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Mutations in zebrafish *pitx2* model congenital malformations in Axenfeld-Rieger syndrome but do not disrupt left-right placement of visceral organs

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Abstract

Pitx2 is a conserved homeodomain transcription factor that has multiple functions during embryonic development. Mutations in human *PITX2* cause autosomal dominant Axenfeld-Rieger syndrome (ARS), characterized by congenital eye and tooth malformations. *Pitx2*$^{-/-}$ knockout mouse models recapitulate aspects of ARS, but are embryonic lethal. To date, ARS treatments remain limited to managing individual symptoms due to an incomplete understanding of PITX2 function. In addition to regulating eye and tooth development, Pitx2 is a target of a conserved Nodal (TGFβ) signaling pathway that mediates left-right (LR) asymmetry of visceral organs. Based on its highly conserved asymmetric expression domain, the Nodal-Pitx2 axis has long been considered a common denominator of LR development in vertebrate embryos. However, functions of Pitx2 during asymmetric organ morphogenesis are not well understood. To gain new insight into Pitx2 function we used genome editing to create mutations in the zebrafish *pitx2* gene. Mutations in the *pitx2* homeodomain caused phenotypes reminiscent of ARS, including aberrant development of the cornea and anterior chamber of the eye and reduced or absent teeth. Intriguingly, LR asymmetric looping of the heart and gut was normal in *pitx2* mutants. These results suggest conserved roles for Pitx2 in eye and tooth development and indicate Pitx2 is not required for asymmetric looping of zebrafish visceral organs. This work establishes zebrafish *pitx2* mutants as a new animal model for investigating mechanisms underlying congenital malformations in ARS and high-throughput drug screening for ARS therapeutics. Additionally, *pitx2* mutants present a unique opportunity to identify new genes involved in vertebrate LR patterning. We show Nodal signaling—independent of Pitx2—controls asymmetric expression of the fatty acid elongase *elovl6* in zebrafish, pointing to a potential novel pathway during LR organogenesis.

Keywords

Pitx2; Axenfeld-Rieger syndrome; Left-right asymmetry; Elovl6; Genome editing; Zebrafish

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Competing interests

No competing interests declared.

Author contributions

Y.J. and J.D.A. designed experiments. Y.J. and S.M.B. performed experiments. Y.J., S.M.B. and J.D.A. analyzed data and prepared the manuscript.

1. Introduction

Pitx2 (Paired-like homeodomain 2) is a member of the bicoid class of homeodomain transcription factors that has been highly conserved during evolution. Vertebrate *Pitx2* genes encode multiple protein isoforms that have different N-termini but share a common C-terminal homeodomain that mediates DNA binding (Chaney et al., 2005). Loss-of-function mutations in the homeodomain of human *PITX2* cause autosomal dominant Axenfeld-Rieger syndrome (ARS). PITX2 haploinsufficiency in ARS patients disrupts development of the anterior segment of the eye and tooth morphogenesis (Footz et al., 2009). Ocular defects found in ARS patients include malformation of the anterior chamber angle, central corneal thickness and iris atrophy with corectopia. A persistence of the endothelial layer on the angular structures and an anterior iris root insertion result in aqueous outflow defects. As a consequence, patients often have high intraocular pressure and are at risk for developing glaucoma that can lead to blindness (Chang et al., 2012; Shields et al., 1985). Dental abnormalities caused by *PITX2* mutations include microdontia, hypodontia and enamel hypoplasia (Jena and Kharbanda, 2005). Additional craniofacial malformations such as hypertelorism, a broad flat nasal bridge and maxillary defects are associated with ARS (Antevil et al., 2009). ARS patients generally have a normal life span and treatment options are currently limited to managing individual symptoms, which require complex multidisciplinary approaches.

Animal models have played an important role in our understanding of Pitx2 function and underlying causes of ARS malformations. Targeting the *Pitx2* homeodomain in knockout mouse models eliminated the activity of all Pitx2 isoforms and caused eye and tooth developmental defects in $Pitx2^{-/-}$ embryos that are consistent with ARS (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). However, global loss of *Pitx2* resulted in additional developmental malformations in the brain, visceral organs and body wall and was embryonic lethal. Most heterozygous *Pitx2* knockout mice developed into normal adults, but ~10% developed ARS phenotypes that included small eyes, tooth defects and reduced body size (Gage et al., 1999). Additional mutant alleles have been used to study *Pitx2* isoforms and gene dosage effects in mouse (Liu et al., 2001, 2002, 2003) and conditional alleles have helped define tissue-specific roles, such as the requirement for *Pitx2* in neural crest cells for normal eye development (Evans and Gage, 2005). However, fundamental gaps in our understanding of *Pitx2* functions and target genes have made it difficult to conceptualize therapeutic approaches for ARS patients. Thus, it will be critical to exploit existing knockout mouse models and develop new animal models to fully understand molecular functions of *PITX2* and identify effective treatments for ARS.

One of the roles of Pitx2 during embryonic development is to function as a downstream effector of an ancient Nodal signaling pathway associated with generating morphological asymmetries in cnidarians (Watanabe et al., 2014), echinoderms (Duboc et al., 2005) and chordates (Boorman and Shimeld, 2002). In vertebrates, the secreted TGFβ-related ligand Nodal triggers asymmetric expression of a specific *Pitx2* isoform, *Pitx2c*, in left lateral plate mesoderm during left-right (LR) patterning of the embryo (Hamada et al., 2002). Unlike Nodal, asymmetric *Pitx2c* expression was found to persist on left side of the developing

heart and gut, where it is thought to regulate genes that mediate asymmetric morphogenesis of these organs. In chicken and frog embryos, misexpression of Pitx2 or Nodal in right lateral plate mesoderm reversed heart and gut looping (Levin et al., 1997; Ryan et al., 1998; Sampath et al., 1997) and blocking Pitx2 or Nodal function randomized looping direction (Toyoizumi et al., 2005; Yu et al., 2001). Further analyses in chick embryos revealed Pitx2c induces cellular condensations during gut looping (Davis et al., 2008; Welsh et al., 2013). Together, these findings suggested Nodal-Pitx2 signaling controls direction of asymmetric organ development. Consistent with this model, Nodal mouse mutants showed randomized heart and gut looping (Brennan et al., 2002). However, the initial looping of the heart and gut occurred normally in *Pitx2* knockout mice (Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). Abnormalities during subsequent steps of asymmetric organ morphogenesis in Pitx2 knockout mice, and mice specifically lacking Pitx2c expression (Liu et al., 2001; Shiratori et al., 2006), resulted in organ-specific LR defects that included lung isomerism, abnormal looping of the duodenum and cardiac malformations. Cardiac outflow tract defects were subsequently linked to Pitx2 functions in the second heart field that contributes to outflow tract development (Ai et al., 2006). These studies revealed that the roles of Pitx2 downstream of Nodal in LR patterning remain unclear and may not be completely conserved.

The zebrafish provides a useful model system to investigate gene function and evolution, and the small size and external development of the zebrafish embryo makes it ideal for high-throughput chemical screening (Zon and Peterson, 2005). Since *pitx2* mutations have not been identified in genetic screens, we generated the first zebrafish *pitx2* mutants via genome editing to gain insight into Pitx2 functions and develop a new ARS animal model. We found that mutations that truncate the zebrafish homeodomain, which is 100% identical to human PITX2, caused eye and tooth developmental defects consistent with phenotypes observed in ARS patients. Importantly, homozygous $pitx2^{-/-}$ zebrafish grow to adulthood and thereby provide a new model of adult ARS. Interestingly, analysis of the heart and gut looping in mutant embryos revealed Pitx2 is dispensable for correct LR asymmetric orientation of these organs. These results identify functions for Pitx2 in eye and tooth development that appear conserved from fish to mammals, and indicate Pitx2 is not required for LR asymmetric looping of visceral organs in zebrafish. Mutant *pitx2* zebrafish are an important new disease model that can be used in chemical screens for therapeutic treatments of ARS and can be leveraged to discover new pathways that guide LR morphogenesis. To begin to identify new candidate molecules that may regulate LR development, we show asymmetric expression of the *ELOVL fatty acid elongase 6* (*elovl6*) gene is controlled by LR Nodal signaling and is independent of Pitx2 function in zebrafish.

2. Material and methods

2.1. Zebrafish

Wild-type TAB zebrafish, obtained from Zebrafish International Resource Center, were used to generate *pitx2* mutants and for microinjections. $cftr^{pd1048}$ mutants were provided by the Bagnat lab (Duke University). All animal studies were approved by the Institutional Animal Care and Use Committee at SUNY Upstate Medical University.

2.2. Generation of pitx2 mutant zebrafish

pitx2HD and *Pitx2c* TALENs were designed using TAL Effector-Nucleotide Targeter 2.0 and assembled using golden gate assembly (Cermak et al., 2011). TALEN mRNAs were synthesized using the Ambion mMessage Machine kit and purified by LiCl precipitation. 150–200 pg of each TALEN mRNA was injected into wild-type zebrafish embryos at the one cell stage. Injected embryos were raised to adulthood and then outcrossed with wild-type fish to identify founders that transmitted mutations through the germ line. Mutations were identified via digestion of PCR products with SalI (*pitx2HD* TALEN) or BamHI (*pitx2c* TALEN) and confirmed by sequencing. The PCR primers used for the pitx2HD alleles were forward primer 5′-TGAAGCTTGTTCCTCTGC-3′ and Reverse primer 5′-AAAATTTAGGGTTATATCACATA-3′ and for the pitx2c allele were forward primer 5′ GGAGTG TCGCTTTTAGTGG-3′ and reverse primer 5′-ACTAGAGGCCATCGAAAGC-3′.

2.3.. mRNA synthesis and microinjection

For cloning of *pitx2* cDNAs, total RNA was extracted from pooled embryos of *pitx2HD*^{sny15} intercross at 6 dpf and RT-PCR was used to amplify full-length *pitx2c* (primers: 5′-agatctatgacctctat-gaaggatcc 3′ and 5′-tctagattacaccggtctatccactg-3′). The product was inserted into a Topo vector (Invitrogen) and confirmed by sequencing. Confirmed *pitx2c* cDNAs were transferred into pCS2 vectors and then linearized for mRNA synthesis using the Sp6 mMessage mMachine kit (Ambion). 50 pg of wild-type or mutant *pitx2c* mRNA was injected into wild-type embryos at the 1-cell stage. Full-length *elovl6* was amplified from total RNA from 1 dpf wild type embryos (primers: 5′-cgcggatccatgtcggtgctggcattg-3′ and ccgcctcgagttattggcttttcttggctgc-3′) and cloned using a pCS2 vector. mRNA was prepared the same as *pitx2c* and 300 pg was injected into 1-cell stage embryos.

2.4. Eye sectioning

5 day old zebrafish were euthanized and flash frozen in O.C.T (Sakura) and cryosectioned as described (Uribe and Gross, 2007). Sections for every 5 μm or 10 μm were collected for 5 dpf and 3 month fish respectively. Slides were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet (Sigma) 5 min at room temperature. Stained sections were dehydrated using a series of ethanol rinses, cleared with xylene and mounted in Poly-Mount medium (Polysciences).

2.5. Tooth staining

Staining of mineralized pharyngeal teeth was performed as described (Walker and Kimmel, 2007). Larvae at 5 dpf were fixed in 4% paraformaldehyde in PBS for 2 h. After 50% ethanol dehydration, embryos were stained with alizarin red solution (0.005% alizarin red and 60 mM $MgCL_2$ in 70% ethanol) overnight. Embryos were cleared with 0.25% KOH in glycerol and imaged using a stereo microscope.

2.6. RNA in situ hybridizations

Whole mount RNA in situ hybridizations were conducted as described (Gao et al., 2010). In addition to previously described probes (*cmlc2*, *foxa3* and *spaw*), *pitx2c* and *elovl6* probes

were made from corresponding cDNAs amplified from a zebrafish cDNA library (see primer sequences above) and cloned using a TOPO TA cloning Kit (invitrogen). *elovl6* expression was analyzed in *cftr*^{pd1048} mutants (Navis et al., 2013) or embryos injected with Lgl2 morpholino (5′-gcccatgacgcctgaacctcttcat-3′) (Tay et al., 2013). *gh1* (primers: 5′-atggctagagcattggtgc-3′ and 5′-tacagggtacagttggaatc-3′) and *pomca* (primers: 5′-atggtgaggggagtgaggatg-3′ and tcact-catccttcctcggttg-3′) cDNA fragments were cloned using a TOPO TA cloning kit with a pCRII-TOPO vector (Invitrogen) and used as templates for antisense probes. The areas of *gh1* and *pomca* expression domains were measured using Zeiss AxioVision software. To genotype embryos following in situ hybridization analysis, DNA was extracted from individual embryos by boiling in 100 µl 50 mM NaOH and analyzed via PCR and restriction digest as described above.

2.7. Small molecule treatments

Embryos were cultured in 40 µM of SB-505142 (Sigma) in 0.2% DMSO between the tailbud and 18 somite stages. Embryos were then fixed at 18 somite stage for *pitx2c* or *elovl6* in situ hybridizations. Control embryos were treated with 0.2% DMSO alone.

3. Results

3.1.. Generation of mutant pitx2 zebrafish

The zebrafish genome contains a single *pitx2* gene that encodes two isoforms that correspond to human PITX2A and PITX2C. To disrupt zebrafish Pitx2 function, we used TALEN-mediated genome editing (Joung and Sander, 2013) to create small insertion or deletion (indel) mutations in the *pitx2* gene. We targeted the homeodomain to disrupt both *pitx2a* and *pitx2c* or the first exon of *pitx2c* to generate *pitx2c*-specific mutations (Fig. 1A). Three different indel mutations in the homeodomain were recovered, which we refer to as *pitx2HD* alleles designated as *pitx2HD*^{sny6}, *pitx2HD*^{sny7} and *pitx2HD*^{sny15} (Fig. 1B). All three *pitx2HD* mutations result in a frameshift and premature stop codon in the second helix of the homeodomain (Fig. 1C; Fig. S1A), which is predicted to eliminate DNA binding (Chaney et al., 2005) and result in loss of function of both Pitx2a and Pitx2c proteins. In addition, we recovered one *pitx2c*-specific mutation, designated as *pitx2c*^{sny3} (Fig. 1D), that causes a frameshift after the third amino acid and a stop codon in the N-terminus of the protein (Fig. 1E). Both *pitx2HD* and *pitx2c* mutations were identified by loss of a restriction enzyme cut site in the target sequence, which provides an efficient assay for genotyping (Fig. 1F and G).

To characterize the effect of these mutations on *pitx2* expression, we first analyzed *pitx2* transcripts in mutant embryos. RNA in situ hybridizations revealed *pitx2* mRNAs were present in mutants, including asymmetric expression of *pitx2c* in left lateral plate mesoderm (Fig. 2A). RT-PCR and sequencing confirmed only mutant *pitx2* mRNAs were present in the homozygous mutants (Fig. S1B). Attempts to detect zebrafish Pitx2 protein using commercially available antibodies raised against human PITX2 were not successful in immunohistochemistry or Western blot experiments, so we cloned the mutant *pitx2HD*^{sny15} transcripts for functional analysis. Similar to previous observations (Essner et al., 2000), injecting wild-type *pitx2a* or *pitx2c* mRNA into wild-type embryos resulted in severe

malformations that included axial defects (Fig. 2B). In contrast, most embryos injected with mutant *pitx2c* mRNA that contained the *HD*^{sny15} frameshift mutation developed normally (Fig. 2B). These results suggested *pitx2HD* mutations were loss-of-function alleles.

We next characterized development of embryos that inherited the edited *pitx2* alleles. Expression of *pitx2a* and *pitx2c* is not maternally supplied (Fig. S2), but both isoforms are expressed during gastrulation in mesendoderm and/or prechordal plate (Essner et al., 2000). These early expression domains suggested loss-of-function might cause early patterning defects in the embryo. However, all offspring from *pitx2HD*$^{sny7/+}$ parents were grossly indistinguishable through the first 5 days of development (Fig. 3A). Genotyping individual embryos revealed the expected Mendelian ratio of heterozygous and homozygous genotypes. Normal gross morphology was also observed in *pitx2HD*$^{sny6/sny6}$ and *pitx2HD*$^{sny15/sny15}$ embryos at 5 days post-fertilization (dpf) (Fig. S3A). Homozygous mutant *pitx2HD* fish survived to adulthood at variable rates (Table S1). Adult *pitx2HD* fish were smaller overall than wild-type siblings (Fig. 3B) and had variable eye defects that appeared to affect the iris and cornea (Fig. 3C, Fig. S3B). Bilateral eye phenotypes were observed in nearly all *pitx2HD*^{sny7} (n=17/17), *pitx2HD*^{sny6} (n=4/4) and *pitx2HD*^{sny15} (n=5/6) affected embryos. Intriguingly, 1/6 mutant *pitx2HD*^{sny15} embryos had a defect in only one eye. Similar to *pitx2HD* mutants, embryos homozygous for the *pitx2c*-specific mutation (*pitx2c*$^{sny3/sny3}$) were indistinguishable from wild-type at 5 dpf (Fig. 3A) and survived to adulthood. However, adult *pitx2c*$^{sny3/sny3}$ fish were the same size as wild-type siblings (Fig. 3B) and eye phenotypes were not observed (Fig. 3C). Interestingly, based on external morphological features, we have recovered only male *pitx2c*$^{sny3/sny3}$ adults (10 homozygous mutant males out of 57 adults genotyped). Male sex was confirmed by the presence of testes (Fig. S4) and outcrosses with wild-type females resulted in viable embryos (7/16 crosses were successful) indicating *pitx2c*$^{sny3/sny3}$ males are fertile. This finding raises the possibility that Pitx2c may be involved in zebrafish sex determination or gonad development as described in chicken (Guioli and Lovell-Badge, 2007; Rodriguez-Leon et al., 2008).

3.2. pitx2HD mutant zebrafish develop defects associated with ARS

Patients with *PITX2* homeodomain mutations at the same amino acid as our *pitx2HD* alleles developed ARS phenotypes, including malformations of the anterior segment of the eye (Yin et al., 2014). To investigate eye phenotypes observed in *pitx2HD* mutant zebrafish, we analyzed eye morphology during embryogenesis. At 5 dpf, sectioned of homozygous mutant *pitx2HD* ^{sny7} eyes (identified by genotyping) were smaller and malformed relative to wild-type eyes (Fig. 4A and B). We observed a reduced anterior chamber, an increased thickness of the mesenchyme surrounding the cornea and malformation of the iridocorneal angle (Fig. 4B′). The same anterior segment defects were present in mutant *pitx2HD*^{sny6} embryos (Fig. S5A), but were not observed in *pitx2c*^{sny3} mutants (Fig. 4C, C′). Higher resolution images indicated that the layers of the retina were intact in *pitx2HD* and *pitx2c* mutants (Fig. S6). These findings are reminiscent of eye phenotypes in ARS patients and Pitx2 homeodomain mutant mouse embryos (Gage et al., 1999; Lu et al., 1999), suggesting the role for Pitx2 in development of the anterior segment of the eye is conserved from zebrafish to mammals.

In addition to ocular defects, *Pitx2* homeodomain mutations cause dental hypoplasia, which manifests as small, missing or malformed teeth in ARS patients (Dressler et al., 2010; Semina et al., 1996) and knockout mice (Lin et al., 1999; Lu et al., 1999) Zebrafish lack oral teeth but have pharyngeal teeth that have all the features of teeth in other vertebrates (Yelick and Schilling, 2002). At 5 dpf, three pairs of mineralized teeth, denoted as $3V^1$, $4V^1$ and $5V^1$, are attached to fifth branchial arches (Van der Heyden and Huysseune, 2000; Wise and Stock, 2010). Using alizarin red to stain developing teeth at 5 dpf, we found aberrant tooth morphogenesis in *pitx2HD* mutants. Compared to normal tooth development in wild-type siblings (Fig. 5A), the $3V^1$ and $5V^1$ teeth were absent and $4V^1$ teeth were malformed and smaller in *pitx2HD* mutants (Fig. 5B, D; Fig S5B). Similar tooth morphogenesis defects were observed at 9 dpf (Fig. 5D), indicating tooth development is not just delayed. In contrast to *pitx2HD* mutants, $pitx2c^{sny3/sny3}$ embryos had normal teeth (Fig. 5C, D). Taken together, these results suggest a conserved role for Pitx2 in vertebrate tooth development.

To begin to test whether *pitx2HD* mutants develop additional phenotypes found in ARS patients, we analyzed pituitary development. Abnormalities associated with pituitary defects are found in ARS patients (Chang et al., 2012; Tumer and Bach-Holm, 2009) and *pitx2* knockout mice (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Suh et al., 2002). To assess the pituitary in zebrafish *pitx2* mutants, RNA in situ hybridizations were performed using markers of different cell types in the pituitary, which included *gh1* (*growth hormone 1*) that marks somatotropes and *pomca* (*proopiomelanocortin a*) that marks corticotropes and melanotropes (Herzog et al., 2003). By quantifying these expression domains at 5 dpf, we found that the area of both *gh1* and *pomca* staining was reduced in $pitx2HD^{+/-}$ and $pitx2HD^{-/-}$ embryos as compared to wild-type siblings (Fig. S7). These results suggest a reduction of somatotropes, corticotropes and melanotropes in $pitx2HD^{-/-}$ embryos and a slight reduction of somatotropes and corticotropes in $pitx2HD^{+/-}$ embryos relative to wild-type. This analysis provides evidence that some zebrafish pituitary phenotypes are Pitx2 dose-dependent, as observed in mammals. In *pitx2* knockout mice, a dose-dependent effect was reported that moderately reduced *Gh* staining in mutants, but unlike *pitx2HD* zebrafish, *Pomc* expression was not changed (Suh et al., 2002). These results suggest Pitx2 has a function in zebrafish pituitary development that may be different than in mouse and provide the foundation for future work analyzing Pitx2 functions in the pituitary.

3.3.. The heart and gut undergo normal left-right asymmetric looping and placement in pitx2 mutants

We next wanted to determine the role of Pitx2 in establishing LR asymmetry of visceral organs in zebrafish. The zebrafish Nodal homolog *southpaw* (*spaw*) is asymmetrically expressed in left lateral plate mesoderm and is required for asymmetric *pitx2c* expression (Long et al., 2003). Homozygous *spaw* mutants—in which *pitx2* expression is absent in lateral plate mesoderm—have LR patterning defects: heart looping was altered in 30% of mutant embryos and gut looping was abnormal in 50% of the mutants (Noel et al., 2013). This suggested Pitx2 may function downstream of Nodal/Spaw to regulate asymmetric morphogenesis of the zebrafish heart and gut. To test this, we first analyzed asymmetric heart development in *pitx2* mutants via RNA in situ hybridizations using the heart-specific

marker *cmlc2*. At 30 hours post-fertilization (hpf), the developing heart tube undergoes asymmetric movements referred to as heart 'jogging' (Chen et al., 1997). The heart typically jogs to the left in wild-type embryos, but jogging can occur along the midline or to the right (Fig. 6A) when LR patterning cues are disrupted. Analysis of *pitx2HD* and *pitx2c* mutants revealed normal leftward jogging of the heart at 30 hpf (Fig. 6B; Table S2). Since *pitx2* mutants could not be distinguished from wild-type siblings, each embryo was genotyped after RNA in situ analysis. Following asymmetric jogging, the heart tube undergoes rightward looping as observed in other vertebrate embryos. We visualized asymmetric looping of the zebrafish heart tube at 50 hpf and found heart morphology and looping direction in *pitx2HD* and *pitx2c* mutants were similar to wild-type siblings (Fig. 6C and D; Table S2). Examination of the heart in live embryos did not reveal gross defects in heart formation or function through 5 dpf, which is consistent with survival of *pitx2HD* and *pitx2c* mutants to adulthood. These results indicated loss of Pitx2 function did not alter cardiac jogging or looping morphogenesis.

Next, asymmetric development of the gut was analyzed using *foxa3* RNA in situ hybridization probes that mark the developing gut tube. Leftward looping of the zebrafish gut occurs between 26 and 30 hpf and is driven by movements of adjacent lateral plate mesoderm (Horne-Badovinac et al., 2003). Similar to the heart, the usual left-sided gut looping can remain at the midline or be reversed to the right (Fig. 6E) if embryo laterality is perturbed. When analyzed at 30 hpf, we found no differences in gut looping among wild-type, *pitx2HD* and *pitx2c* mutant embryos (Fig. 6F; Table S3). At 50 hpf, normal asymmetric looping of the gut places the liver to the left of the midline and the pancreas to the right (Fig. 6G). The orientation of these organs was reversed in nearly 50% of *spaw* mutants (Noel et al., 2013). However, consistent with normal gut looping, asymmetric positioning of the liver and pancreas developed normally in *pitx2HD* and *pitx2c* mutants (Fig. 6G and H; Table S3). At 7 dpf, visualization of gut auto-fluorescence in living embryos confirmed the LR orientation of visceral organs was unaffected in *pitx2HD and pitx2c* larvae (Fig. S8). RNA in situ analysis of the Nodal-Pitx2 cascade in lateral plate mesoderm revealed left-sided expression of *spaw* and *pitx2c* was expressed correctly in both *pitx2HD* and *pitx2c* mutants (Fig. S9), indicating early LR patterning of these embryos is normal. Taken together, these results indicate Pitx2 is dispensable for LR orientation of the zebrafish heart and gut and suggest molecules other than Pitx2 function downstream of Nodal to regulate heart and gut looping morphogenesis.

3.4.. Asymmetric expression of elovl6 is linked to left-right patterning information generated by Kupffer's vesicle

Our finding that Pitx2 is not required to establish heart or gut laterality in zebrafish led us to search for new Nodal-responsive genes that may function in LR development. We found that the fatty acid elongase *elovl6*, which lies immediately adjacent to *pitx2* on chromosome 14, was identified in a large-scale RNA in situ screen to be asymmetrically expressed during LR patterning stages (Thisse et al., 2001). Similar to *pitx2c*, we found asymmetric *elovl6* mRNA expression in what appears to be left lateral plate mesoderm at the 18 somite stage and expression in the left brain at the 20 somite stage (Fig. 7A). To test whether asymmetric *elovl6* expression changes when LR patterning is perturbed, we used previously

characterized approaches to alter asymmetric Nodal/Spaw signaling. In zebrafish, correct left-sided asymmetric gene expression depends on the function of motile cilia in Kupffer's vesicle (KV) that generate a directional fluid flow that is required to orient the LR body axis (Essner et al., 2005; Kramer-Zucker et al., 2005). We first analyzed *elovl6* expression in embryos injected with antisense morpholinos that knockdown expression of the Lethal giant larvae (Lgl2) protein. Lgl2 depletion disrupts KV development and results in bilateral *spaw* expression in ~50% of the embryos (Tay et al., 2013). Similarly, we observed bilateral *elovl6* in ~50% of Lgl2 depleted embryos (Fig. 7B). In a few cases, *elovl6* expression was right-sided or absent. We also tested *elovl6* expression in $cftr^{pd1048}$ mutant embryos, in which KV fails to develop and *spaw* expression is also predominantly bilateral (Navis et al., 2013). Again, we observed altered *elovl6* expression that was primarily bilateral in $cftr^{pd1048}$ mutants that were confirmed by genotyping (Fig. 7B). Thus, similar to Nodal/Spaw signaling, correct asymmetric expression of *elovl6* depends on LR patterning information generated by Kupffer's vesicle.

3.5. Asymmetric expression of elovl6 depends on Nodal signaling but not Pitx2

To determine whether asymmetric *elovl6* expression requires Nodal/Spaw signaling, we used the small molecule inhibitor SB-505124 that effectively blocks TGFβ signaling in zebrafish (Lenhart et al., 2013). As expected, SB-505124 treatments between the bud stage and 18 somite stage eliminated asymmetric expression of *pitx2c* in lateral plate mesoderm (Fig. 8A), which is a known target of Nodal/Spaw signaling. Similarly, SB-505124 treatments abolished asymmetric *elovl6* expression in lateral plate (Fig. 8B), suggesting *elovl6* is a new Spaw target gene. To test whether *elovl6* expression depends on Pitx2 transcriptional activity in lateral plate mesoderm, we assessed *elovl6* mRNA in *pitx2* mutants. Similar to wild-type siblings, *elovl6* expression was present in left lateral plate mesoderm in $pitx2HD^{sny15/sny15}$ mutants (Fig. 8C). Together, these analyses indicate asymmetric *elovl6* expression requires Nodal/Spaw signaling but is independent of Pitx2. To begin to test Elovl6 function, we injected mRNA encoding full-length Elovl6 into wild-type embryos. These embryos showed severe malformations—including axial defects—that precluded analysis of heart and gut looping (Fig. S10). These results suggest Elovl6 is functional in the early zebrafish embryo. Future studies will need to address whether *elovl6* functions downstream of Nodal signaling to impact LR patterning of the heart and gut.

4. Discussion

Here we present the initial characterization of the first zebra-fish mutant *pitx2* alleles. Our results show zebrafish Pitx2 is required for proper development of the anterior segment of the eye and morphogenesis of teeth, which likely represent conserved functions for Pitx2 in vertebrate embryos. Since eye and tooth phenotypes in zebrafish *pitx2HD* mutants are similar to malformations observed in ARS patients, these zebrafish provide an important new animal model that can be used for investigating Pitx2 functions during early embryogenesis and for high-throughput screening of small molecules for ARS therapeutics. Intriguingly, although *pitx2c* is asymmetrically expressed in left lateral plate mesoderm, our analyses indicate Pitx2 is not required for asymmetric looping of the zebrafish heart and gut. This finding indicates Pitx2 is not an essential downstream effector of Nodal signaling

during looping of visceral organs in zebrafish. The mutant *pitx2* zebrafish background presents a new opportunity to characterize novel molecules and pathways that impact organ LR asymmetry.

Disrupting the zebrafish Pitx2 homeodomain resulted in tooth hypoplasia and ocular defects, which are features of ARS patients with heterozygous *PITX2* mutations. In contrast to humans, eye and tooth phenotypes were only observed in homozygous *pitx2HD*$^{-/-}$ zebrafish. In previous work, transient knockdown of Pitx2 expression using morpholino oligonucleotides caused defects in eye, craniofacial and asymmetric brain development (Bohnsack et al., 2012; Garric et al., 2014; Liu and Semina, 2012). *pitx2HD* mutants developed eye phenotypes that were consistent with Pitx2 morpholino injected embryos, but did not develop other defects (e.g. pericardial edema) and could survive to adulthood. Defects in the iridocorneal angle observed in *pitx2HD* mutants are reminiscent of characteristic anterior chamber angle defects in ARS patients (Tumer and Bach-Holm, 2009) that commonly develop glaucoma in late childhood or early adulthood. In addition, we uncovered a requirement for Pitx2 in development of zebrafish pharyngeal teeth, which is consistent with *pitx2* expression during tooth formation (Jackman et al., 2004). However, the function of Pitx2 in zebrafish tooth development may be different than in the mouse. In mice, Fgf8 and Bmp4 are expressed during tooth morphogenesis and it has been found that Ffg8 expression in the oral ectoderm is diminished and Bmp4 is expanded in pitx2$^{-/-}$ mice (Lin et al., 1999; Liu et al., 2003; Lu et al., 1999). In zebrafish, Bmp4 is dispensable for tooth development (Wise and Stock, 2010) and Fgf8 is not expressed in tooth-forming regions at any stage (Jackman et al., 2004). It will be interesting to compare and contrast Pitx2 functions in mouse and zebrafish models in future work. Our analysis of pituitary development also suggests differential effects in mouse and fish *pitx2* mutants. These differences provide an opportunity to explore the evolution of Pitx2 functions. Interestingly, zebrafish with a *pitx2c*-specific mutation grew to adulthood with no apparent morphological abnormalities, but we have only recovered males. In birds, Pitx2 has been implicated in asymmetric gonad morphogenesis (Guioli and Lovell-Badge, 2007; Rodriguez-Leon et al., 2008), making it tempting to speculate that Pitx2c may have a role in gonad development in zebrafish. However, since we recovered a single *pitx2c* mutant allele we cannot rule out off-target effects of the TALEN mutagenesis. Thus, further studies are needed to investigate the possible role of Pitx2c in zebrafish gonad development.

In contrast to mouse *Pitx2*$^{-/-}$ knockouts that are embryonic lethal, *pitx2HD*$^{-/-}$ zebrafish can survive to adulthood. It is interesting to consider why the *pitx2* knockout phenotype is less severe in zebrafish. Our results suggest Pitx2 has essential functions in the mouse embryo—such as establishing LR asymmetry in the heart and gut—that are not conserved in zebrafish. Additional problems that likely contribute to embryonic lethality, including body wall closure defects, are also specific to the mouse. It is also possible that compensatory mechanisms exist in *pitx2HD*$^{-/-}$ zebrafish that are absent in mice. This could include the presence of redundant transcription factors and/or the upregulation of factors to take the place of Pitx2 in some tissues. Nonetheless, adult *pitx2HD*$^{-/-}$ zebrafish provide an important new animal model that can be exploited to develop new strategies to treat or prevent eye and tooth diseases associated with ARS. The zebrafish is well suited for efficient high-throughput screening of thousands of small molecules, and mutant alleles have been

successfully used to identify compounds that suppress disease phenotypes. In addition to using *pitx2* mutant to screen for compounds that treat congenital eye and tooth malformations, Pitx2 has been implicated in other processes, such as myogenesis (Knopp et al., 2013; L'Honore et al., 2007), cardiac arrhythmia (Tao et al., 2014) and cancer (Vinarskaja et al., 2013). Although more work is needed to investigate these and other phenotypes, *pitx2* mutant zebrafish may provide a screening platform for drug discovery for a broad range of maladies. Additionally, combining mutant alleles with zebrafish transgenes that label specific cell types provides a useful approach to identifying genes that are misregulated in a particular organ or tissue of the mutant fish. Since defects in neural crest development likely contribute to eye, tooth and craniofacial malformations in ARS patients and zebrafish *pitx2* mutants, transgenic zebrafish that label neural crest cells (Kwak et al., 2013) will allow real-time tracking and transcriptome analyses of neural crest cells in *pitx2* mutant embryos. This approach provides an opportunity to characterize $Pitx2^{-/-}$ cell behaviors and identify cell-specific target genes of Pitx2 to get at molecular mechanisms of Pitx2 function. Discovery of new genes and pathways downstream of Pitx2 may suggest new therapeutic targets.

Based on the highly conserved asymmetric expression of Nodal-Pitx2 axis in left lateral plate mesoderm, we predicted Pitx2 would be required for asymmetric development of visceral organs in zebrafish. However, we did not observe defects in LR asymmetry of the heart or gut in zebrafish *pitx2* mutants. In *Pitx2* mutant mice, heart looping direction is also normal and cardiac LR defects largely encompass defects in septation of the outflow tract that is not conserved in the zebrafish heart. Analysis of zebrafish *spaw* (Nodal) mutants (Noel et al., 2013), in which asymmetric *pitx2c* expression is lost in lateral plate mesoderm, revealed predominantly normal cardiac looping. However, in contrast to mild effects on the heart, loss of *spaw* resulted in complete randomization of gut laterality. Since we observed normal gut asymmetries in zebrafish *pitx2* mutants, this indicates molecules other than Pitx2 mediate signaling downstream of Spaw/Nodal during zebrafish gut LR development. In mouse and chick embryos, gut looping depends on the mechanical forces generated from the dorsal mesentery that attaches to the gut (Savin et al., 2011). Pitx2c expression in the left dorsal mesentery controls Wnt signaling to generate asymmetric cellular condensations that mediate gut looping (Davis et al., 2008; Welsh et al., 2013). The gut looping process in zebrafish results from the asymmetric migration of the neighboring lateral plate mesoderm (Horne-Badovinac et al., 2003). Thus, differences in anatomy provide a plausible explanation for how zebrafish heart and gut LR morphogenesis could be independent of Pitx2.

If Pitx2 is not required for asymmetric looping of visceral organs in zebrafish, what are alternative Nodal pathway target molecules? We found that *elovl6*, which is adjacent to *pitx2* on chromosome 14, is asymmetrically expressed with *pitx2* and that normal left-sided *elovl6* expression depends on Kupffer's vesicle function and Nodal signaling. Taking advantage of our *pitx2* mutants, we determined asymmetric *elovl6* expression is independent of Pitx2 function. The close proximity of *pitx2* and *elovl6* suggests left-sided expression of these genes is controlled by a Nodal-responsive enhancer on chromosome 14. Intriguingly, *Elovl6* is asymmetrically expressed opposite of *Pitx2* in the right dorsal mesentery in the chicken embryo during gut looping (Welsh et al., 2015). It is not clear whether asymmetric *Elovl6*

expression in chicken is Nodal-dependent, but studies in chicken and mouse indicate chromatin conformation at the *Pitx2* locus plays an important role in directing left-sided expression of *Pitx2* and right-sided expression of downstream genes *Enpep* and *Elovl6* (Welsh et al., 2015). In contrast to chicken and mouse, the zebrafish *pitx2* locus lacks an *enpep* gene and the *pitx2* and *elovl6* genes are very close together. The distinct arrangement of genes at *pitx2* loci provides a likely explanation for the differential regulation of *elovl6* expression in zebrafish and chicken.

The findings of asymmetric *elovl6* expression in chicken (right-sided) and zebrafish (left-sided) raise the question of whether Elovl6 plays roles during LR development. Elovl6 belongs to a family of rate-limiting enzymes that catalyze the synthesis of saturated and monounsaturated fatty acids and have been implicated in lipid metabolism related diseases (Matsuzaka and Shimano, 2009). Elovl6 may be involved in the synthesis of fatty acid molecules that participate in cell signaling. In zebrafish, sphingosine-1-phosphate receptors are required for myocardial migration and endoderm convergence (Kupperman et al., 2000; Ye and Lin, 2013), indicating regulation of fatty acids plays important roles during heart and gut morphogenesis. To test whether Elovl6 impacts LR asymmetry in zebrafish we first conducted gain-of-function experiments by expressing *elovl6* ubiquitously (bilaterally) in the zebrafish embryo. These injections caused severe malformations and, due to the possibility of secondary effects, it was not possible to cleanly determine effects on visceral organ asymmetry. We also took a loss-of-function approach using the CRISPR-Cas9 system (Gagnon et al., 2014), which we have used to successfully generate mutations in several zebrafish genes. However, attempts to edit the *elovl6* locus (using 5 different guide RNAs) have thus far been unsuccessful. This suggests alternative approaches (e.g. TALENs) may be needed to generate *elovl6* mutations. Thus, additional work is still needed to test whether Eovl6 plays a role in establishing LR asymmetry in vertebrate embryos.

Taken together, these results establish a new animal model of ARS, provide new insight on the conservation of Pitx2 functions in vertebrate embryogenesis and raise the possibility that previously unrecognized molecules regulate zebrafish LR asymmetry. We propose *pitx2* mutant zebrafish will be a useful model for elucidating molecular mechanisms of Pitx2 functions in embryos and adults, understanding the basis of congenital eye and tooth malformations in ARS patients and ultimately identifying new drugs to treat or prevent ARS symptoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.06.010.

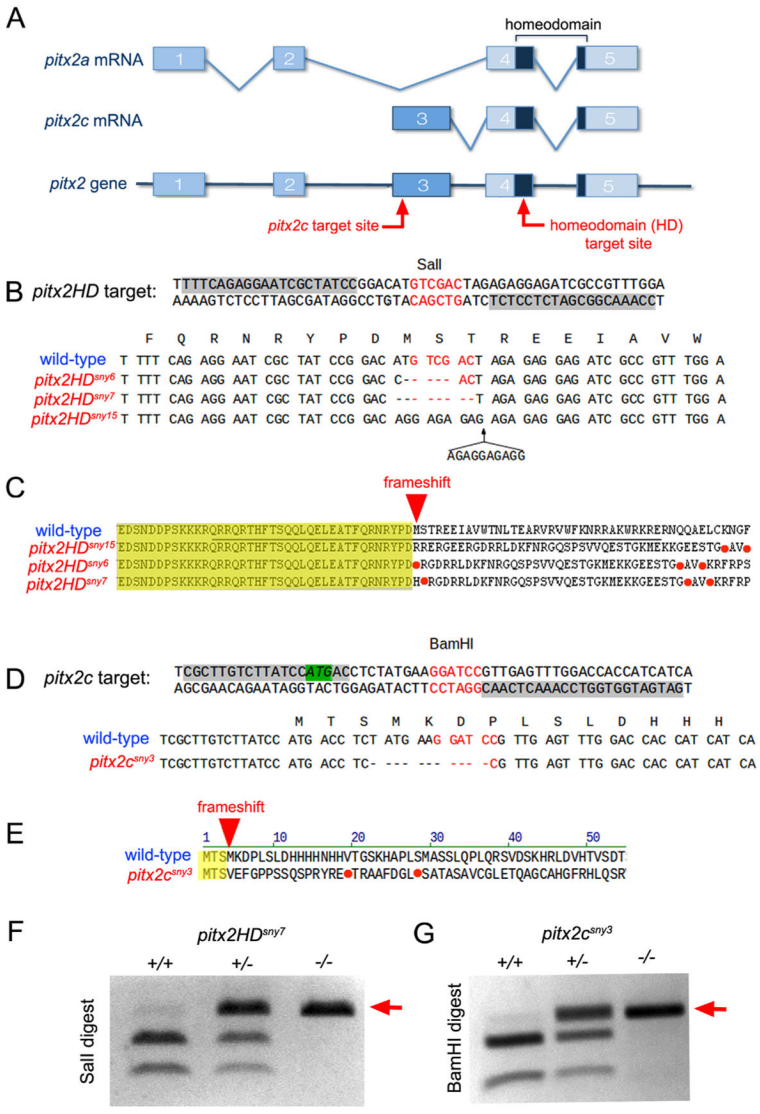


Fig. 1.

Genome editing of *pitx2* in zebrafish. (A) Intron and exon (boxes) structure of zebrafish *pitx2a* and *pitx2c* transcripts and the *pitx2* gene. The homeodomain encoded by exons 4–5 is dark blue. Red arrows indicate TALEN target sites. (B) Sequence of the *pitx2HD* target site and *pitx2HD* alleles. TALEN binding sites are highlighted gray and a *SalI* restriction site in the spacer is red. *pitx2HD* alleles are aligned with the wild-type sequence; deleted nucleotides are indicated by dashes. *pitx2HD*^{sny15} has an insertion. (C) Predicted peptide sequences for wild-type and *pitx2HD* alleles. A red arrow indicates a frameshift and a red dot indicates a premature stop codon. The HD sequence is underlined. (D) Sequence of the *pitx2c* target site and *pitx2c* allele. The *pitx2c* start codon is highlighted green and a BamHI restriction site is red. The mutant *pitx2c*^{sny3} allele is aligned with the wild-type sequence. (E) The predicted peptide sequence of the *pitx2c*^{sny3} allele revealed a frameshift after the third amino acid in Pitx2c. (F, G) Genotyping results using loss of restriction digest to identify mutant *pitx2HD* (F) or *pitx2c* (G) alleles (arrows). (For interpretation of the

references to color in this figure legend, the reader is referred to the web version of this article.)

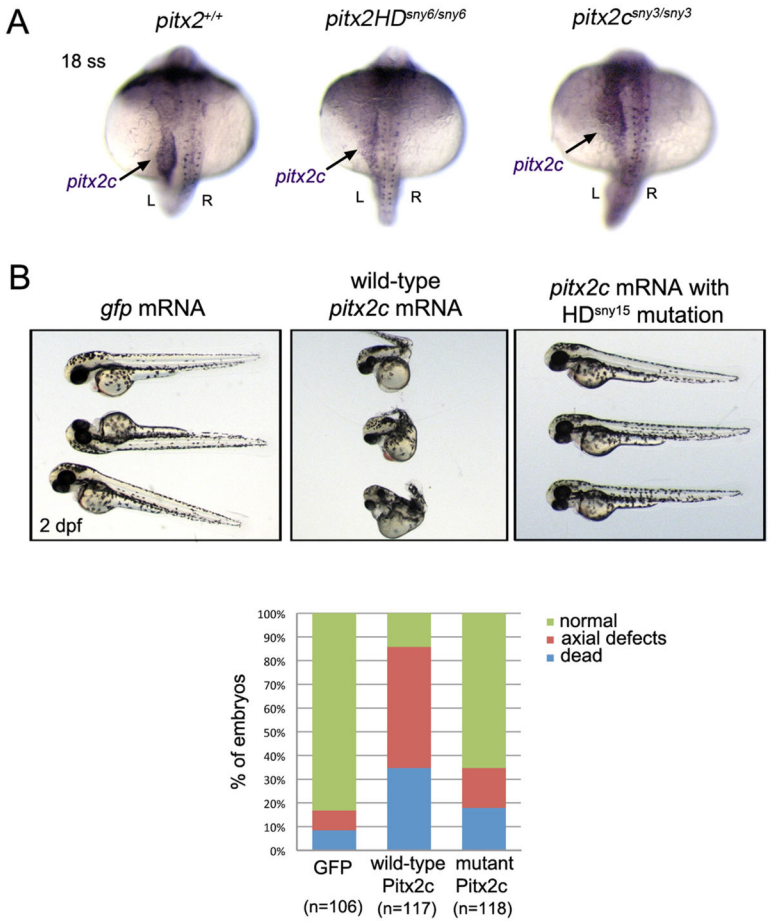


Fig. 2.

Functional analysis of mutant *pitx2HD* transcripts. (A) RNA in situ hybridizations revealed normal *pitx2* mRNA expression in wild-type, *pitx2HD*$^{-/-}$ and *pitx2c*$^{-/-}$ embryos, including asymmetric *pitx2c* in lateral plate mesoderm (arrow) at the 18 somite stage (18 ss). L=left; R=right. (B) Microinjection of wild-type *pitx2c* mRNA into wild-type embryos resulted in severe developmental defects or lethality at 2 dpf, which was not frequently observed in controls injected with *gfp* mRNA. Most embryos injected with *pitx2c* mRNA containing the HD^{sny15} mutation developed normally. n=number of embryos analyzed (pooled from three experiments).

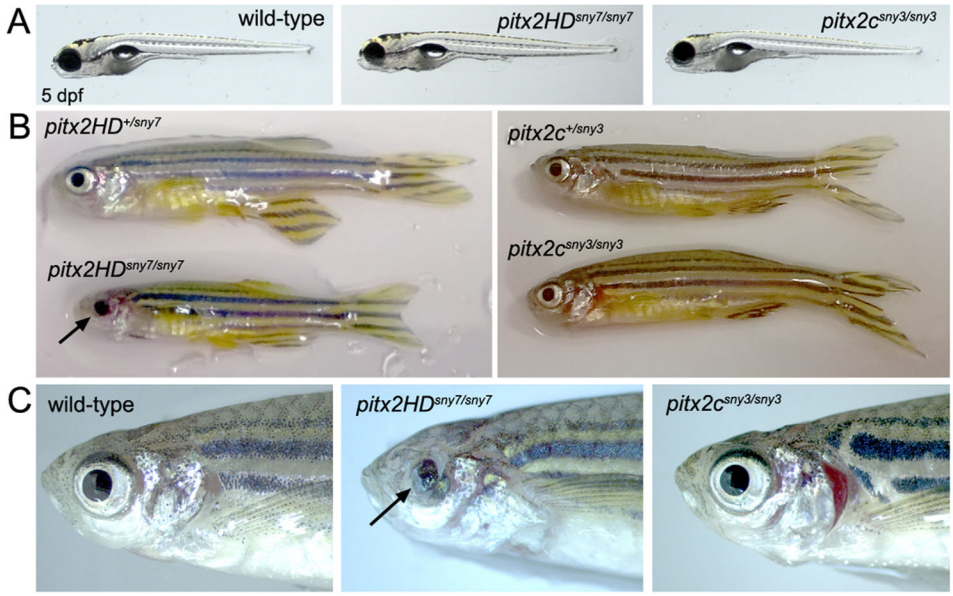


Fig. 3.

Pitx2HD mutants grow to adulthood and develop eye defects. (A) Normal gross appearance of wild-type, *pitx2HD*$^{sny7/sny7}$ and *pitx2c*$^{sny3/sny3}$ fish at 5 dpf. (B) Homozygous *pitx2HD* mutants reached adulthood, but were smaller than heterozygous siblings and had malformed eyes (arrow). Adult homozygous *pitx2c* mutants were indistinguishable from wild-type siblings. (C) Eye and craniofacial phenotypes in adult fish. Ocular defects of the iris and cornea (arrow) were observed in *pitx2HD* mutants, but not *pitx2c* mutants.

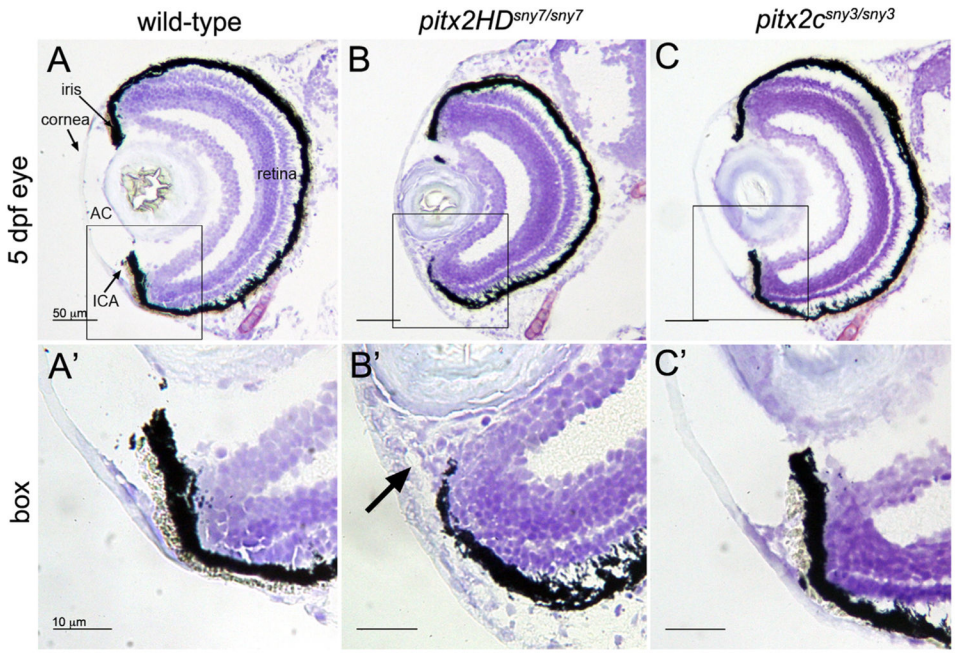
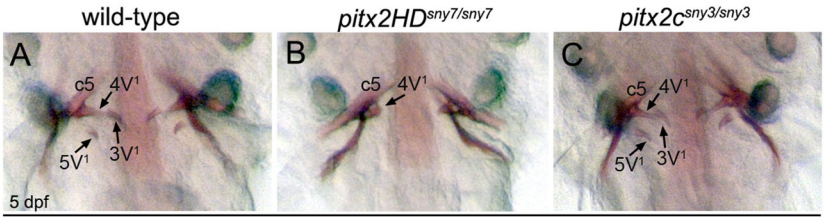


Fig. 4.

Malformations of the anterior segment of the eye in *Pitx2HD* mutant embryos. (A–C) Cryosections of the eye from 5 dpf wild-type (A), *pitx2HD*$^{sny7/sny7}$ (B) and *pitx2c*$^{sny3/sny3}$ (C) fish stained with crystal violet. AC=anterior chamber; ICA=iridocorneal angle. Scale bars=50 μm. (A′–C′) Enlarged view of box 1 in A–C showing anterior segment structures. In *pitx2HD* mutants (B′), the anterior chamber was reduced and the iridocorneal angle (arrow) was malformed. Scale bars=10 μm.



D

Stage	Allele	Genotype	# embryos with tooth malformations / total # of embryos scored
5 dpf	*pitx2HD*^{sny6}	+/+	0/12
		+/-	0/15
		-/-	2/3
	pitx2HD^{sny7}	+/+	0/14
		+/-	1/34
		-/-	16/17
	pitx2c^{sny3}	+/+	0/13
		+/-	0/40
		-/-	0/12
9 dpf	*pitx2HD*^{sny15}	+/+	1/10
		+/-	0/16
		-/-	8/8

Fig. 5.

Tooth morphogenesis is disrupted in *Pitx2HD* mutants. (A–C) Ventral views of alizarin red staining of teeth at 5 dpf in wild-type (A), *pitx2HD*$^{sny7/sny7}$ (B) and *pitx2c*$^{sny3/sny3}$ (C) fish. c5: fifth ceratobranchial; $4V^1$, $3V^1$ and $5V^1$ designate individual teeth. (D) Quantification of tooth defects observed in *pitx2HD*^{sny7} and *pitx2c*^{sny} fish at 5 dpf. Tooth malformations were defined as a reduced number of teeth and incomplete tooth formation as shown in (B).

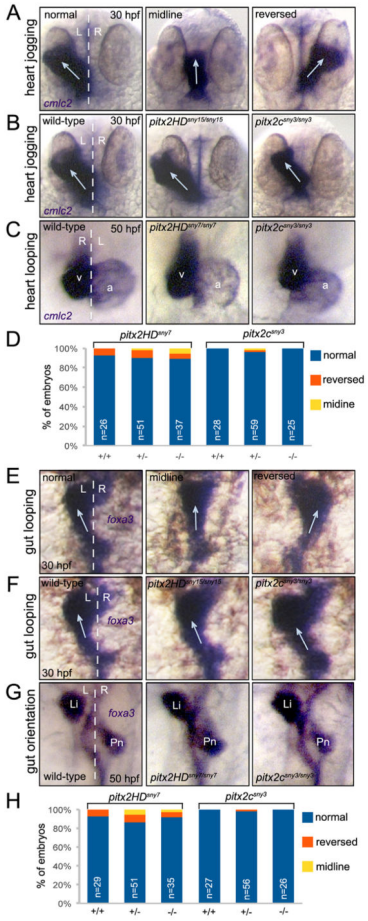


Fig. 6.

Direction of asymmetric heart and gut morphogenesis is normal in *pitx2* mutants. (A–C) RNA in situ hybridizations using *cmlc2* probes labels the developing heart tube. (A) Selected wild-type embryos that show the potential outcomes of heart jogging at 30 hpf, which include normal left-sided jogging, jogging along the midline and reversed jogging. (B) Representative images of heart jogging in wild-type and *pitx2HD* and *pitx2c* mutant embryos. Arrows indicate direction of heart jogging. (C) Normal asymmetric heart looping in wild-type and *pitx2HD* and *pitx2c* mutant embryos placed the ventricle (v) to the right of the atrium (a) at 50 hpf. (D) Heart looping direction was scored as either normal, reversed or along the midline for individual embryos at 50 hpf and the genotype (+/+, +/− or −/−) was then determined for each embryo. (E–G) *foxa3* in situ hybridizations marked the developing gut tube. (E) Potential outcomes of gut looping at 30 hpf include normal left-sided looping, remaining at the midline and reversed looping. (F) Direction of gut looping was similar in wild-type and *pitx2HD* and *pitx2c* mutant embryos. Arrows indicate direction of gut looping. (G) Normal asymmetric placement of the liver (Li) and pancreas (Pn) at 50 hpf. (H) Gut asymmetry was scored at 50 hpf and then each embryo was genotyped. No statistical differences in heart looping (D) or gut asymmetry (H) were identified using single factor one way ANOVA. White dashed lines represent the midline; L=left; R=right; n=number of embryos analyzed.

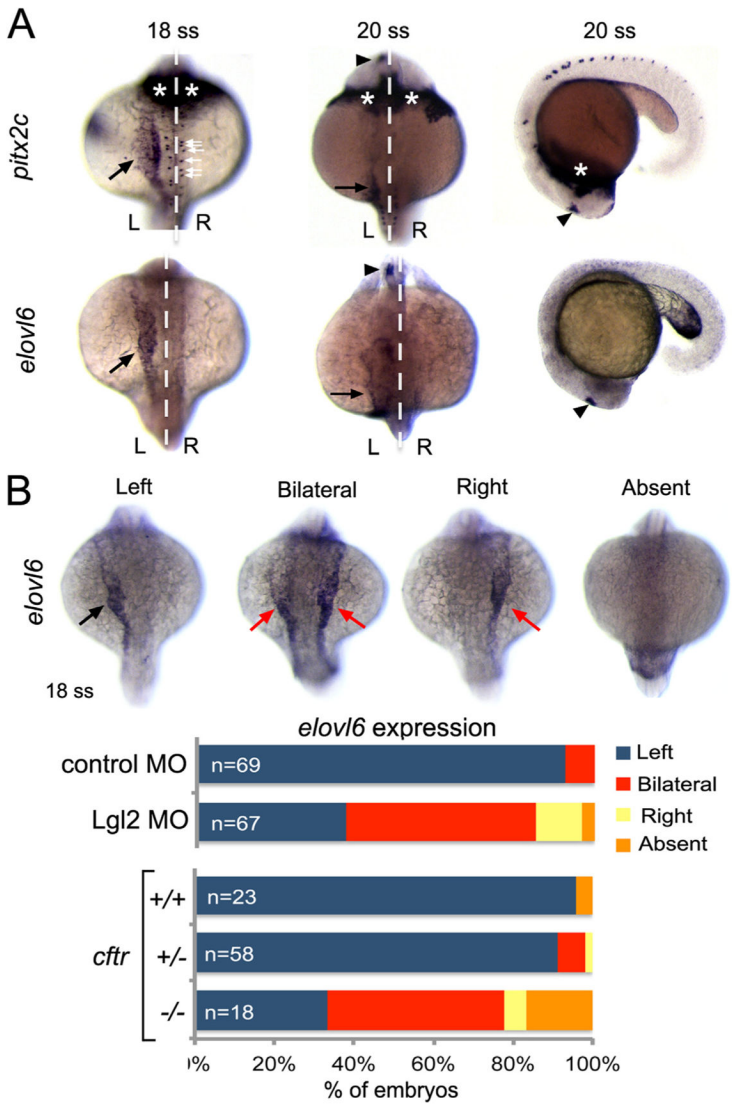


Fig. 7.

Asymmetric expression of *elovl6* depends on Kupffer's vesicle. (A) RNA in situ hybridizations revealed similar asymmetric expression patterns for *pitx2c* and *evolv6* in left lateral plate mesoderm (black arrows) and brain (arrowheads) at 18–20 somite stages (ss). *pitx2c* mRNA was detected in hatching gland (asterisks) and neurons (white arrows), whereas *elovl6* was not expressed in these domains. White dashed line is the midline; L=left and R=right. (B) Representative images are shown of left-sided, bilateral, right-sided and absent *elovl6* expression in lateral plate mesoderm (arrows) at 18 somite stage. The graph shows quantification of asymmetric *elovl6* expression profiles in control, Lgl2 depleted and genotyped *cftr*^{pd1048} mutant embryos.

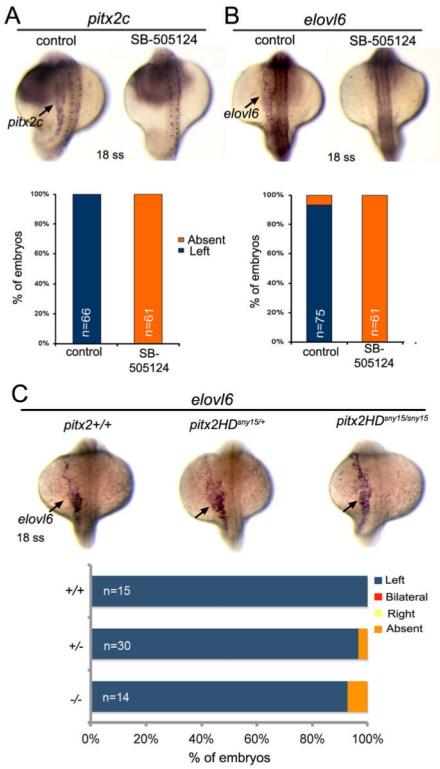


Fig. 8.

Asymmetric expression of *elovl6* requires Nodal signaling but does not depend on Pitx2. (A, B) Analysis of asymmetric *pitx2c* (A) or *elovl6* (B) expression (arrows) at the 18 somite stage (ss) in control embryos and embryos treated with SB-505124 to block Nodal signaling. The graphs indicate the percentage of embryos with normal left-sided expression or absent expression. (C) Asymmetric *elovl6* expression (arrows) in wild-type and *pitx2HD* mutant embryos. n=number of embryos analyzed.