ABSTRACT

The cellular and genetic mechanisms that coordinate formation of facial sensory structures with surrounding skeletal and soft tissue elements remain poorly understood. Alx1, a homeobox transcription factor, is a key regulator of midfacial morphogenesis. ALX1 mutations in humans are linked to severe congenital anomalies of the facial skeleton (frontonasal dysplasia, FND) with malformation or absence of eyes and orbital contents (micro- and anophthalmia). Zebras with loss-of-function alx1 mutations develop with craniofacial and ocular defects of variable penetrance, likely due to compensatory upregulation in expression of a paralogous gene, alx3. Here we show that zebrafish alx1;alx3 mutants develop with highly penetrant cranial and ocular defects that resemble human ALX1-linked FND. alx1 and alx3 are expressed in anterior cranial neural crest (aCNC), which gives rise to the anterior neurocranium (ANC), anterior segment structures of the eye and vascular pericytes. Consistent with a functional requirement for alx genes in aCNC, alx1; alx3 mutants develop with nearly absent ANC and grossly aberrant hyaloid vasculature and ocular anterior segment, but normal retina. In vivo lineage labeling identified a requirement for alx1 and alx3 during aCNC migration, and transcriptomic analysis suggested oxidative stress response as a key target mechanism of this function. Oxidative stress is a hallmark of fetal alcohol toxicity, and we found increased penetrance of facial and ocular malformations in alx1 mutants exposed to ethanol, consistent with a protective role for alx1 against ethanol toxicity. Collectively, these data demonstrate a conserved role for zebrafish alx genes in controlling ocular and facial development, and a novel role in protecting these key midfacial structures from ethanol toxicity during embryogenesis. These data also reveal novel roles for alx genes in ocular anterior segment formation and vascular development and suggest that retinal deficits in alx mutants may be secondary to aberrant ocular vascularization and anterior segment defects. This study establishes robust zebrafish models for interrogating conserved genetic mechanisms that coordinate facial and ocular development, and for exploring gene–environment interactions relevant to fetal alcohol syndrome.

KEY WORDS: Neural crest, ALX, Frontonasal dysplasia, Ocular anterior segment, Fetal alcohol syndrome

INTRODUCTION

The vertebrate face comprises ocular, vascular and skeletal cell lineages that are closely apposed during development. The anterior cranial neural crest (aCNC) originates from the anterior–dorsal neural tube (future forebrain and midbrain), migrates cephalad to the eyes and turns caudally toward the nascent mouth opening to give rise to facial chondrocytes (Kague et al., 2012; Kish et al., 2011; Kuratani, 2018; Serbedzija et al., 1992; Wada et al., 2005), ocular anterior segments, e.g. the cornea and iris (Cavodeassi et al., 2019; Cvekl and Tamm, 2004; Ma and Lwigale, 2019; Williams and Bohnsack, 2015), and vascular pericytes of ocular blood vessels (Gage et al., 2005; Trost et al., 2013). Despite their importance, the gene networks that regulate these aCNC-derived lineages are poorly understood.

The Aristless-less (alx) gene family consists of ALX1, ALX3 and ALX4 in human, mouse and zebrafish; ALX4 is represented by duplicated alx4a and alx4b in zebrafish (Dee et al., 2013). In humans, ALX1 mutations are linked to severe frontonasal dysplasia (FND) and extreme microphthalmia, and ALX3 and ALX4 genes are associated with a phenotypic spectrum of hypertelorism and nasal-tip duplications (Pini et al., 2020; Uz et al., 2010). These functions of alx genes are conserved in other vertebrates, but the pathogenic developmental mechanisms leading to the observed craniofacial malformations remain largely unknown (Beverdam et al., 2001; Dee et al., 2013; Forsthoefer, 1963; Lakhwani et al., 2010; Lyons et al., 2016; Pini et al., 2020; Qu et al., 1999; Zhao et al., 1996). In mice, disruption of alx1 results in anencephaly, precluding analysis of its role in facial and ocular morphogenesis, while compound mutants of alx3 and alx4 present with FND-like defects (Beverdam et al., 2001; Lakhwani et al., 2010).

Mouse alx family members, alx1, alx3 and alx4, are co-expressed in aCNC that gives rise to the anterior neurocranium (ANC), the primary palate (Dee et al., 2013; McGonnell et al., 2011; Mitchell et al., 2021; Qu et al., 1999; ten Berge et al., 1998), anterior segment and periorcular neural crest (Ma and Lwigale, 2019; Sedykh et al., 2017). Recent reports demonstrate that zebrafish alx1 is required for regulating neural crest differentiation and migration in vitro and in vivo (Pini et al., 2020) and that zebrafish alx3 controls ANC chondrocyte differentiation in zebrafish (Mitchell et al., 2021).
However, these studies do not address the role for alx function in the developing eye, nor do they examine the potential for compensatory interactions between alx1 and alx3.

While hereditary FND is rare, similar facial and ocular malformations are commonly associated with fetal alcohol exposure in humans (Farlie et al., 2016; Lovely et al., 2017). Genetic susceptibility to ethanol toxicity is actively studied in model organisms (Boschen et al., 2021; de la Morena-Barrio et al., 2018; Eberhart and Pamela, 2016; Kaminen-Ahola, 2020; Lovely et al., 2017), but much remains to be learned about the mechanisms of gene–environment interactions, particularly at the level of transcription.

Here we report that ALX1 homozygous loss-of-function mutation is associated with severe ocular malformation in humans and show that alx1 mutant zebrafish also develop with ocular impairments. Targeted mutagenesis of a paralogous gene, alx3, was carried out to generate alx1alx3 double-mutant zebrafish and to demonstrate that variable penetrance of alx1 mutant defects in zebrafish is due to functional redundancy with alx3. Our data demonstrate genetic requirements for alx1 and alx3 in craniofacial cartilage development, at least in part through regulating migratory aCNC that gives rise to the median element of the ANC. Importantly, we report evidence of unexpected roles for alx1 and alx3 in ocular vasculature and identify a role for alx1 and alx3 in regulating oxidative stress response and ribosome biogenesis. Consistent with these findings, zebrafish alx1 protects ocular and facial embryonic lineages against the effects of ethanol toxicity, which acts through ethanol-induced oxidative stress in neural crest cells. Collectively, these data establish the alx1 and alx1alx3 zebrafish models as unique new tools for dissecting both the genetic controls and gene–environment interactions during craniofacial and ocular development.

RESULTS

Human ALX1 and zebrafish alx1 are required for ocular development

We recently reported a pedigree with consanguineous parents and 13 children, four of whom were born with FND linked to a missense gene variant, I166F, in the homeodomain of alx1 (Pini et al., 2020) (Fig. 1A). All four ALX1-homozygous patients presented with oblique orofacial clefts together with severe bilateral ocular deficits (Fig. 1B,C). These findings are consistent with a previous study that described patients with deletions that encompassed ALX1 and other loci, and a patient with splice-site mutation at the ALX1 locus (Uz et al., 2010). While the mechanism of ALX1 function in the facial cartilage is coming to light through active investigation, the role for ALX1 during ocular development remains unknown. We sought to address this knowledge gap by establishing zebrafish models of ALX1-linked FND. Using CRISPR/Cas9-mediated targeted mutagenesis, we generated loss-of-function alleles in zebrafish alx1 and showed that the majority of alx1 homozygotes were viable as adults, with a small proportion exhibiting craniofacial malformations that manifest during larval stages (Pini et al., 2020).

On gross anatomical examination, we observed a range of ocular impairments in alx1^{iow} adults. Histological analysis of four representative affected adults revealed aberrant pupils, missing lenses (aphakia) and iridial dysplasia (Fig. 1D-K), but largely normal retinae. These ocular anomalies occurred in at least one eye in a significant proportion of alx1^{iow} adults (21 of 99 total, P<0.0001, Fig. 1L). These findings suggest a post-embryonic requirement for zebrafish alx1 function, which may be restricted to the anterior segment lineages of the eye.

Zebrafish alx1 and alx3 function redundantly during craniofacial lineage formation

alx1 and its homologs, alx3 and alx4, are co-expressed in aCNC, which gives rise to the median element of the ANC (Dee et al., 2013; McGonnell et al., 2011; Mitchell et al., 2021; Qu et al., 1999; ten Berge et al., 1998), as well as to the ocular anterior segment and periorcular neural crest (Ma and Lwigale, 2019; Sedykh et al., 2017). All four single-locus mutants have been analyzed but only alx1 mutants (Pini et al., 2020) and alx3 mutants (Mitchell et al., 2021) exhibited embryonic defects. These defects were far milder than those reported in antisense morpholino-mediated knockdown assays (D ee et al., 2013) and did not recapitulate the severe craniofacial and ocular phenotypes in ALX1-linked (Pini et al., 2020; Uz et al., 2010) or ALX3-linked (T wigg et al., 2009) FND patients. These findings point to redundant or compensatory functions of alx genes in the embryonic zebrafish.

A recent study used single cell RNA-seq to show that all four zebrafish alx genes are expressed in frontonasal neural crest at 24 hours post fertilization (hpf) (Mitchell et al., 2021). We have also shown that alx3 transcription is increased in alx1 mutants (Pini et al., 2020), likely through the process of transcriptional adaptation (El-Brolisy et al., 2019). We hypothesized that the low penetrance of craniofacial malformations in alx1 mutants is due to functional compensation by alx3 and used CRISPR/Cas9 mutagenesis to mutagenize alx3 in the alx1^{iow} background (see Materials and Methods). Three frame-shift mutant alleles were recovered; since homozygotes for each allele developed with similar phenotypes, we chose one of the alleles, a 13-nucleotide insertion allele named alx3^{2113}, for in-depth analysis. alx3^{2113} is predicted to encode a non-functional protein due to premature translation termination that results from a frame-shift mutation upstream of the conserved homeodomain and transactivation domains of alx3 (Fig. S1).

alx1^{iow};alx3^{2113} and alx1^{iow};alx3^{2113} (abbreviated alx1;alx3/+) and alx1^{iow};alx3^{2113} zebrafish were viable as adults and fertile, with alx1;alx3 adults exhibiting a range of facial anomalies (data not shown). alx1;alx3 larvae developed with a distinctive facial phenotype consistent with hypoplastic ANC at 5 days post-fertilization (dpf) (Fig. 2A,B). Alcian Blue staining, which labels neural crest-derived chondrocytes, confirmed ANC hypoplasia with various degrees of severity in the mutant larvae (Fig. 2C-F).

Notably, alx1;alx3/+ embryos also developed with anomalous ANC primordia, albeit milder and at a lower penetrance (Fig. 2G).

We next sought to define the earliest time that craniofacial cartilage defects manifest in alx1;alx3 embryos. To do this, we examined expression of col2a1a, an early chondrocyte marker (Yan et al., 1995) in wildtype, alx1^{iow} (abbreviated alx1), and alx1;alx3 embryos at 2 dpf. The majority of alx1 embryos (88%) exhibited normal expression of col2a1a (Fig. 2H,M), consistent with the low penetrance of deficits by 5 dpf. In contrast, only 24% of embryos derived from an alx1;alx3/+ incross developed with normal ANC, while 76% had truncated or missing ANC at 5 dpf (Fig. 2I,L,M). This phenotypic ratio indicates that the ANC primordium is aberrant in both alx1;alx3 and alx1;alx3/+ embryos by 2 dpf. col2a1a expression was also detected in the presumptive sclera, the cartilaginous outer shell of the eye globe contiguous with the cornea, which is derived from neural crest (Gestri et al., 2012). col2a1a expression in putative scleral precursors was reduced in alx1 and alx1;alx3 embryos (Fig. 2K,L), suggesting an early requirement for alx1 and alx3 function in the scleral lineage.
Zebrafish *alx1* and *alx3* function redundantly during ocular morphogenesis

We next asked if *alx3* function compensated for the loss of *alx1* during ocular morphogenesis. Embryos obtained from a cross between *alx1;alx3/*+ parents exhibited aberrantly shaped (elongated) eye capsules covered by abnormally patchy iridophores at 5 dpf (Fig. 3A-C). Genotypic analysis confirmed this phenotype, which we termed severe, to be strongly linked to the *alx1;alx3* genotype. We also observed a milder manifestation of this ocular phenotype in *alx1;alx3* larvae (Fig. 3D).

Unexpectedly, we also observed sporadic intraocular and periocular hemorrhage in living *alx1* and *alx1;alx3* embryos at 2-3 dpf. We employed o-dianisidine, a histological stain for erythrocytes, to visualize the aberrantly pooled erythrocytes and to delineate the vascular network. At this stage in development, ocular blood supply is carried by a branched hyaloid network that starts forming around the lens at 32 hpf (Hartsock et al., 2014). In wildtype embryos, erythrocytes were distributed in a pattern that resembled normal hyaloid vasculature (Fig. 3E) (Detrich et al., 1995). This was also true for most *alx1* mutants, with a minority exhibiting patches of erythrocytes suggestive of hyaloid hemorrhaging (Fig. 3F-H). In *alx1;alx3* homozygotes, o-dianisidine staining revealed extensive and penetrant ocular and periocular hemorrhage (Fig. 3I-K). Ocular hemorrhage was present in ~50%
of \textit{alx1;alx3/+} incross progeny and was strongly associated with ocular dysmorphology (Fig. 3L). Notably, this defect emerges contemporaneously with the loss of \textit{col2a1a} expression in ANC precursors and in the sclera (see Fig. 2L, asterisk).

To examine hyaloid vasculature directly, we took advantage of endogenous alkaline phosphatase activity in the vascular endothelium surrounding the lens (Alvarez et al., 2007). Larvae derived from an \textit{alx1;alx3/+} incross were sorted by ocular morphology at 5 dpf and stained for alkaline phosphatase. Examination of dissected lenses revealed a range of vascular defects, from mild disorganization to complete absence (Fig. S2). Surprisingly, severity of vascular defects did not correlate with presence or severity of ocular dysmorphology. Together, these data indicate a novel function for the \textit{alx} family in the developing hyaloid vasculature.

**Fig. 2. \textit{alx1} and \textit{alx3} function redundantly to control anterior neurocranium morphogenesis in zebrafish.** Larvae derived from a cross between \textit{alx1;alx3/+} parents were fixed at 5 dpf. (A,B) Representative siblings with normal facial structures (A) and with aberrantly short neurocranium (asterisk) and abnormally protruding jaw cartilage (arrowhead; B). (C-F) Alcian Blue staining revealed EP anomalies that range from mild (reduced median ethmoid plate width in D), moderate (absent median and reduced lateral ethmoid plate in E), to severe (absent ethmoid plate in F). (G) Penetration and severity of cartilage defects is increased in \textit{alx1;alx3} compared to \textit{alx1;alx3+} embryos (two trials). (H-M) Embryos derived from wildtype, \textit{alx1}, or \textit{alx1;alx3/+} parental crosses were fixed at 2 dpf and stained for \textit{col2a1a} expression using whole-mount in situ hybridization. (H,H’) Normal \textit{col2a1a} expression in a wildtype embryo. (I,I’) A truncated EP in an \textit{alx1;alx3} embryo (arrow). (J) Normal \textit{col2a1a} expression in a wildtype embryo. (K) Normal ethmoid plate and reduced presumptive scleral staining (asterisk) in an \textit{alx1} mutant. (L) Hypoplastic ethmoid plate (arrow) and absent presumptive scleral staining (asterisk) in an \textit{alx1;alx3} mutant. ANC, anterior neurocranium; OV, optic vesicle; PA, pharyngeal arches; FB, fin bud; SC, sclera. M: 75% of the embryos derived from \textit{alx1;alx3/+} have aberrant ANC and reduced scleral precursors (**P=0.0097, ****P<0.0001, Fisher’s exact test; two trials for wildtype and \textit{alx1;alx3/+}, and one trial for \textit{alx1}). Embryos in A,B, and H-I’ are shown in lateral views, anterior to the left. Embryos in J-L are shown in ventral views, anterior to the left.

\textit{alx1} and \textit{alx3} are largely dispensable for early retinal development

Having documented ocular defects in supporting ocular tissues, we asked if retinal development depended on \textit{alx1} and \textit{alx3} function. \textit{alx1;alx3/+} incross progeny were fixed at 2 dpf and immunostained with an anti-zn5/alcama antibody, which labeled retinal ganglion cell (RGC) neurons, and counter-stained with the nuclear stain DAPI to reveal retinal layers. Confocal imaging revealed a normal pattern of RGCs and correct overall layering in the retinae of \textit{alx1}, \textit{alx1;alx3/+}, and \textit{alx1;alx3} larvae (Fig. 4A-D).

Next, we tested visually triggered behaviors to assess retinal function in \textit{alx1;alx3} larvae at 5 dpf. When presented with an abrupt absence of light (a dark flash), larval zebrafish execute a turning behavior termed the ‘O-bend’, in which they turn their bodies ~180 degrees (Burgess and Granato, 2007) (Fig. 4I). We exposed 5 dpf larvae to 10 dark flashes and evaluated the probability of O-bend initiation in response to the abrupt absence...
examined for the presence of the 13 base-pair insertion in the alx3 gene. We harvested from individual siblings without pooling at 5 dpf, (Fig. 4J), while visually impaired mutants showed a marked reduction in O-bend initiation compared to wildtypes (Fig. 5A, Table S1), and 34 genes differentially expressed in siblings (***P<0.0001, Fisher’s exact test, two trials). (D) The severe ocular defect shown in B is strongly associated with the alx1;alx3 genotype, while the majority of alx1;alx3+ embryos present with a milder misshapen eye phenotype (not shown) (***P<0.0002 using Fisher’s exact test, two trials). (E-L) Embryos derived from wildtype or alx1;alx3+ parents were stained with o-dianisidine at 2-3 dpf to label hemoglobin in red blood cells. Normal hemoglobin distribution in the eye of a wildtype embryo (E) versus ocular hemorrhage (arrowhead in F) and periocular hemorrhage (asterisk in G) in alx1 mutants. (H) Hemorrhaging is restricted to a subset of alx1 mutants (****P<0.0001, Fisher’s exact test, five trials). Normal hemoglobin distribution in the pharyngeal region of a wildtype embryo (I) versus evidence of hemorrhaging in an alx1;alx3 embryo (J). Intraocular and periocular hemorrhages (asterisk) near the optic artery and optic vein in an alx1;alx3 embryo (K). (L) Hemorrhaging is strongly associated with eye defects in alx1;alx3+ and alx1;alx3 embryos (****P<0.0001, Chi-square test, two trials). Embryos are shown in lateral views, anterior to the left except in I and J, which are ventral views, anterior to the top. OA, optic artery; OV, optic vein; PLV, palatocerebral vein.

We next set out to identify transcriptional targets that depend on alx1 and alx3 functions in the post-embryonic zebrafish larvae. Embryos were derived from an alx1;alx3/+ cross and RNA was harvested from individual siblings without pooling at 5 dpf, followed by RNA-seq. For each sample, sequence tracks were examined for the presence of the 13 base-pair insertion in the alx3allele to deduce the embryonic genotype. 12 samples (four each of alx1, alx1;alx3/+ and alx1;alx3) were selected for pairwise comparisons as detailed in Materials and Methods. This approach identified a compact set of 275 differentially expressed genes (DEGs) between alx1;alx3 and their alx1 siblings (Fig. 5A, Table S1), and 34 genes differentially expressed between alx1;alx3/+ larvae and alx1;alx3 siblings (Fig. S3); six of the differentially expressed genes were shared between the two sets. Among DEGs with lineage-restricted expression patterns were several retinal markers that were significantly increased in alx1;alx3 versus alx1 homozygotes at 5 dpf. These differentially expressed genes included cels3, expressed in the amacrine and ganglion cells of the zebrafish retina (Lewis et al., 2011), and rdh5, expressed in retinal pigment epithelium (RPE) (Nadauld et al., 2006). Rdh5 is a retina-specific retinol dehydrogenase required for ventral retinal morphogenesis; its upregulation in alx1;alx3 mutants is consistent with expansion of the RPE. igfpb7, which is highly expressed in vascular endothelium (Abu-Safieh et al., 2011), was also upregulated in alx1;alx3 and in alx1;alx3/+ siblings (Fig. 5B, Fig. S3).

The downregulated DEG list was enriched for pancreatic and liver markers, namely, serping1, hmxox1a, ela2l, cpa3, ctrl, ela5l, ins and amy2a (Cheng et al., 2006; Holowiecki et al., 2017) suggesting an unexpected role for alx1 and alx3 in these primordia. Two of these genes, serping1 and ela2l, were also downregulated in alx1;alx3/+ versus alx1 siblings (Fig. 5B, Fig. S3).

We next used gene set enrichment analysis (GSEA) (Subramanian et al., 2005) to deeply analyze molecular pathways that require alx1 and alx3 function. This analysis identified several molecular pathways that were suppressed or activated in alx1;alx3 larvae. Ribosome biogenesis was identified as significantly suppressed (Fig. 5C, Fig. S4), while brain and head development, neuronal development and eye morphogenesis were notable among those activated in alx1;alx3 larvae compared to alx1 siblings (Fig. 5D, Fig. S5, Table S2).
Zebrafish alx1 and alx3 function during migration of presumptive anterior neurocranium progenitors

To gain additional insight into the requirement for alx1 and alx3 in the aCNC lineage, we performed in vivo lineage-tracing analysis. To do this efficiently, we adapted CRISPR-driven G0 knockout in zebrafish (Wu et al., 2018) to generate somatic mutations in the alx3 locus in the alx1 homozygous mutant background (alx1;alx3CR, also referred to as ‘crispants’). The alx1;alx3CR larvae exhibited midline clefts, abnormal trabeculae and abnormal anterior neurocranium (ANC) formation (Fig. S6) that resembled the severe defects observed in alx1;alx3 embryos (see Fig. 2A-F). In contrast, alx1 mutants and alx3CR in the wildtype background developed with normal craniofacial cartilages (Fig. S6). Groups of cells in the anterior/frontonasal (aCNC) or maxillary stream (MxCNC), fated to give rise to the ANC (Fig. 6A) (Knight and Schilling, 2006), were labeled by kaede photoconversion in Tg(sox10:kaede) (Fig. 6B,D,F,H) or alx1;alx3CR; Tg(sox10:kaede) embryos (Fig. 6J,L,N,P) at the 20 somite stage and imaged immediately. At 4 dpf, the larvae were imaged again to determine labeled aCNC locations post-migration. Wildtype larvae developed with normal cartilages (labeled in green) and showed normal post-migratory localization of the photo-converted red-kaede-labeled cells (magenta channel) in the median and lateral element of the ANC (Fig. S6). These observations suggest that...
*alx1* and *alx3* affect the migration of the frontonasal and maxillary streams of cranial neural crest cells. There also appeared to be fewer crispant cells reaching their destination, consistent with a potential proliferative deficit. Notably, while expression of *alx1* and *alx3* is primarily NCC-restricted (Dee et al., 2013; Sedykh et al., 2017), these findings do not rule out a non-cell-autonomous function of these transcription factors in tissues adjacent to the migrating neural crest.

**Transcriptomic analysis identifies candidate targets of Alx1 and Alx3 in the developing embryo**

Having demonstrated essential roles for zebrafish *alx1* and *alx3* in the forming frontonasal cartilage, anterior ocular segment and ocular vasculature, we sought to address the underlying molecular mechanisms through transcriptomic analysis. We surveyed transcriptome changes that result from loss of *alx1* and *alx3* at 3 dpf, when ocular and ANC defects in mutant embryos become evident. *alx1;alx3CR* embryos were generated as detailed above, and age-matched wildtype controls were subjected to RNA isolation and high-throughput sequencing. Bioinformatic analysis identified a large set of differentially expressed genes (4426 genes with adjusted $P$-value <0.05), which included 207 strongly upregulated (Log2 fold change >1) and 93 downregulated (Log2 fold change $<$ −1) genes (Fig. 7A, Table S3).

GSEA analysis of this data set showed suppression of lens development, the only pathway relevant to ocular development, in crispants (Fig. 7B, Fig. S7), while a group of pathways that include monoxygenase activity and xenobiotic metabolism was activated.
in crisps (Fig. 7C, Fig. S8, Table S4). Notably, several of the leading-edge gene sets contain *heme oxygenase 1* (*hmox1a*), also known for its critical functions in the cellular response to oxidative stress in several tissues, including the vascular endothelium (Fuse et al., 2015; Holowiecki et al., 2017). On further examination of the DEG set, a number of key oxidative-stress-response genes (Nakajima et al., 2011), summarized in the heatmap in Fig. 7D, were found to be upregulated in crisps. These data indicate novel requirements for *alx1* and *alx3* function in the developing lens, and in regulating the oxidative stress response program.

**Zebrafish alx1 protects frontonasal and ocular lineages against ethanol toxicity**

The aCNC is particularly sensitive to environmental influences and ethanol, a common environmental toxin and teratogen, is known to impact neural-crest-derived lineages through increasing oxidative stress and disrupting ribosome biogenesis (Kiecker, 2016; Lovely, 2020; Smith et al., 2014). We hypothesized that *alx1* function protects aCNC lineages against ethanol toxicity and took advantage of the low penetrance of defects in zebrafish *alx1* mutants to test this hypothesis. Embryos derived from *alx1*/*alx1*+ crosses were exposed to 0.5% ethanol from 6 hpf until 48 hpf and stained with Alcian Blue at 5 dpf to visualize craniofacial cartilage (Fig. 8). The exposure dose and duration were selected based on a previous study that demonstrated their minimal effects on embryogenesis in wildtype embryos (McCarthy et al., 2013). As expected, a small proportion of untreated *alx1* larva developed with hypoplastic ANC (Fig. 8A-D). Notably, the penetrance of ANC defects increased significantly in ethanol-exposed *alx1* larva (Fig. 2D, *P*=0.024), while wildtype ANC formation was not significantly affected by ethanol exposure (Fig. 2D, *P*=0.24).

*alx1*–*alx1*+ mutants, supports a function for *alx1* in the zebrafish ANC and in several ocular lineages, but this difference did not rise to significance (*P*=0.10, Fig. 8J). Together, these data support a novel role for *alx1* in conferring robustness to ethanol toxicity on both craniofacial and ocular lineages. Collectively, our findings support crucial functions for *alx1* and *alx3* in the zebrafish ANC and in several ocular lineages and identify a robust set of candidate molecular mechanisms that underlie these important functions.

**DISCUSSION**

Here, we show a redundant genetic requirement for zebrafish *alx1* and *alx3* in several midface structures derived from the aCNC: the ANC, the ocular anterior segment, and ocular blood vessels. We identify a protective role for zebrafish *alx1* in ocular and facial embryonic lineages against ethanol toxicity, which, together with the evidence from transcriptome analysis of *alx1-/+;alx3* mutants, supports a function for *alx1* and *alx3* in regulating oxidative stress response and ribosome biogenesis. These findings extend and complement our previous human iPSC studies (Pini et al., 2020) and validate the first animal model of ALX-linked FND with ocular involvement, crucial for understanding conserved roles for *ALX* genes during vertebrate development.
Fig. 7. RNAseq transcriptome analysis identifies a role for *alx1* and *alx3* in oxidative stress response regulation. *alx1<sup>1<sup>we2016</sup>;Tg(sox10:kaede)* embryos were injected with *alx3* CRISPR RNPs to induce somatic mutations at the *alx3* locus to generate *alx1*<sup>1<sup>we2016</sup>;Tg(sox10:kaede);*alx3<sup>CR</sup>* embryos. At 3 dpf, *alx1*<sup>1<sup>we2016</sup>;Tg(sox10:kaede);*alx3<sup>CR</sup>* embryos with characteristic craniofacial defects and age-matched *Tg(sox10:kaede)* embryos were lysed. A total of six replicate groups of 20 embryos from each genotype were collected in two independent experiments for RNA extraction and Illumina high-throughput sequencing. (A) Volcano plot shows the distribution of genes that are differentially expressed in *alx1*<sup>1<sup>we2016</sup>;Tg(sox10:kaede);*alx3<sup>CR</sup>;sox10:GFP* embryos relative to *sox10:GFP* embryos at 3 dpf. Vertical lines indicate −1.5-fold and +1.5-fold change cut-offs, and the horizontal line indicates the FDR=0.05 cutoff. (B,C) Molecular pathways that are significantly suppressed (B) or activated (C) in *alx1*<sup>1<sup>we2016</sup>;Tg(sox10:kaede);*alx3<sup>CR</sup>;sox10:GFP* embryos were identified using GSEA, with top 20 activated and top 20 suppressed terms shown as a 2D map to emphasize overlaps. (D) Heatmap summary of differentially expressed oxidative stress response genes shows increased expression levels of these genes in *alx1*<sup>1<sup>we2016</sup>;Tg(sox10:kaede);*alx3<sup>CR</sup>;sox10:GFP* embryos.
Complex roles of alx genes during development

Loss of alx1 function has distinct consequences in different vertebrate species. Humans homozygous for ALX1 mutations develop with fully penetrant, severe FND and extreme microphthalmia (Uz et al., 2010; Pini et al., 2020), as do alx1-mutant cats (Lyons et al., 2016). In contrast, mouse alx1 mutants exhibit an earlier and more profound deficit, failure of cranial neural tube closure (Zhao et al., 1996), while zebrafish alx1 mutants develop with variably penetrant, mild craniofacial phenotypes (Pini et al., 2020). Thus, loss of ALX1 function always affects midface
development in these vertebrates, but with varying severity and penetrance. These differences may reflect evolutionary divergence in functional specializations of individual ALX genes, common after gene duplication. Comparative genomic studies reveal substantial variability among chordates in the number of alx paralogs, ranging from one in hemichordates to four in zebrafish, likely a result of gene duplication events (Khor and Ettensohn, 2020; Lamichhaney et al., 2015; McGonnell et al., 2011). This variability is thought to have driven emergence of skeletogenesis strategies during chordate evolution (Khor and Ettensohn, 2020). Allelic variation at the alx1 locus is associated with diversity in beak morphologies in Darwin’s finch species, thus contributing to rapid evolution (Lamichhaney et al., 2015).

Species-specific differences in alx1 gene functions may also reflect divergence in genetic compensation strategies employed by these organisms. In mouse, alx4 compensates for loss of alx3 since alx3;alx4 double mutants develop with severe orooral clefts and single mutants do not (Beverdam et al., 2001; Lakhwani et al., 2010). In zebrafish, single alx4a and alx4b mutants develop normally, while alx3 mutants exhibit medial ANC defects (Mitchell et al., 2021). Notably, while Mitchell et al. did not observe craniofacial phenotypes in alx1 mutants (Mitchell et al., 2021), we showed that alx1 mutants develop with partially penetrant ANC defects (Pini et al., 2020). This discrepancy may be due to genetic background variation, which implies the existence of genetic modifiers of alx1 function that will be important to examine in future studies.

Here we show that genetic removal of both alx1 and alx3 functions significantly increases the penetrance and severity of craniofacial defects compared to alx1 (Pini et al., 2020) and to alx3 single mutants (Mitchell et al., 2021). It is important to note that we did not examine the phenotype of alx3 single mutants in our genetic background, leaving open the possibility that alx3 interacts strongly with genetic modifiers and that the deficits we report here are due entirely to alx3 loss-of-function. However, the absence of defects in alx3 crispsants (Fig. S6) argues strongly against this possibility. It is more likely that the relative severity of alx1;alx3 mutant phenotypes reflect functional compensation between alx1 and alx3, potentially through the mechanism of transcriptional adaptation triggered by nonsense-mediated RNA decay (El-Brolosy et al., 2019; Jakutis and Stainier, 2015; Kontarakis and Stainier, 2020; Sztal and Stainier, 2020). This mechanism may also explain why stearins, 2021; Kontarakis and Stainier, 2020; Sztal and Stainier, 2020). Functional compensation by the remaining zebrafish paralogs, alx4a and alx4b, is a plausible explanation for this discrepancy that can be tested by generating triple and quadruple alx mutants.

It is perhaps not surprising that defects in ocular vasculature have not been observed in ALX1-linked FND patients, since hyaloid vasculature is transient in mammals and regresses prior to birth (Selvam et al., 2018). Our demonstration of a novel role for alx function in hyaloid vessel formation suggests the possibility of a transient defect during early ocular vascular development of FND patients, which could in turn impact retinal development by disrupting endothelial-to-retina signaling recently shown to be required for retinal neurogenesis in zebrafish (Dhakal et al., 2021, 2015). Impaired ocular blood flow may also affect retinal development in mammals which, unlike zebrafish, rely on ocular blood flow for early retinal gas exchange (Dhakal et al., 2015; Pelster and Burggren, 1996).

Neural crest that populates the anterior segment is highly heterogeneous; its influence on retinal development during embryogenesis is poorly understood and likely to vary across vertebrates (Takamiya et al., 2020; Van Der Meulen et al., 2020; Williams and Bohnsack, 2015, 2020). It is also likely that the ocular anterior segment offers differing degrees of protection to the developing retina in humans versus lower vertebrates. However, congenital anterior segment dysgenesis (ASD) (Evans and Gage, 2005; Hendee et al., 2018; Portal et al., 2019; Reis and Seminna, 2011) is associated with postnatal death of retinal ganglion cells, but not with prenatal retinal defects (Mao et al., 2011, 2017; Martino et al., 2016).

alx1 is also expressed in mouse and chick sclera, a largely overlooked ocular lineage that derives from neural crest and forms adjacent to the retinal pigmentation epithelium (Thompson et al., 2010). The sclera produces cartilage in birds, while mouse embryos initiate the program but stop short of cartilage production in the sclera (Thompson et al., 2010). Notably, microcalcification of the sclera and cystic lesions in the anterior chamber have been reported in a patient with splice-site mutation in ALX1 (Uz et al., 2010), lending support for the hypothesis that a role for alx genes in these ocular lineages has been conserved and can be effectively modeled in animals.

**Fetal alcohol syndrome and ALX function**

Fetal alcohol spectrum disorder (FASD) is a common condition estimated to affect 5% of live births in the US. It presents with disabling neurological defects and is frequently associated with facial and ocular deficits that contribute to the heavy health burden of FASD (Brennan and Giles, 2014; Gupta et al., 2016; Lovely et al., 2017; Lovely et al., 2017; Lovely, 2020). Emerging evidence indicates that fetal genotype strongly influences the outcome of prenatal ethanol exposure, since many children exposed to high levels of ethanol in utero do not develop FASD (Lovely, 2020). Naturally occurring alleles that confer robustness or sensitivity to alcohol toxicity can be identified epidemiologically, but model organism studies are essential for sustained progress toward a complete understanding of gene-environment interactions that contribute to FASD (de la Morena-Barrio et al., 2018; Eberhart and Parnell, 2016; Kaminen-Ahola, 2020; Lovely et al., 2017).
Model organism studies have shown that neural crest-derived lineages are particularly vulnerable to ethanol toxicity (Smith et al., 2014). Neural crest cells give rise to most craniofacial structures, including the face and the anterior segment of the eyes, and indirectly influence formation of the retina and early brain development. In other words, neural crest vulnerability to ethanol toxicity may account for the majority of defects observed in FASD. Studies in animal models have offered several mechanistic hypotheses for fetal ethanol toxicity: an increase in reactive oxygen species (ROS), resulting in increased oxidative stress (Brocardo et al., 2011; Dong et al., 2010; Lovely et al., 2017; Wang et al., 2015; Wentzel and Eriksson, 2011), disruption of ribosome biogenesis (Garic et al., 2014) interference with retinoic acid signaling (Williams and Bohnsack, 2019) and apoptosis (McCarthy et al., 2013). Here we show increased vulnerability of facial and ocular lineages to ethanol toxicity in the absence of functional alx1 in zebrafish, and a requirement for alx1 and alx3 function in regulating two of the pathways implicated in FASD: ribosome biogenesis (Fig. 5) and oxidative stress response (Fig. 7). Moreover, mouse Alx3 has been shown to regulate oxidative stress response through activating transcription of Foxo1, a key regulator of the antioxidant pathway (Garic et al., 2014). Notably, the penetrance of ocular defects is lower than the penetrance of craniofacial defects in ethanol treated alx1 mutant embryos, consistent with reports that anterior segment lineages are less susceptible to oxidative stress than craniofacial neural crest (Eason et al., 2017). Our study is the first to demonstrate a protective function for alx1 in the zebrafish model. Real-time in vivo imaging with lineage-specific transgenic reporters, together with pharmacological manipulation of the oxidative stress response pathway, will allow us to rigorously test these functions in future studies. Ultimately, animal models studies will be essential for elucidating the complex, poorly understood processes that contribute to frontonasal development (Farlie et al., 2016).

MATERIALS AND METHODS

Zebrafish strains and embryo manipulation

Adult zebrafish were maintained according to established methods (Westerfield, 1993). All experimental protocols using zebrafish, including adult animal care, were approved by the University of Wisconsin Animal Care and Use Committee and carried out in accordance with the institutional animal care protocols. Embryos were obtained from natural mating and staged according to Kimmel (Kimmel et al., 1995). The following mutant strains of zebrafish were used: alx1moon16 (Pini et al., 2020) and alx1mo16:alx3mo2113+/- generated in the course of this study (see below) and pappaap170 (Wolman et al., 2015). Embryos were exposed to 0.5% ethanol diluted in E3 starting at 6 hpf. At 2 dpf, embryos were transferred to fresh E3 and raised until 5 dpf to score for craniofacial defects, or until 2 months to score for ocular defects.

CRISPR/Cas9 mutagenesis and mutant allele identification

alx1mo16:alx3mo2113+/- zebrafish were obtained through alx3-targeted CRISPR/Cas9 mutagenesis in the alx1moon16 mutant background. Design of alx3 CRISPR site 5′-GGAGTCCCGATGCGGCATGC-3′ in exon 2 and mutagenesis were carried out as previously described (Sedykh et al., 2016, 2017), and the recovered mutant alleles were initially characterized by sequencing PCR-amplified genomic DNA. To detect the presence of alx1moon16 and alx3mo2113+/- mutant alleles, the relevant region was PCR amplified from genomic DNA extracted from adult tail clips or individual embryos, and subjected to PCR with the following primers: alx1 forward: 5′-CGTGACCACTGGCCTCCTCT-3′ (Pini et al., 2020), alx1 reverse: 5′-CGAGTTCGTCGAGGTCTGTT-3′ (Pini et al., 2020), alx3 forward: 5′-CTATTCCTGCTGCGGACT-3′, alx3 reverse: 5′-TCTTCAGGTGGAAAAGT-3′. To characterize mutant alleles, alx3 PCR fragments were subcloned via TA cloning into pGemT-Easy (Promega) and sequenced. To determine pappaap170 genotype, DNA was extracted from individual larvae and subjected to PCR with the following primers: pappaap170 forward: 5′-CTCTCTTAGGACTCCCTCCAGTTGC-3′ and pappaap170 reverse: 5′-TTGTCGACGTTGATGAGC-3′ (Wolman et al., 2015). The PCR product was digested with MseI (New England Biolabs), cleaving the mutant allele and producing a 245 bp fragment distinguishable from the 271 bp wildtype allele. Subsequently, metaphor (Lonza) gel electrophoresis was used to genotype individual embryos and adult fish.

Immunohistochemistry

alx1;alx3+/+ incross progeny were fixed at 2 dpf in 4% paraformaldehyde (PFA)/PBS overnight. Their trunk/tail portions were reserved for genotyping and the heads were exposed to 1% KOH/6% H2O2 to remove pigmentation and washed with PBST-X (1X PBS with 0.5% Triton-X-100), followed by a 5-min proteinase-K (1:5000) treatment and 30-min 4% PFA/PBS post-fix at room temperature. Embryos were blocked with PBS/0.1% Tween with 0.1% BSA (1% BSA, 10% goat serum) for 2 h, at room temperature, incubated with mouse-anti-zn5 primary antibody (Zebrafish International Resource Center A28175, 1:100) and then Alexa-488-conjugated goat anti-mouse secondary antibody (Invitrogen AB_10013770, 1:500). Nuclei were labeled with DAPI (Vector probes, 1:5000). For imaging, embryos were mounted in VectaShield (Vector laboratories) and imaged on an Olympus IX81 inverted confocal microscope with the Fluoview 1000 confocal package, using a 60x water immersion objective (NA 1.10).

In situ hybridization and Alcian Blue staining

In situ hybridization was carried out as previously described (Paffett-Lugassy and Zon, 2005). To visualize the intraocular red blood cell population, retinal pigments were removed by exposure to 1% KOH/6% H2O2 and embryos were mounted in 100% glycerol and imaged on a Leica MZ FLIII stereo microscope equipped with Leica DFC310 FX camera and LAS v4.0 software. For cartilage staining, larvae were fixed at 5 dpf in 4% PFA/PBS overnight and stained with Alcian Blue (Zebrafish International Resource Center A28175, 1:100). Stained larvae were further dissected using forceps to isolate jaw cartilage and anterior neurocranium, and imaged using a Nikon Eclipse E600 microscope equipped with Q Imaging Qiclick 1.4MP CCD Monochrome Microscope camera and NIS-elements software.

O-dianisidine staining and alkaline phosphatase staining

Staining erythrocytes with o-dianisidine was carried out as previously described (Pauflet-Lugassy and Zon, 2005). To visualize the intraocular red blood cell population, retinal pigments were removed by exposure to 1% KOH/6% H2O2 and embryos were mounted in 100% glycerol and imaged on a Leica MZ FLIII stereo microscope equipped with Leica DFC310 FX camera and LAS v4.0 software. Endogenous alkaline phosphatase activity in the anterior segment and hyaloid vessels was detected as previously described (Alvarez et al., 2007; Goehlert et al., 1981; Lessell and Kuwabara, 1964; Vorontsova et al., 2019). Briefly, zebrafish larvae were exposed to 0.5% PFA/PBS overnight and eyes were pierced with a sharpened tungsten microneedle and forceps prior to staining. After staining, the retina and sclera were removed with tungsten needles to release the lens, and dissected lenses were imaged using a Nikon Eclipse E600 microscope equipped with Q Imaging Qiclick 1.4MP CCD Monochrome Microscope camera and NIS-elements software.

Adult ocular histology

Adult alx1moon16 zebrafish were euthanized and preserved in 4% paraformaldehyde fixative. Each fish was photographed on both sides to document eye morphology, and the entire body was immersed for 30 min in a solution of 12% hydrochloric acid (Decal II, Surgipath) to decalcify the bone. Transverse sections were made through the head. The resulting head blocks containing the eyes were submitted for paraffin embedding and step sectioned at 100 μm to sample beyond the midway of the globes. Extra slides were saved at each step. The selected sections were stained using standard protocol with hematoxylin and eosin. Rough measurements were not reported.
made of each globe in the axial plane and in the vertical plane using the histology slide.

**Lineage tracing and fluorescent imaging**

Embryos derived from alx1<sup>uw2016</sup>; Tg(soxi10:kaede) were injected with ∼2 nl of 3 mM gRNA:Cas9 RNP complex at the 1-cell stage, as described in a previous study (Hoshijima et al., 2019) to generate alx3 crispsants. Injected embryos were raised until the 20somite stage, whereupon they were dechorionated, selected for strong fluorescent expression, and mounted in low melting agarose containing 0.013% tricaine. Crispsants were photoconverted using the 405 nm UV laser on a Leica scanning confocal microscope, with regional selectivity accomplished by using region of interest (ROI) features. Selected cells were exposed to the photoconverting laser at full power for 3–5 min, depending on the size of the region, or until red kaede was observed. Crispsants were imaged to record initial position of the photoconverted cells and raised until 4 dpf, when they were once again mounted in low melting agarose with tricaine and imaged on the confocal microscope. For fluorescent imaging of the ANC and Meckel’s cartilage, both uninjected siblings and crispsants at 4 dpf with different genotypes on a Tg(soxi10:kaede) background were dissected and flat-mounted for imaging.

**RNA-Seq transcriptome analysis**

For the alx1;alx3 mutant dataset, embryos produced from a cross between alx1;alx3<sup>+/+</sup> parents were flash-frozen individually at 5 dpf, and RNA was prepared using the RNeasy Plus Mini Kit (Qiagen 74134) and a protocol adapted from (de Jong et al., 2010). Genomic DNA contamination was removed using the DNase Max Kit (Qiagen 15200-50). cDNA libraries were prepared using the TruSeq stranded mRNA library preparation protocol with poly-A selection and sequenced on the Illumina HiSeq 2500. Prior to analysis, each sample was genotyped from raw sequencing data alignments using the Integrative Genomics Viewer (Robinson et al., 2011). For the alx1;alx3<sup>CR</sup> dataset, embryos derived from alx1<sup>uw2016</sup>; Tg(soxi10:kaede) parents were injected with alx3 sgRNA:Cas9 RNP complex, raised until 3 dpf and screened under fluorescence to select those exhibiting malformed ANC. Embryos derived from Tg(soxi10:kaede), fertilized within 2 h and raised under the same conditions were used as age-matched controls. Twenty embryos were combined into each biological replicate, and six replicates for each genotype were collected in two independent experiments. RNA was prepared using the RNeasy Plus Mini Kit (Qiagen 74134) and RNA quality was assessed by Agilent High Sensitivity RNA ScreenTape (Agilent, 5067-5580). cDNA libraries were prepared using the NEBNext<sup>®</sup> Ultra™ II Directional RNA Library Kit (NEB, E7765S) with NEBNext<sup>®</sup> Poly(A) mRNA Magnetic Isolation Module (E7490S) and sequenced (50 bp paired-end) on the Illumina Nextseq 550.

Bioinformatic analysis of transcriptomic data adhere to recommended ENCODE guidelines and best practices for RNA-Seq (Encode Consortium, 2016). For the alx1;alx3-mutant RNA-seq dataset, alignment of adapter-trimmed (Jiang et al., 2014) (Skever v0.1.123) 2×150 (paired-end; PE) bp strand-specific Illumina reads to the Danio rerio GRCz11 genome (assembly accession GCF_000002035.6) was achieved with the Spliced Transcripts Alignment to a Reference (STAR v2.5.3a) software (Dobin et al., 2013), a splice-junction aware aligner. Expression estimation was performed with RSEM v1.3.0 (RNASeq by Expectation Maximization) (Li et al., 2013), a splice-junction aware aligner. Expression estimation was performed with the Integrative Genomics Viewer (Robinson et al., 2011). For the alx1<sup>uw2016</sup>; Tg(soxi10:kaede) dataset, alignment of adapter-trimmed (Jiang et al., 2014) (Skever v0.1.123) 2×150 (paired-end; PE) bp strand-specific Illumina reads to the Danio rerio GRCz11 genome (assembly accession GCF_000002035.6) was achieved with the Spliced Transcripts Alignment to a Reference (STAR v2.5.3a) software (Dobin et al., 2013), a splice-junction aware aligner. Expression estimation was performed with RSEM v1.3.0 (RNASeq by Expectation Maximization) (Li and Dewey, 2011). To test for differential gene expression among individual group contrasts, expected read counts obtained from RSEM were used as input into Deseq2 (Love et al., 2014). Pairwise contrasts between CRISPants and age-matched wildtype were conducted to generate differentially expressed gene lists. Gene set enrichment was then determined by gene set enrichment analysis (GSEA) (Subramanian et al., 2005) using the Gene Ontology database (Ashburner et al., 2000; Gene Ontology Consortium, 2020).

**Behavior testing, video recording and analysis**

Zebrafish larvae were raised at 29°C in E3, on a 14:10 h light/dark cycle starting at 1 dpf. On the day of behavioral testing (5 dpf), 60-mm-wide Petri dishes with 25 larvae/dish in 10 ml E3, were placed on a white light box (800 µW/cm²) for at least 1 h. Larvae were transferred to a 4×4 grid and illuminated from above with a mounted LED light (MCWHL5 6500K LED, powered by LEDD1B driver, Thorlabs), and from below with an infrared light source (IR Illuminator CM-IR200B, C&M Vision Technologies). In the grid, larvae were acclimated to the illuminated testing stage (85 µW/cm²) for 5 min before exposure to a series of 10 dark flash stimuli. The O-bend behavior was elicited using an automated behavioral platform in which the mounted LED light and the timing of dark flashes could be controlled (Burgess and Granato, 2007; Wolman et al., 2011). Stimuli were presented at 30 s interstimulus intervals, with each stimulus lasting for 1 s. We used Flote to analyze video images responses in an experimenter-independent, automated manner (Burgess and Granato, 2007). Flote tracks the position of individual larval frame by frame and characterizes locomotor maneuvers according to predefined kinematic parameters that distinguish these maneuvers. After testing in the 4×4 grid, larvae were transferred to a 96-well plate and genotyped for alx1<sup>+</sup>, alx3<sup>+</sup>, or Pappar<sup>+/−</sup> post-hoc.

**Statistics**

Statistical analyses, including calculation of means, SEM, and ANOVA, Fisher’s exact test and the Chi-square test, were performed using GraphPad Prism software (GraphPad Software). P-values below 0.05 were considered statistically significant.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: Y.G.; Methodology: B.Y., P.Y., L.E.B., K.K., Y.G.; Investigation: B.Y., P.Y., N.S., L.E.B., K.K., J.K., R.D., J.V.O., J.C., Resources: Y.G.; Writing - original draft: B.Y., Y.G.; Writing - review & editing: Y.G., E.C.L.; Supervision: Y.G., B.Y., P.Y., N.S., L.E.B., K.K., J.K., R.D., J.V.O., J.C.; Funding acquisition: Y.G., B.Y., E.C.L.

**References**

Abu-Safieh, L., Abboud, E. B., Alkuraya, H., Shamseldin, H., Al-Enzi, S., Al-Abdi, L., Hashem, M., Colak, D., Jarallah, A., Ahmad, H. et al. (2011). Mutation of IGFBP7 causes upregulation of BRAF/MEK/ERK pathway and familial retinal arterial macroaneurysms. Am. J. Hum. Genet. 89, 313-319. doi:10.1016/j.ajhg.2011.07.010
of ALX1 causes extreme microphthalmia and severe facial clefting: expanding the spectrum of autosomal-recessive ALX-related frontonasal dysplasia. Am. J. Hum. Genet. 86, 789-796. doi:10.1016/j.ajhg.2010.04.002

Van Der Meulen, K. L., Vöckling, O., Weaver, M. L., Meshram, N. N. and Famulski, J. K. (2020). Spatiotemporal characterization of anterior segment mesenchyme heterogeneity during zebrafish ocular anterior segment development. Front. Cell Dev. Biol. 8, 379. doi:10.3389/fcell.2020.00379

Vorontsova, I., Hall, J. E. and Schilling, T. F. (2019). Assessment of zebrafish lens nucleus localization and sutural integrity. J. Vis. Exp. 147, e59528. doi:10.3791/59528

Wada, N., Javidan, Y., Nelson, S., Carney, T. J., Kelsh, R. N. and Schilling, T. F. (2005). Hedgehog signaling is required for cranial neural crest morphogenesis and chondrogenesis at the midline in the zebrafish skull. Development 132, 3977-3988. doi:10.1242/dev.01943

Wang, X.-Y., Li, S., Wang, G., Ma, Z.-L., Chuai, M., Cao, L. and Yang, X. (2015). High glucose environment inhibits cranial neural crest survival by activating excessive autophagy in the chick embryo. Sci. Rep. 5, 18321. doi:10.1038/srep18321

Wentzel, P. and Eriksson, U. J. (2011). Altered gene expression in rat cranial neural crest cells exposed to a teratogenic glucose concentration in vitro-paradoxical downregulation of antioxidative defense genes. Birth Defects Res. B Dev. Reprod. Toxicol. 92, 487-497. doi:10.1002/bdr.20321

Westerfield, M. (1993). The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio). Eugene, OR: M. Westerfield.

Wolman, M. A., Jain, R. A., Liss, L. and Granato, M. (2011). Chemical modulation of memory formation in larval zebrafish. Proc. Natl. Acad. Sci. USA 108, 15468-15473. doi:10.1073/pnas.1107156108

Wolman, M. A., Jain, R. A., Marsden, K. C., Bell, H., Skinner, J., Hayer, K. E., Hogenesch, J. B. and Granato, M. (2015). A genome-wide screen identifies PAPP-AA-mediated IGFR signaling as a novel regulator of habituation learning. Neuron 85, 1200-1211. doi:10.1016/j.neuron.2015.02.025

Wu, R. S., Lam, I. I., Clay, H., Duong, D. N., Deo, R. C. and Coughlin, S. R. (2018). A rapid method for directed gene knockout for screening in G0 zebrafish. Dev. Cell 46, 112-125.e114. doi:10.1016/j.devcel.2018.06.003

Yan, Y.-L., Hatta, K., Riggelman, B. and Postlethwait, J. H. (1995). Expression of a type II collagen gene in the zebrafish embryonic axis. Dev. Dyn. 203, 363-376. doi:10.1002/aja.1002030308

Zhao, Q., Behringer, R. R. and de Crombrugghe, B. (1996). Prenatal folic acid treatment suppresses acrania and meroanencephaly in mice mutant for the Cart1 homeobox gene. Nat. Genet. 13, 275-283. doi:10.1038/ng0796-275