Defective neural crest migration revealed by a Zebrafish model of Alx1-related frontonasal dysplasia

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Frontonasal dysplasia (FND) refers to a class of midline facial malformations caused by abnormal development of the facial primordia. The term encompasses a spectrum of severities but characteristic features include combinations of ocular hypertelorism, malformations of the nose and forehead and clefting of the facial midline. Several recent studies have drawn attention to the importance of Alx homeobox transcription factors during craniofacial development. Most notably, loss of Alx1 has devastating consequences resulting in severe orofacial clefting and extreme microphthalmia. In contrast, mutations of Alx3 or Alx4 cause milder forms of FND. Whilst Alx1, Alx3 and Alx4 are all known to be expressed in the facial mesenchyme of vertebrate embryos, little is known about the function of these proteins during development. Here, we report the establishment of a zebrafish model of Alx-related FND. Morpholino knock-down of zebrafish alx1 expression causes a profound craniofacial phenotype including loss of the facial cartilages and defective ocular development. We demonstrate for the first time that Alx1 plays a crucial role in regulating the migration of cranial neural crest (CNC) cells into the frontonasal primordia. Abnormal neural crest migration is coincident with aberrant expression of foxd3 and sox10, two genes previously suggested to play key roles during neural crest development, including migration, differentiation and the maintenance of progenitor cells. This novel function is specific to Alx1, and likely explains the marked clinical severity of Alx1 mutation within the spectrum of Alx-related FND.

INTRODUCTION

The development of the vertebrate face is a remarkably dynamic and intricate process, requiring the tightly regulated growth, fusion and patterning of the facial primordia. These primordia consist of frontonasal, maxillary and mandibular prominences which are derived primarily from migratory neural crest (1). The formation, outgrowth and maturation of the various prominences are orchestrated by multiple tissue interactions and a complex network of genes and regulatory molecules that we are only just beginning to elucidate (2–5). In this context, it is perhaps unsurprising that facial malformations are among the most common of developmental defects, causing considerable and lifelong morbidity to those affected.

Frontonasal dysplasia (FND), or median facial cleft syndrome (OMIM 136760), is a well-documented class of developmental abnormalities caused by incomplete growth and fusion of the facial prominences. The term includes a broad range of clinical presentations varying from mild ocular hypertelorism to severe clefting of the facial midline (6,7). Additional diagnostic findings may include broad nasal root, malformed nasal tip, anterior cranium bifidum occultum or a widow’s peak hairline. Whilst many cases are regarded as sporadic, several recent studies have demonstrated the importance of members of the Alx gene family in craniofacial development (8–11). This small family of genes encoding paired-class homeobox transcription factors consists of only three members, namely Alx1 (formerly known as Cart1), Alx3 and Alx4 (12). However, a new class of recessively inherited Alx-related FND has now been defined, and includes a full spectrum of clinical severities with associated phenotypes (11).

Most striking of these is the extremely severe phenotype of frontofacionasal dysplasia caused by homozygous mutation of Alx1, which has so far been reported as loss-of-function
mutations in two separate Turkish families (11). Alx1-related FND manifests as severe oblique facial cleft (including complete cleft palate), marked hypertelorism, and extreme microphthalmia. In addition, these are associated with a variety of malformations of the upper face such as sparse eyelashes, lack of eyebrows, wide nasal bridge and hypoplasia of the ala nasi. Although only a small number of cases have been described to date, an individual affected by this disfigurement can survive well into childhood (and possibly beyond). It is interesting to note that the developmental abnormalities in these patients appear largely restricted to the craniofacial region, with mild retardation and relatively unaffected motor development (11). Taken together, such findings indicate a specific and early role for Alx1 in the maturation and fusion of the frontonasal, nasomedical, nasolateral (which are both derived from the frontonasal process) and maxillary prominences.

In comparison to Alx1, it is clear that mutations in Alx3 and Alx4 result in much milder, albeit clinically distinctive malformations of the frontonasal region. Homozygous mutations in the Alx3 gene are known to underlie the autosomal recessive disorder known as frontorhiny (Alx3-related FND) (10,13), which is characterized by hypertelorism and a distinctive broad, bifid nasal morphology indicative of incomplete fusion of the frontonasal and nasomedical prominences. Alx4-related FND can be associated with significant non-facial findings such as total alopecia and genital abnormalities, as well as significant defects of the skull. However, whilst most reported cases of Alx4-related FND are more severe in terms of the level of hypertelorism and abnormal eye development, the distinct nasal phenotype is very comparable with that of frontorhiny (8,9).

Despite its clear importance, little is known about the function of Alx1 during craniofacial development. In the mouse, Alx1, 3 and 4 are all robustly expressed in specific, but overlapping aspects of facial mesenchyme, suggesting that they may be partially redundant in function (12,14–17). Consequently, it has long been proposed that Alx genes might represent key regulators of chondrocyte development. However, whilst mouse Alx1 null mutant mice have severe defects of the cranial skeleton, these appear to be a secondary consequence of a neural tube closure defect not found in human cases (18,19). Certainly, abnormal fusion of the facial prominences is not reported and the phenotype does not appear to be directly comparable with that described in human patients. In addition, a similar neural tube phenotype has recently been reported in Alx3 knock-out mice (20), although many Alx3 mutant mice are born with no obvious abnormalities (21). Alx4-mutant mice have previously attracted interest primarily due to a role in patterning of the limb bud, but exhibit only mild skull abnormalities, most notably a delay in the development of the parietal bone (22). This observation is at least partially consistent with reports that Alx4 haploinsufficiency can result in skull ossification defects and parietal foramina (23–25). More interestingly, varying degrees of midfacial clefting has been reported in compound mutants of Alx1 and Alx4 (19), and similarly in an Alx3/Alx4 double mutant (21), which the authors have ascribed to impaired survival of the frontonasal mesenchyme. These data further indicate how the phenotype of Alx1 mutation is likely to be moderated by overlapping function of these genes. However, the developmental mechanisms underlying the extreme nature of the human Alx1-mutant phenotype remain unclear.

The zebrafish has emerged as an increasingly valuable model of human craniofacial development and disease (1, 26–29). In particular, the early developmental events are conserved, and several recent studies have demonstrated that the development of the zebrafish palate, or anterior neurocranium, shares considerable homology to the palatal skeleton of mammals in terms of both cellular origin (30,31) and the programme of genetic control (32). Frontonasal and maxillary populations of mesenchyme have been identified and are derived from a population of cranial neural crest (CNC) which is homologous to that found in mammals (32).

Here, we employ the advantages of the zebrafish model to gain further insights into the molecular pathology of Alx1-related FND. We report a novel function for alx1 in the development of migratory CNC, which directly influences the development of the palate. We show that expression of all zebrafish alx genes, alx1, alx3, alx4a and alx4b, is remarkably well conserved with both mouse and chicken in specific and overlapping domains of neural crest-derived facial mesenchyme. Importantly, the alx1 gene is the only member of this family to be expressed during the early stages of CNC development. Morpholino knock-down of zebrafish alx1 expression results in a severe craniofacial phenotype including drastically reduced eyes, catastrophic loss of facial cartilage and abnormal development of the palate. We show that alx1 does not affect the formation of the CNC, but reduced alx1 expression causes aberrant expression of foxd3 and sox10 coincident with inhibition of neural crest migration in the frontonasal stream. By comparison, morpholino knock-down of alx3 expression produces no obvious craniofacial phenotype. We propose a model whereby this non-redundant role of alx1 in migratory neural crest contributes to the severe facial malformations seen in zebrafish and humans, and provides an explanation for the striking clinical severity of Alx1 mutation amongst the spectrum of Alx-related FND.

RESULTS

Conserved developmental expression of zebrafish alx genes

A search of the genome database identified the predicted gene sequences for zebrafish alx1 (NM_001045074) and alx3 (XM_690238). As a result of a whole genome duplication event in the teleost lineage (12), two paralogues of Alx4 can be identified designated alx4a (XM_001340930) and alx4b (NM_001089357). The protein-coding regions of all four genes were cloned by RT-PCR and used to generate probes for in situ hybridization. The Alx1, Alx3 and Alx4 genes have previously been reported to be expressed in specific regions of the neural crest-derived facial mesenchyme of mouse (14–17), and we have recently described similar patterns of Alx expression in chick and the western clawed frog Xenopus tropicalis (12). The expression of zebrafish alx genes is remarkably well conserved with that of mammals and other vertebrates with expression in both distinct and overlapping domains within the frontonasal mesenchyme, periocular mesenchyme (POM), mandible arch and the prospective palate (summarized in Table 1).
In 24hpf stage embryos, zebrafish alx1 is expressed strongly in the POM (Fig. 1A), which will ultimately give rise to components of the eye and orbit including the orbital skeleton (33).

By 48hpf alx1 expression is maintained in the POM towards the anterior of the head, with weaker expression in the nasal region and the prospective palate (Fig. 1B, arrow, and Supplementary Material, Fig. S1). In addition, alx1 is expressed in a discrete region at the distal tip of the mandible arch (Fig. 1B, arrowhead, and Supplementary Material, Fig. S1).

Expression of alx3 is also observed specifically in the anterior domain of POM at 24hpf, with strong expression in the frontonasal mass (Fig. 1C) as has been previously described in mouse (12,15). Expression surrounding the nasal placodes is maintained at 48hpf at which time alx3 is expressed strongly in the developing palate (Fig. 1D, arrow, and Supplementary Material, Fig. S1). The alx4a gene is also expressed in the frontonasal region at 24hpf (Fig. 1E). At 48hpf alx4a expression is weaker than alx3 in the mesenchyme surrounding the nasal placodes, but is expressed strongly throughout the mandible arch (Fig. 1F, arrow and Supplementary Material, Fig. S1). In contrast, alx4b is expressed only weakly in the nasal mesenchyme at 24hpf (Fig. 1G). Interestingly, at 48hpf alx4b is absent from the mandible arch, but expressed strongly in the hyoid arch which in fish (but not mammals) also gives rise to specific components of the jaw skeleton (Fig. 1H, arrow, and Supplementary Material, Fig. S1) (1). In addition, alx4b is expressed widely in the pigment cells located on the surface of the eye (Fig. 1H), which, like the facial mesenchyme, are initially derived from neural crest.

Given that Alx4 (and to a lesser extent Alx1) has been reported to play an important role in the patterning of the developing limb in mouse and chick (19,22,34–36), it is also worth noting that both alx4a and alx1 are expressed in the pectoral fin bud primordia of zebrafish (Fig. 1A and E). However, developmental defects of the limb have not been reported in human cases (8,9,11).

The alx1 gene is expressed during the early stages of neural crest development

Given the extreme severity of Alx1-related FND, we were particularly interested to note a previously unreported feature of alx1 expression, namely, that alx1 is expressed during the early stages of neural crest development. As in other species, the neural crest is initially induced from within the ectoderm (37,38), and by the three somite stage (3ss) the expression of neural crest genes is clearly recognizable at the border of the neural plate. Expression of alx1 is initially detected at the tailbud stage (immediately following gastrulation), when it is expressed weakly in the cephalic mesoderm (Fig. 2A, arrow), and this mesoderm expression is maintained.
during early stages (Fig. 2B, C, E). Expression of alx1 is initiated in the neural crest by the 5ss, and is interestingly restricted to CNC cells migrating towards the frontonasal and maxillary regions (asterisk marks the position of the eye). (E) Section of the 5ss embryo (D, dotted line) showing expression in the neural crest and cephalic mesoderm. (F) Section of 12ss embryo (D, dotted line) showing expression of alx1 in the migrating neural crest (arrows indicate the direction of migration). (G–I) Fluorescent in situ hybridization for alx1 in sox10:GFP embryos at 10–11ss, viewed as coronal section, anterior to the left. Transcription of alx1 is detected in the nucleus of CNC cells in the midbrain (G, green) and is specific to GFP-positive cells (H, red; merged in I). (J–M) Expression of alx1 (J), alx3 (K), alx4a (L) and alx4b (M) in migrating CNC cells at 18hpf. (A, D, J–M) Lateral views, anterior to the left. (B and C) Dorsal views, anterior to the top. nc (neural crest), m (mesoderm).

Alx1 is essential for craniofacial development in zebrafish

Although there are considerable differences in the morphology of the mammalian and fish facial skeletons, it is clear that the underlying developmental events are conserved. For example, the zebrafish palate, or anterior neurocranium, is composed of an ethmoid plate and paired trabeculae, which as with the mammalian palate, are derived from the maxillary and frontonasal progenitor domains (30–32).

To analyze the function of Alx1 in craniofacial development, we designed a morpholino antisense oligonucleotide (MO) to target the first intron–exon boundary of alx1 and inhibit normal processing of the transcript (alx1i1e2). Analysis using RT-PCR confirmed that injection of alx1i1e2 caused substantial knock-down of the alx1 transcript (Fig. 3A). To validate this approach, a second MO was designed to block translation of the alx1 transcript by targeting the translation start codon (alx1AUG).

Analysis of embryos injected with the alx1i1e2 MO revealed a profound craniofacial phenotype with features suggesting considerable homology of function between human and zebrafish. By 4dpf, alx1 morphtants could clearly be seen to have a small, misshapen head, eyes significantly reduced in size and loss of the jaw (Fig. 3B and C). These features were accompanied by a pericardial oedema, a common secondary effect presumably resulting from altered circulation (Fig. 3C, asterisk). The same phenotype was obtained by injection of the alx1AUG MO (Fig. 3D).

Given that extreme microphthalmia is a consistent yet understudied feature of human Alx1 loss-of-function in FND, we more closely examined the eye phenotype in zebrafish. This revealed several features consistent with defective development of the neural crest-derived POM (39–41). We observed microphthalmia in morphant embryos (61/65) and macroscopically visible retinal colobomas (severe coloboma 46/65, mild coloboma 15/65; Fig. 3E–G). Histological sections revealed massive disorganization of the retina at the
posterior of the eye (Fig. 3H and I, white arrow). In the anterior segment, the lens appeared small and staining shows reduced differentiation of lens fibres (Fig. 3I). In addition, the corneal layers appear to be disorganized (black arrow in Fig. 3I).

Alcian blue stain of alx1 morphants revealed significant loss of the cartilaginous facial skeleton (Fig. 4). Embryos injected with a control MO (cMO) are indistinguishable from wild-type embryos (Fig. 4A and B). Embryos injected with the alx1i1e2 MO have been classified into three categories of severity. The majority of alx1 morphants exhibit total loss of the cartilage elements of the jaw and almost complete loss of the palatal structures, although remnants of the paired trabeculae remain (85/136; Fig. 4C and D). A second substantial group retains the elements of the larval palate, the ethmoid plate and the trabeculae, but both are substantially reduced in size. The jaw is almost completely absent, but some residual fragments of jaw cartilage remain (45/136; Fig. 4E and F). A few embryos exhibit a milder phenotype, retaining skeletal elements of both the palate and the jaw, but all are reduced in size and the Meckel’s cartilage is deformed (6/136; Fig. 4G and H). In all cases, the ceratobranchial cartilages (which support the gill slits of fish) are also absent. We also performed the standard control of injecting the cMO and alx1i1e2 into embryos homozygous for a p53 null mutation (p53<sup>tm124k</sup>) (42,43). This resulted in identical phenotypes to those described above (Supplementary Material, Fig. S2), further demonstrating the specificity of the phenotype. Identical phenotypes were also obtained by injecting various doses of alx1AUG (Supplementary Material, Fig. S3).

For comparison, we also examined the effect of inhibiting Alx3 function in zebrafish. Whilst loss of Alx1 causes severe impairment of craniofacial development in both humans and zebrafish, loss of Alx3 results in a considerably milder facial malformation in human and the direct effect on craniofacial development is reportedly minimal in mouse (10,20,21). We designed MOs to block the expression of zebrafish Alx3. The alx3e1i1 MO targets the exon1/intron1 splice donor site, whilst alx3i1e2 targets the intron1/exon2 splice acceptor site. RT-PCR analysis indicates that injection of either of these MOs results in effective knock-down of alx3 with the transcript undetectable in either case (Supplementary Material, Fig. S3). No abnormal splice products could be detected following injection of either alx3e1i1 or alx3i1e2, suggesting that the transcript has been removed by nonsense-mediated RNA decay. Alcian blue stain of alx3 morphants revealed no significant abnormalities of the facial skeleton (Fig. 4I and J; Supplementary Material, Fig. S3). This is consistent with the observation that whilst zebrafish alx3 is enhanced in the frontonasal mesenchyme and the developing palate, these domains are shared by weaker expression of alx1 and alx4a, which may be able to compensate for the loss of alx3.

Overall, these data demonstrate that in zebrafish, as in human, Alx1 is essential for the development of the facial skeleton and cannot be compensated for by the action of other Alx transcription factors.
Enhanced expression of Figure 4. For example, roles in different phases of development and in different neural cated network of regulatory factors, which often play multiple factors. The expression analysis presents phases of development including neural crest specification, alx1 morphants could result from defects during a number of known neural crest gene which marks this same midbrain migration of the CNC in zebrafish at this stage. At the 13ss, migration, the expression of both foxd3 and sox10 remains intact in alx1 morphants compared with wild-type controls (Fig. 5A–H). By the 12ss, CNC migration has initiated in wild-type or control morphant embryos and the expression of foxd3 has been heavily down-regulated in the midbrain, alx1 expressing population of CNC (Fig. 5I and K, arrowheads). Expression of sox10 is also down-regulated at this stage, although it continues to be expressed at lower levels in the migrating neural crest (Fig. 5M and O, arrowheads). In alx1 morphant embryos, however, foxd3 expression is clearly maintained ectopically at the 12ss (50/53; Fig. 5J and L, arrows) and sox10 expression also appears to be enhanced with a distribution suggesting that CNC cell migration has been inhibited (38/38, Fig. 5N and P, arrows). Taken together, these data suggest that alx1 is required for normal expression of two key neural crest genes implicated in regulating the onset of CNC migration.

Alx1 knock-down results in defective migration of the CNC

The severe disruption of craniofacial development observed in alx1 morphants could result from defects during a number of phases of development including neural crest specification, migration and differentiation. The expression analysis presents the tantalizing possibility that Alx1 may function both in early neural crest and also later maturation of mesenchyme of the upper face and mandible. A potential role in early neural crest is of particular interest as this is exclusive to Alx1 and, therefore, not modulated by redundancy with other Alx factors.

The development of neural crest is regulated by a complicated network of regulatory factors, which often play multiple roles in different phases of development and in different neural crest populations (37,38). For example, sox10 and foxd3 are both involved in the formation and specification of the neural crest (44–48), but are down-regulated immediately prior to the onset of CNC migration (49). Foxd3 is potentially interesting in this context, as it is expressed in the same midbrain population of CNC (see Fig. 5) that expresses alx1 and can inhibit cell migration in both CNC (49,50) and also in the comparable context of melanoma progression (51).

Expression of alx1 in the neural crest is first detected at the 5ss, after the expression of many neural crest genes has already been induced, and we initially found that the expression of various neural crest markers (sox10, snai1b, foxd3) was unaffected by alx1 knock-down at 5–6ss (Supplementary Material, Fig. S4). This is maintained during early development so that by the 10ss, immediately prior to the onset of migration, the expression of both foxd3 and sox10 remains intact in alx1 morphants compared with wild-type controls (Fig. 5A–H). We initially examined the expression of snai1b, a well-known neural crest gene which marks this same midbrain population of CNC in zebrafish at this stage. At the 13ss, migration of snai1b expressing CNC cells could clearly be seen to be inhibited in alx1 morphant embryos compared with wild-type controls (Supplementary Material, Fig. S5). To examine this effect in more detail, we again employed the sox10-GFP transgenic line to visualize CNC cells during migration (31). Carefully staged embryos injected with either a control or alx1 MO were fixed at various time points during the early stages of CNC migration. At the 10ss, immediately prior to the onset of migration, CNC cells are indistinguishable between control and alx1 morphant embryos (Fig. 6A and E), confirming our previous observations regarding the expression of foxd3 and sox10 (Fig. 5). At the 12ss, 14ss and 16ss, CNC

Figure 4. Inhibition of alx1 causes loss of the facial cartilages. Alcian blue stained larvae viewed laterally (A, C, E, I) or ventrally (B, D, F, H, J) at 5dpf, anterior to the left. (A and B) Control morphant larvae are identical to wild-type. (C–H) Morpholino (alx1i1e2)-mediated knock-down of Alx1 resulted in three classes of craniofacial defects. (C and D) Class I shows severe loss of the palate (arrows indicate position of trabeculae) and ventral cartilage (n = 85/136). (E and F) Class II shows heavily reduced palate (arrowheads in F) and severe loss of ventral cartilage (45/136). (G and H) Class III is less affected, but remaining cartilage elements are malformed (6/136). (I and J) alx3 morphant (alx3e111) larvae are indistinguishable from the controls. e (ethmoid plate), t (trabeculae), m (Meckel’s cartilage).
cells can be seen to have migrated rostral and caudal to the eye (Fig. 6B–D). Inhibition of CNC migration in \textit{alx1} morphant embryos (Fig. 6F–H) can be seen clearly in the frontonasal stream migrating in a path dorsal to the eye (leading edge of the migrating stream is marked by white arrows; yellow arrows mark the caudal limit of the eye). Migration was measured as the distance from the caudal limit of the eye to the leading edge of the migrating cluster (Fig. 6I and Supplementary Material, Fig. S5E). To account for variation in size and morphology between embryos, we expressed the migration distance as a ratio to the longest diameter of the eye (Fig. 6I), and statistical analysis confirmed significant inhibition of CNC migration in the frontonasal stream (Fig. 6J, \( P < 0.001 \)). High magnification imaging of CNC cells migrating dorsal to the eye at the 13ss revealed cell projections primarily orientated in the direction of migration in controls (Fig. 6K, small white arrows). In \textit{alx1} morphants, CNC cells at the leading edge appeared disorganized and projections were reduced (Fig. 6L). Altogether, these data demonstrate significant inhibition of CNC migration emanating from the midbrain.

### Defective palate development in \textit{alx1} morphants is a consequence of abnormal CNC migration

Consistent with what has been described for the mammalian palate, the cellular precursors of the zebrafish ethmoid plate and trabeculae can be divided into frontonasal and maxillary populations (31,32,52). By 24hpf, these can be seen to reside at locations beneath the eye in \textit{sox10:GFP} embryos injected with the cMO (Fig. 7A and C, white arrows), and are separated from the mandible arch by the stomodaeum (arrowheads in Fig. 7A–D). In \textit{alx1} morphants, the frontonasal and maxillary neural crest cells fail to occupy these positions and there is a substantial space separating the frontonasal and maxillary populations (Fig. 7B and D). In \textit{alx1} morphants, the frontonasal and maxillary neural crest cells fail to occupy these positions and there is a substantial space separating the frontonasal and maxillary populations (Fig. 7B and D). 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strongly expressed in the frontonasal mesenchyme at 24hpf (Fig. 7E and F). In many cases, neural crest cells are seen to have migrated aberrantly in a path across the surface of the eye (Fig. 7B and D, yellow arrowheads; Fig. 7F, black arrowhead). The failure of development of the palate can be seen clearly by 48hpf, by which time the mesenchymal cells have begun to condense to form the cartilaginous elements of the palate. In 48hpf control morphant embryos, the paired trabeculae can already be identified by expression of sox10 (Fig. 7G and I, arrows). In alx1 morphant embryos, the trabeculae have failed to extend (Fig. 7H and J). By 3dpf, alcian blue stain clearly reveals the cartilaginous ethmoid plate and paired trabeculae in control embryos (Fig. 7K), but these have not formed in alx1 morphants (Fig. 7L).

Taken together, our results show that the abnormal CNC migration caused by zebrafish alx1 knock-down results in failure of the development of the derivatives of the frontonasal and maxillary mesenchyme.

**DISCUSSION**

Although many cases of FND have been described as sporadic, a significant number of familial cases have been described (53–59). However, various patterns of inheritance have been reported and the analysis is often complicated by variable penetrance of the phenotypes. These observations are consistent with the subtle complexity of facial development, and identifying the molecular pathways involved constitutes a major challenge. The discovery that disruption of three Alx transcription factors can cause different variations within the FND spectrum is therefore of considerable interest.

In this study, we have established the zebrafish model as a means to explore the biology of Alx-related FND. Our results strongly support the findings of Uz et al. (2010), who reported that homozygous mutation of human Alx1 results in a severe form of FND including marked hypertelorism, severe clefting of the facial midline and extreme microphthalmia (11). The severity of these malformations suggests that Alx1 plays a crucial role in the early development of the facial primordia. The data presented here demonstrate that Alx1 is also essential for the early events of craniofacial development in the zebrafish model, providing an opportunity to explore the underlying developmental mechanisms.

We have shown that depletion of zebrafish alx1 causes a severe craniofacial phenotype including defects of eye development and substantial reduction of the viscerocranial...
cartilages. In addition, we have shown for the first time that Alx1 is required for normal migration of the CNC into the frontonasal region. These observations by no means exclude the possibility that Alx1 performs an equally important function in the later maturation of the facial mesenchyme. However, it seems likely that the role of Alx1 in these tissues overlaps with that of Alx3 and Alx4 (Alx4a and Alx4b in zebrafish) in regions where they are co-expressed. This notion is supported by our observation that Alx3 depletion causes no detectable phenotype in zebrafish, acts redundantly with Alx4 in mouse (21) and is absent from the genomes of several model species including chicken and Xenopus (12). The function of zebrafish Alx1 in early CNC is specific to Alx1, and we therefore propose that this non-redundant role likely offers an explanation for the exceptional clinical severity of Alx1-related FND in comparison to mutations in Alx3 or Alx4.

Our results are consistent with several studies which have used the zebrafish model to show that abnormal neural crest migration can underlie human craniofacial malformations, including oblique facial clefting or cleft palate (28,29,60). To date, EPHB1, mutation of which causes X-linked craniofrontonasal syndrome (OMIM 304110), is the only other gene which has been directly linked to a subclass of FND (55,56). The mechanism of this disorder is not well understood (61), and the gene product, Ephrin-B1, belongs to a family of receptor tyrosine kinase ligands known to be involved in a variety of cellular and developmental processes (62). However, in the light of our study, it is worth considering that Ephrin-B1 is known to be important for both neural crest induction and migration (63–65). The Brachyrrhine (3H1 Br)-mutant mouse, which results in the loss of the transcription factor Six2, has been reported to display a phenotype comparable with human FND, although in this case the authors have described reduced proliferation of mesenchymal cells in the craniofacial region (66–68). Our results are also interesting in the light of the recent study by Phillips et al. (2012), in which the authors employed the conditional

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**Figure 7.** Inhibition of CNC migration causes abnormal development of the palate. (A–D) Control and alx1 morphant sox10:GFP embryos at 24hpf (lateral view, anterior to the left) viewed with (C and D) and without (A and B) brightfield images. (A and C) In embryos injected with the cMO, the precursors of the zebrafish palate are positioned beneath the eye (white arrows) and anterior to the stomodeum (white arrowheads) at this stage. CNC cells have also populated the frontonasal region and surround the nasal placodes (asterisks). (B and D) In alx1 morphant embryos, the frontonasal and maxillary CNC are separated by an unpopulated region ventral to the eye (yellow arrows). The frontonasal region is sparsely populated and nasal placodes (asterisks) not fully surrounded by CNC cells. CNC cells sometimes migrate abnormally across the surface of the eye (yellow arrowhead, see also the black arrowhead in F). (E and F) In situ hybridization for alx3 expression in control and alx1 morphant embryos at 24hpf confirms these observations (lateral view, anterior to the left). (G–J) At 48hpf, sox10 expression reveals the development of the trabeculae (arrows) in control embryos (G and I), which have not extended in alx1 morphants (H and J). (K and L) Alcian blue stain at 3dpf shows the ethmoid plate and trabeculae (K) in control embryos, but these have failed to develop in alx1 morphant embryos (L). The parachordal cartilages are indicated by arrowheads. Embryos are lateral view, anterior to the left (G and H) or ventral view, anterior upwards (I–L). Ethmoid plate (ep), trabeculae (t), notochord (n).
expression of a dominant-negative form of Rho kinase to independently block neural crest development (69). This resulted in increased cell death and severe hypoplasia of the facial prominences and ultimately reduced maxilla, nasal bones and midline facial clefting reminiscent of human FND. The establishment of a zebrafish model, which has clear advantages in terms of powerful genetics and tracing of migrating cells, provides an exciting opportunity to further explore these mechanisms, which may be more widely applicable to severe forms of frontonasal malformation that are not caused by mutation in Alx1.

Alx1-related FND belongs to a subclass of FND associated with defects of ocular development, and the symptoms have been described as combining features of autosomal-recessive frontofactional dysplasia and Fryns microphthalmia syndrome (11). The zebrafish model described here exhibits microphthalmia along with coloboma and defects of the retina, lens and cornea. To date, the eye phenotype caused by human Alx1 mutation has not been described in detail, although coloboma is an ocular defect reported in some cases of FND (54). Correct ocular development is dependent in part on migratory CNC cells which contribute to correct orbital and anterior segment development (33,70), and several human ocular disorders have been linked to defects in genes which control migration/differentiation of the neural crest (39,41,71,72). For example, mutation of Pitx2 underlies certain cases of Axenfeld–Rieger syndrome, a class of malformations which primarily affect the eye, but are also associated with craniofacial abnormalities (73). As in the present study, comparable phenotypes are obtained by inhibiting pitx2 expression in zebrafish (39,41). Given the strong expression of alx1 in migrating CNC and POM, we propose that loss of alx1 in these cells directly underlies the observed eye defects.

Whilst the zebrafish model presents many advantages, mouse null mutants have previously been reported for all three of Alx1, Alx3 and Alx4 (18,20,22,74). In each case, the phenotypes described do not appear to directly recapitulate those reported in human cases, but it is interesting to re-evaluate this information in the light of the data available in human and zebrafish. In part, an evaluation of the mouse model is complicated by the significant neural tube defects of any kind, and this disparity may be explained by the differences in the biology of mouse and human neural tube closure. For example, it is thought that in mouse, the mechanics of neural tube closure may be more complicated than in human, with the mouse requiring an additional closure site in the midbrain region (75). The defects observed in the Alx1 mutant mouse were reported to stem from a failure to initiate closure in the midbrain (18). In contrast, the zebrafish neural tube forms by an alternative mechanism which does not involve the closure of neural folds. Instead, the neural tube initially forms as a solid rod, the neural keel, which later develops a lumen by cavitation (76,77). Thus, the relevance of these mouse phenotypes to human development is currently unclear, although we note that certain cases of FND are associated with neural tube defects (78–80).

Perhaps, the most interesting observation in mouse is that whilst single Alx mutants do not generate phenotypes comparable with FND, combining an Alx1 null mutation with Alx4 knock-out does result in hypoplasia of the facial prominences (19). It is possible that the increased cell death reported in the facial mesenchyme of these mice is a consequence of abnormal CNC migration, and it is interesting to note that something similar is also observed in Alx3/Alx4 double mutants (21). The implication is of functional redundancy in mouse, which differs from human or zebrafish. Such disparities between human and mouse are not uncommon, reflecting the remarkable complexity underlying craniofacial development. This is perhaps also manifested in the observation that the severity of the mouse phenotypes often depends on the genetic background (81). Indeed, subtle variations in the expression of genes and regulatory molecules involved in the development of the face is believed to be responsible for generating the remarkable morphological variation seen between different species (82). This further emphasizes the value of the zebrafish model and that combining information from multiple model organisms is essential.

Given the clinical findings described previously (11), and the developmental basis described here, Alx1-related FND bares many of the hallmarks of disorders previously classified as neurocristopathies (83) and we suggest that this should be considered in the future stratification of the FND spectrum. The zebrafish model should be valuable for further exploring the function of Alx1 and for elucidating the relationship between Alx genes and the genetic pathways underlying other human craniofacial disorders.

MATERIALS AND METHODS

Zebrafish maintenance

Zebrafish embryos were collected and staged according to established protocols (84,85). The sox10:GFP transgenic fish Tg(4.9sox10:eGFP)982 (31) and the tp53 null-mutant line (42) have been described previously.

Cloning and RT-PCR

The full-length-coding regions of zebrafish alx genes were cloned by RT-PCR using the following primers: alx1 forward 5′-TTGAGACGAGCCAGAGAC-3′ and reverse 5′-CCTGGCTCTGTGAAATTACAG-3′, alx3 forward 5′-ACGAGGATGAGACGAGGA-3′ and reverse 5′-GTGAAATCTGGGATCTGCTG-3′, alx4a forward 5′-ATGAACGAGGGCTGTC-3′, alx4b forward 5′-ATGAAACGCGAGACTGTGC-3′ and reverse 5′-TCATGTAGCCCTAAAGAGATGGGC-3′, alx4b forward 5′-ATGAAACGCGAGCTGCTG-3′ and reverse 5′-TCATGTAGCCCTAAAGAGATGGGC-3′. PCR fragments were cloned into the vector pGEMT-easy (Promega) and used for the transcription of riboprobes for in situ hybridization.

To assess transcript knock-down, wild-type or morphant RNA was isolated using Trizol (Invitrogen), treated with DNaseI (New England Biolabs) and purified using the RNAeasy mini kit (Qiagen). Equal concentrations of wild-type and morphant RNA were reverse transcribed using Superscript
Ill first strand (Invitrogen). Primers were designed to amplify a 491 bp fragment of alx1 (forward 5′-ACTCTCCCATGGACAGATCG-3′, reverse 5′-CGGCCCCGTCCATAGGTTCAT-3′), a 552 bp fragment of alx3 (forward 5′-CGACGCACCTCCAAAAGTCTC-3′, reverse 5′-CATCTTTCTCAGTTACAATTTATA-3′) and a 559 bp product of β-actin (forward 5′-TGTTTTCCCTCCATTGGTGTG-3′, reverse 5′-CTTCTCCC TTGATGTCACGGAC-3′).

**In situ hybridization, immunohistochemistry and alcian blue stain**

Whole mount in situ hybridization was carried out as previously described (86). Probes used were: foxd3 (48), snai1 (48), sox10 (45) and myoD (87). Fluorescent detection of the alx1 probe used the tyramide (Cy5) amplification kit (Perkin Elmer). Immunohistochemistry for enhanced GFP (EGFP) was detected in fixed sox10:GFP embryos according to standard protocols using anti-GFP (1/200; Roche) and anti-mouse Alexa488 (1/500; Molecular Probes).

Staining of cartilage with alcian blue was performed as described previously (88).

**Cryosectioning and histology**

For ocular histology, embryos were fixed in 4% paraformaldehyde and sectioned at a thickness of 20 μm on a Leica CM3050 S cryostat after embedding in 25% fish gelatine/15% sucrose. Haemotoxylin and eosin stain was performed on a Leica CM3050 S cryostat after embedding in 25% fish gelatine/15% sucrose. Immunohistochemistry for enhanced GFP (EGFP) was carried out as previously described (88).

**Morpholino microinjection**

MOs were obtained from Gene Tools, LLC (Philomath, OR, USA). MOs for alx1 were designed to target the intron1–exon2 splice acceptor site (alx1i1e2) 5′-CAGCCTGAGGGAGTACAGAAAT-3′ or the initiating AUG (alx1AUUG) 5′-GTCTCTGGCCTGCTCAGATCGACAT-3′, and for alx3 to target the exon1–intron1 splice donor site (alx3i1e1) 5′-GAACAGATTCTCACCACATCGGAA-3′ or the intron1–exon2 splice acceptor site (alx3i2e2) 5′-ATCTCTGCTGTA CAAAACACTCGA-3′. The standard cMO is 5′-CCTCTACCTCAGTTACAATTATA-3′. MOs were injected at the following concentrations as 1 nl into the yolk: alx1i1e2 (0.4 mm), alx1AUUG (0.4 mm or 0.8 mm), alx3i1e1 (0.6 mm), alx3i2e2 (0.4 mm).

**Imaging, migration distance and statistics**

Fixed Tg(-4.9sox10:eGFP)embryos were imaged using a Nicon A1R confocal microscope. Migration distance and the longitudinal diameter of the eye were measured using NIH Image J. Datasets were analyzed using a two-way repeated-measures analysis of variance test with the Bonferroni correction. In all cases, the significance was set at P < 0.05.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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