds a certain threshold, alx1 reverses its activity and becomes a repressor of its own transcription (Damle & Davidson, 2011). As a transcription factor, alx1 was found to be essential for the regulation of epithelial–mesenchymal transition, a process of great importance for the ability of NCC to delaminate and initiate migration (Ettensohn et al., 2003).

The specific role of Alx1 in craniofacial development was investigated in different animal models. Targeted gene ablation of Alx1 in mice resulted in neural tube closure defects in the majority of the pups, a phenotype not observed in any reported case report of mice. This study utilized germline mutants to investigate the effect of Alx1 loss-of-function, downstream transcription factor, alx1 was found to be essential for the regulation of epithelial–mesenchymal transition, a process of great importance for the ability of NCC to delaminate and initiate migration (Ettensohn et al., 2003).

Informed consent was obtained from the parents of the patients prior to all sample collections. All experimental protocols used for human samples were approved by the Animal Care and Use Committees of Massachusetts General Hospital (IACUC No. 2010N00106) and the University of Wisconsin and carried out in accordance with institutional animal care protocols.

iPSC and EB generation

Peripheral blood mononuclear cells were isolated using whole blood from two individuals (subjects 5 and 6: ALX1<sup>165F/165F</sup>), the unaffected father (subject 1: ALX1<sup>165L/165F</sup>), and three unrelated healthy individuals (controls: ALX1<sup>165L/165L</sup>). Samples were diluted in an equal volume of PBS and gently transferred to a tube containing 4 ml of FICOLL. After centrifuging the sample for 10 min at 350 g, the FICOLL-plasma interface containing the PBMCs was recovered and washed several times in PBS. After 24 h of recovery in StemPro-34 SFM medium (Invitrogen) supplemented with 100 ng/ml Stem Cell Factor (SCF, PeproTech, Rocky Hill, NJ), 100 mg/ml Fms-related tyrosine kinase 3 ligand (Flt3L, PeproTech), 20 ng/ml Interleukin-3 (IL-3, PeproTech) and 20 ng/ml IL-6 (PeproTech), 1 million PBMC were processed using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Invitrogen, Carlsbad, CA), following manufacturers’ instructions, for iPSC generation. One million PBMCs were infected with three different Sendai Viruses containing the Yamanaka reprogramming factors, OCT4, SOX2, KLF4, and c-MYC, in StemPro-34 SFM medium supplemented with cytokines. Starting on day 21, individual iPSC clones were picked based on morphologic criteria. Subsequently, the iPSC were maintained in StemFlex medium and passaged 1–2 a week using ReLSR (STEMCELL Technologies, Vancouver, BC, Canada) dissociation buffer. Since iPSC can exhibit genetic instability after reprogramming, the clones were expanded up to passage 10 before characterizing each cell line. The genetic stability of the cells was assessed analyzing copy number variants through genome-wide microarray analysis (Thermo Fisher). Epigenetic differences were controlled for in a limited manner by ensuring that all major experiments were performed in both biologic and technical triplicate at the identical passage number.

To form embryoid bodies (EB), iPSC were harvested using ReLSR dissociation buffer and clumps were transferred to a low adherent 6-well plate in differentiation medium containing 80% DMEM-F12, 20% Knock out Serum Replacer (Invitrogen), 1 mM nonessential amino acids, 1 mM Penicillin/Streptomycin, and 100 μM 2-mercaptoethanol. The medium was changed daily. After 14 days of differentiation, cells were recovered for RNA extraction and subsequent qPCR analysis of markers of the ectoderm, endoderm, and mesoderm.

Derivation of NCC and multilineage differentiation

In order to derive NCC, a previously published protocol for mesenchymal differentiation was adapted (Pini et al., 2018). iPSC medium was replaced by NCC-inducing medium containing DMEM-F12, 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1 mM Penicillin/Streptomycin, 1 mM nonessential amino acid, 110 μM 2-mercaptoethanol, and 10 ng/ml epidermal growth factor (EGF). The medium was changed every 2 days. After 1 week, cells were recovered using 0.25% trypsin-EDTA and transferred to new cultureware for an additional week. Following this, cells were harvested,
phenotypically characterized by flow cytometry for their expression of NCC markers and assayed for their mesenchymal differentiation ability.

Schwann cell differentiation was performed as previously described (Kawano et al., 2017). NCC were plated on glass coverslips in 24-well tissue culture plates (0.2 × 10^5 cells per well) in neuronal differentiation medium consisting of a 3:1 ratio of DMEM-F12 and neurobasal medium supplemented with 0.25% B-27, 1 mM glutamine, and 1 mM Penicillin/Streptomycin for 5 weeks. The medium was changed weekly. At the end of the differentiation, cells were fixed in 4% formaldehyde and analyzed by immunohistochemistry for S100B (Thermo Fisher, Waltham, MA) and GFAP expression (Abcam, Cambridge, United Kingdom).

Adipocyte and chondrocyte differentiation was performed as previously described (Pini et al., 2018). Adipogenesis was investigated using the StemPro adipogenesis differentiation kit (Life Technologies, Carlsbad, CA). NCC were seeded at 5 × 10^4 per well, in a 24-well plate, and cultured for 2 weeks, in complete adipogenesis differentiation medium. Lipid deposits were observed following staining with Oil Red O (MilliporeSigma, St. Louis, MO), according to manufacturers’ instructions. After washing, cells were counterstained with Mayer’s hematoxylin.

Chondrogenic differentiation was performed using the StemPro chondrogenesis differentiation kit (Life Technologies). NCC were seeded in a 12-well plate, in aggregates containing 8 × 10^4 cells, in 5 μl of NCC medium, and placed in a 37°C, 5% CO2 incubator for 1 h. Following this, the NCC medium was replaced by chondrogenesis differentiation medium, and cultured for 20 days. The medium was changed once a week. Chondrogenic matrix formation was observed following Alcian blue, Safranin O and Toluidine Blue staining.

Osteoblast differentiation was performed using the StemPro osteogenesis differentiation kit (Life Technologies). NCC were plated in 12-well tissue culture plates (5 × 10^5 cells per well) in osteogenesis differentiation medium for 14 days. At the end of the differentiation, the presence of mineralized nodules was assessed using Alizarin Red S, Von Kossa (silver nitrate) and Alkaline Phosphatase staining.

Images were acquired using the RETIGA OEM fast camera and Qcapture software (Teledyne QImaging, Surrey, BC, Canada).

Genomic DNA extraction and sequencing
Genomic DNA was extracted with the REDExtract-N-Amp tissue PCR kit (MilliporeSigma), following the manufacturer’s instructions. Sanger sequencing of ALX1 to ensure ALX1 sequence integrity in all iPSCs clones was carried out as previously described (Umm-e-Kaloom et al., 2012). The four ALX1 exons encoding the open reading frame were amplified using the CloneAmp HiFi PCR premix (Takara Bio Inc., Kusatsu, Shiga, Japan) and exon-specific ALX1 oligonucleotides. All exon-specific PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) prior to sequencing.

Whole-exome sequencing and analysis
Whole-exome sequencing (WES) of the affected subjects 3 and 4, an unaffected sibling, and the parents was performed and analyzed assuming a recessive mode of inheritance given the presence of multiple affected siblings. Three compound heterozygous variants and one homozygous recessive variant were identified in the affected siblings (ALX1 c.493C>T). This variant was predicted to be causative of the phenotype based on known gene function, the previously identified role of ALX1 in frontonasal development, and the effect of the variant (substitution of phenylalanine for the highly conserved leucine in a DNA-binding domain). Polymorphism Phenotyping v2, Sorting Intolerant from Tolerant, MutationTaster, and Functional Analysis through Hidden Markov Models (v2.3) were used for functional variant consequence prediction (Lowe, 1999; Adzhubei et al., 2010; Schwarz et al., 2014; Shihab et al., 2014). The gnomAD platform was used to identify any other missense variants at the location identified in the subjects (preprint: Karczewski et al., 2019). Clustal Omega was used for multiple sequence alignment (Sievers et al., 2011). Domain Graph was used to create the annotated schematic diagrams of ALX1 and ALX1 (Ren et al., 2009).

RNA extraction and processing
RNA was isolated using the RNAeasy Plus mini kit (Qiagen), following the manufacturer’s recommendations. One μg of RNA was reverse-transcribed using the SuperScript III first-strand synthesis system (Thermo Fisher). All PCR reactions on cDNA were performed using the GoTaq DNA polymerase (Promega, Madison, WI) unless otherwise noted. For zebrafish RNA extraction, 24 hpf Tübingen zebrafish embryos were harvested and homogenized using a microprobe in TRIzol reagent (Thermo Fisher), following manufacturer’s instructions. Total RNA was then purified using phenol-chloroform. One μg of total RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis Kit (Thermo Fisher), following manufacturer’s recommendations.

Flow cytometry analysis
Neutral crest cells were harvested and suspended in FACS buffer solution consisting of PBS with Ca2+ and Mg2+, 0.1% bovine serum albumin (BSA), and 0.1% sodium azide. Approximately 2 × 10^5 cells were incubated with the desired cell surface marker antibodies or isotype controls at 4°C for 15 min. Specific antibodies for CD90, CD73, CD105, and CD57 (BD Biosciences, San Jose, CA), and isotype control immunoglobulin IgG1 (BD Biosciences) were used for labeling. Antibodies were diluted in FACS buffer. After three washes in FACS buffer, samples were fixed in 0.4% formaldehyde and processed using an LSR II flow cytometer (BD Biosciences). The data acquired were analyzed using FlowJo software (FlowJo, LLC).

Immunohistochemical analysis of iPSC
Cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature, permeabilized with 1% saponin in PBS, and blocked using 3% BSA in PBS for 30 min at room temperature. The cells were then incubated with the primary antibodies for 3 h at room temperature. The following primary antibodies and dilutions were used: rabbit anti-OCT4 (1:100, Life Technologies), mouse anti-SSEA4 (1:100, Life Technologies); rat anti-SOX2 (1:100, Life Technologies), mouse anti-TRA-1-60 (1:100, Life Technologies), rabbit
anti-GFAP (1:500, Abcam), and rabbit anti-S100B (1:500, Thermo Fisher). The cells were then incubated with the secondary antibodies (1:1,000, MilliporeSigma) for 1 h at room temperature, washed with PBS, and counterstained with DAPI (MilliporeSigma). Secondary antibodies were Alexa 594 donkey anti-rabbit, Alexa 488 goat anti-mouse, and Alexa 488 donkey anti-rat and Alexa 594 goat anti-mouse (Thermo Fisher). Images were acquired using the RETIGA OEM fast camera and Qcapture software (Teledyne QImaging).

**Staining of iPSC and mesenchymal NCC derivatives**

Alkaline phosphatase activity was measured using the leukocyte alkaline phosphatase staining kit (MilliporeSigma), following the manufacturer instructions. Cells were first fixed with a citrate/acetone/formaldehyde solution for 30 s, washed several times, and stained with Fast Blue for 30 min. After further washing, these cells were counter stained with Mayer’s hematoxylin. Alizarin Red S., Von Kossa, Alcian Blue, and Toluidine Blue staining were performed as described (Pini et al., 2018). Cells were first fixed in 4% formaldehyde at room temperature for 15 min. Following a wash, cells were incubated in either 1% Alizarin Red, 1% Silver nitrate, 0.1% Toluidine Blue, 0.02% Alcian Blue, or 0.1% Safranine O solution. For Von Kossa staining, cells were exposed to UV light until dark staining appeared. Images were acquired using the RETIGA OEM fast camera and Qcapture software (Teledyne QImaging).

**Quantitative and nonquantitative polymerase chain reaction**

Real-time PCR assays were conducted on a StepOnePlus real-time PCR system, using PowerUp SYBR Green Master Mix (Applied Biosystems, Waltham, MA). Transcript expression levels were evaluated using a comparative CT process (ΔΔCT) with human RPLP0 and GAPDH used as reference genes. For zebrafish, elfa and 18S were used as reference genes. Specific primers were used for amplification as noted (Table EV1).

**Apoptosis assay**

2 × 10^5 cells were incubated for 30 min in the dark in 1× Fixable Viability Dye (FVD, Invitrogen) solution. After two washes in FACs buffer and one wash in binding buffer, cells were incubated 10–15 min in 1× Annexin V (BioLegend, San Diego, CA) solution in binding buffer composed of 0.1 M HEPES (pH 7.4), 1.4 M NaCl, and 25 mM CaCl_2_. After one wash in binding buffer, cells were suspended in 200 μl of binding buffer and immediately processed using an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (FlowJo LLC, Ashland, OR). Apoptosis was induced by placing the cell suspension in a water bath at 55°C for 10 min.

**Wound healing assay and analysis**

Migration was investigated using the Radius™ 48-Well Cell Migration Assay (Cell Biolabs, Inc., San Diego, CA), following manufacturer’s instructions. Control or ALX1^165F/165F NCC (1 × 10^5 cells/well) were plated in a 48-well plate containing a Radius™ Gel spot. Before beginning the migration assay, cells were washed three times with medium and incubated with gel removal solution for 30 min at 37°C. Following three subsequent washes in medium, the NCC were placed in a culture chamber for live cell imaging at 37°C and 5% CO2. Rescue experiments were performed through the addition of soluble BMP2, CV2, or a combination of the two at a concentration of 10, 50, or 100 ng/ml to the medium at the beginning of the assay. For fluorescent pictures, cells were stained in serum-free media containing 3.6 μM CellTracker Green CMFDA (Life Technologies) for 30 min at 37°C and allowed to recover for 30 min before starting the experiment. All images were acquired using a Keyence BZ-X800 microscope. The time-lapse film was made by acquiring images every 15 min for 24 h. The fluorescent images were acquired every 6 h. Surface area analyses and percentages of recovery were measured using ImageJ software (NIH, Bethesda, MD).

**Multiplex analysis of BMP concentration**

The concentration of the BMP family in the supernatant of ALX1^165F/165F NCC was measured using a bead-based multiplex array (Forsyth Institute, Cambridge, MA). Manufacturers’ protocols were followed for all panels. Reagents were prepared as per kit instructions. Assay plates (96-well) were loaded with assay buffer, standards, supernatant from the ALX1^165F/165F NCC, and beads and then covered and incubated on a plate shaker (500 rpm) overnight at 4°C. After primary incubation, plates were washed twice. Following this, the detection antibody cocktail, consisting of BMP-specific antibody with biotin (1:10) and the detection antibody streptavidin conjugated with PE (1:25), was added to all wells; the plates were covered and left to incubate at room temperature for 1 h on a plate shaker. After the incubation, streptavidin-phycocerythrin fluorescent reporter was added to all wells, and the plate was covered and incubated for 30 min at room temperature on a plate shaker. Plates were then washed twice, and beads were resuspended in sheath fluid, placed on shaker for 5 min, and then read on a Bio-Plex® 200 following manufacturers’ specifications and analyzed using Bio-Plex Manager software v6.0 (Bio-Rad, Hercules, CA).

**Plasmid construct generation**

The In-Fusion Cloning Kit (Takara) and the In-Fusion Cloning primer design tool were used for primer design. Tübingen zebrafish alx1 was amplified via PCR. Zebrafish alx1 as cloned into the SpeI and PacI (NEB, Ipswich, MA) restriction sites of pCS2 + 8 (Promega). The subsequent reaction product was used to transform One Shot TOP10 competent cells (Thermo Fisher) or Stellar competent cells (Takara).

For the generation of truncated alx1 constructs, the genes were divided into N-terminal and C-terminal sections, with aa181 being designated as the beginning of the C-terminal portion in zebrafish alx1. For all plasmid constructs, individual clones were picked, DNA purified (Qiagen), and validated using Sanger and Next Generation whole plasmid sequencing.

**CRISPR-Cas9 directed mutagenesis of zebrafish**

A CRISPR site on exon 2 of the alx1 gene was selected using the Burgess lab protocol (Varshney et al., 2015, 2016). The single guide
RNA (sgRNA) targeting this site (sequence: GGAGAGCAGCCTG-CACGCGA), and Cas9 or nCas9 mRNA, were prepared as previously described (Gagnon et al., 2014; Shah et al., 2016a,b). Genetically defined wild-type (NIHGR1-1; LaFave et al., 2014) embryos were injected at the one-cell stage with 50–100 pg of sgRNA and 360–400 pg of Cas9 or nCas9 mRNA. Adult F0 animals were intercrossed to produce the F1 generation. F1 mutant carriers were identified by PCR using forward primer GTGGACT-TACTCGCTCCCTA and reverse primer CGAGTTGGTCG-GAGTCTGTT. The PCR products were resolved on a MetaPhor gel (Lonza, Basel, Switzerland) and sequenced. A frameshift allele was identified: a deletion of 16 nucleotides, termed alx1<sup>1609</sup> (Fig EV4).

**Alx1DN expression in zebrafish embryos**

The validated Alx1DN (N-terminal portion of protein product containing homeodomain and nuclear localization domains) clones in pCS2+8 were purified via miniprep (Qiagen) alongside a control (C-terminal portion containing transactivation domain) and digested using NotI (NEB), before being gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Five hundred microgram of purified, digested plasmid DNA served as the input for the mMessage mMachine SP6 Transcription Kit (Thermo Fisher). The resulting mRNA was then further purified using the RNeasy Mini Kit (Qiagen) and frozen in 100 ng/µl aliquots at −80°C. mRNA overexpression was accomplished using microinjections. mRNA stock aliquots were first diluted to the desired concentration with 0.125% Phenol Red in ultrapure water (Invitrogen). A 2 nl drop was then injected into fertilized Tübingen embryos at the single cell stage. At 4 h post-fertilization (hpf), all unfertilized and visibly damaged embryos were removed.

**Alcian blue staining**

All injected and uninjected zebrafish were incubated at 28.5°C for 5 dpf in E3 buffer with 0.0001% methylene blue. At 4 days post-fertilization (dpf), injected and uninjected embryos were fixed overnight at 4°C in 4% formaldehyde, washed stepwise with 1 × PBS and 50% EtOH in PBS before being stained in a solution of 0.02% Alcian blue, 70% EIOH, and 190 mM MgCl₂ overnight at room temperature on a rotating platform. Following this, embryos were washed with ddH₂O before being bleached in a solution of 0.9% H₂O₂, 0.8% KOH, and 0.1% Tween20 for 20 min. Stained embryos were then imaged in 4% methylcellulose in E3 solution and stored in 4% PFA at 4°C. Images were captured using a Nikon DS-Fi3 digital camera.

**Lineage tracing**

The application of Tg:sox10:kaede in cell labeling has previously been reported, where photoconversion of the kaede protein from green to red in selected cells under confocal microscopy can be used to follow distinct NCC migration patterns across time.

Alx1DN injected or control sox10:KAEDER transgenic embryos were imaged using a Leica SP8 confocal microscope at 19 to 20-somite stage to identify neural crest structures in a manner previously described (Dougherty et al., 2012, 2013). The most distal population of the migrating stream of cranial neural crest cells was excited for 15 s for photoconversion with the FRAP module and a 405 nm laser at 25% power. Embryos were then placed back into a 28.5°C incubator. Both the photoconverted red (488 nm) and nonphotoconverted green (572 nm) neural crest populations were captured at 4 days post-fertilization (dpf) using a Leica Sp8 and analyzed with the Leica Application Suite X (Leica Microsystems, Buffalo Grove, IL) software for image capturing. A composite image was subsequently generated using ImageJ (NIH; Fig 8D, Film 3).

**Statistical analysis**

Each experiment was performed on six independent healthy control Alx1<sup>165L/165L</sup> clones, three heterozygous Alx1<sup>165L/165F</sup> clones, and nine homozygous Alx1<sup>165F/165F</sup> clones, and repeated at least three times. The qualitative craniofacial analysis of alx1<sup>−/−</sup> zebrafish and Alx1DN injections was performed three times, on three different clutches of embryos. For RT-qPCR experiments, data from each clone were pooled and the mathematical mean was calculated. SEM was used to determine the standard error. To test statistical significance, Student’s t-test for paired data was used. Statistical analysis of the significance of the qPCR results was performed with an ANOVA test. A P-value < 0.05 was considered to be statistically significant. Statistical analysis was performed using GraphPad Prism 7.0 software. The D’Agostino and Pearson normality test was performed to verify normality. For groups that fulfilled normality and equal variance requirements, a one-way ANOVA with a Sidak comparison test (95% confidence interval to compare all the different groups) was performed. For data sets that did not fulfill normality and equal
variance requirements, a Kruskal–Wallis test was performed. Mean values for each group were compared using two-tailed Student’s test for comparisons of two independent groups.

**Data availability**

The raw sequencing data sets produced in this study are available in the following database: FaceBase Record ID: 25J0 Accession: FB00000907 (https://www.facebase.org/).

**Expanded View** for this article is available online.

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**Author contributions**

JP, JK, and ECL conceived of the project and designed the research studies. JP, JK, YDH, CT, NC, RM, JC, and YG conducted the experiments. JP, JK, CT, NC, RM, JC, YG, and ECL prepared the manuscript. JK, CT, and ECL worked on the revision of the manuscript.

**Conflict of interest**

The author declares that he has no conflict of interest.

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