Research article

Dev Reprod 2024;28(2):55-65
https://doi.org/10.12717/DR.2024.28.2.55

Development & Reproduction

ISSN 2465-9525 (Print)
ISSN 2465-9541 (Online)

A Potential Role of *fgf4*, *fgf24*, and *fgf17* in Pharyngeal Pouch Formation in Zebrafish

Sil Jin[1], †Chong Pyo Choe[2,3]

[1]*Division of Applied Life Science, Gyeongsang National University, Jinju 52828, Korea*
[2]*Division of Life Science, Gyeongsang National University, Jinju 52828, Korea*
[3]*Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 52828, Korea*

Received: March 19, 2024
Revised: April 25, 2024
Accepted: May 15, 2024

†**Corresponding author**
Chong Pyo Choe
Division of Life Science, Gyeongsang National University, Jinju 52828, Korea
Tel: +82-55-772-1367
Fax: +82-55-772-1349
E-mail: cpchoe@gnu.ac.kr

Copyright © 2024 The Korean Society of Developmental Biology.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

ORCID
Sil Jin
https://orcid.org/0000-0003-1177-299X
Chong Pyo Choe
https://orcid.org/0000-0003-0254-9438

Conflict of interests
The authors declare no potential conflict of interest.

Acknowledgements
This work was supported by a grant from Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (2022R1F1A1060199).

Authors' contributions
Conceptualization: Choe CP.
Data curation: Jin S, Choe CP.
Validation: Choe CP.
Writing-original draft: Jin S.
Writing-review & editing: Jin S, Choe CP.

Ethics approval
Animal care and experimental procedures

Abstract
In vertebrates, Fgf signaling is essential for the development of pharyngeal pouches, which controls facial skeletal development. Genetically, *fgf3* and *fgf8* are required for pouch formation in mice and zebrafish. However, loss-of-function phenotypes of *fgf3* and *fgf8* are milder than expected in mice and zebrafish, which suggests that an additional *fgf* gene(s) would be involved in pouch formation. Here, we analyzed the expression, regulation, and function of three *fgfs*, *fgf4*, *fgf24*, and *fgf17*, during pouch development in zebrafish. We find that they are expressed in the distinct regions of pharyngeal endoderm in pouch formation, with *fgf4* and *fgf17* also being expressed in the adjacent mesoderm, in addition to previously reported endodermal *fgf3* and mesodermal *fgf8* expression. The endodermal expression of *fgf4*, *fgf24*, and *fgf17* and the mesodermal expression of *fgf4* and *fgf17* are positively regulated by Tbx1 but not by Fgf3, in pouch formation. Fgf8 is required to express the endodermal expression of *fgf4* and *fgf24*. Interestingly, however, single mutant, all double mutant combinations, and triple mutant for *fgf4*, *fgf24*, and *fgf17* do not show any defects in pouches and facial skeletons. Considering a high degree of genetic redundancy in the Fgf signaling components in craniofacial development in zebrafish, our result suggests that *fgf4*, *fgf24*, and *fgf17* have a potential role for pouch formation, with a redundancy with other *fgf* gene(s).
Keywords: Fgf signaling, Pharyngeal pouch, Facial cartilages, Genetic redundancy, Zebrafish

INTRODUCTION

In vertebrate embryonic head, a series of pouches arises in the pharyngeal endoderm. The pharyngeal pouches are important for craniofacial development. First, they are required for facial skeletons by segmenting the pharyngeal arches and providing survival signals, such as sonic Hedgehog, to the arches (Piotrowski & Nüsslein-Volhard, 2000; Brito et al., 2006). Then, they further differentiate into endocrine glands in the head and neck, including the thymus and parathyroid (Grevellec & Tucker, 2010). In mice and zebrafish, abnormal development of pouches resulted in loss or malformation of facial cartilages and hypoplasia of the thymus and parathyroid (Piotrowski & Nüsslein-Volhard, 2000; Dickmeis et al., 2001; Kikuchi et al., 2001; Grevellec & Tucker, 2010). In humans, defects in pouches are associated with DiGeorge syndrome, a congenital craniofacial disability, with *TBX1* being identified as a key gene

were approved by Gyeongsang National University Institutional Animal Care and Use Committee in accordance with guidelines established by the Korea Food and Drug Administration (GNU-201012-E0074).

in the etiology of the syndrome (Piotrowski & Nüsslein-Volhard, 2000; Lindsay et al., 2001; Tran et al., 2011). Recently, the genetic and cellular basis underlying pouch formation has been revealed in vertebrates. Loss of Tbx1, Pax1, and Foxi1 transcription factors and Fgf ligands led to loss or malformation of pouches and facial skeletons in mice and zebrafish (Lindsay et al., 2001; Nissen et al., 2003; Piotrowski et al., 2003; Tran et al., 2011; Okada et al., 2016; Jin et al., 2018; Liu et al., 2020). In zebrafish, Wnt, Ephrin, and VEGF-C signaling molecules are also required for pouch formation, with Neuropilin signaling being implied in pouch formation (Crump et al., 2004; Ober et al., 2004; Choe et al., 2013; Choe & Crump, 2014; Choe, 2023). To control pouch formation, Pax1 regulates the expression of *tbx1* and *fgf3* in the pharyngeal endoderm in medaka and zebrafish, with Foxi1 regulating *wnt4a* expression in zebrafish (Okada et al., 2016; Jin et al., 2018; Liu et al., 2020). At the cellular level, Tbx1 promotes a collective migration of pouch-forming cells through Wnt11r and Fgf8a, and then the migrating pouch-forming cells are matured into a bi-layered pouch by Wnt4a and Ephrin B2 and B3 ligands in zebrafish (Choe et al., 2013; Choe & Crump, 2014). Besides the roles of these transcription factors and signaling molecules, a lot of genetic and cellular mechanisms of pouch formation have not been uncovered yet. To better understand the genetic basis of pouch formation, here we analyzed the role of Fgf signaling in developing pouches in zebrafish.

In mice, Fgf signaling is implicated in pouch formation. Loss of *Fgf3*, *Fgf8*, *Fgf receptor* (*Fgfr*) *1*, or *2* resulted in abnormal pouches (Aggarwal et al., 2006; Jackson et al., 2014). For pouch formation, *Fgf3* and *Fgf8* were coexpressed in different combinations with *Tbx1* and were strongly downregulated in *Tbx1* mutant embryos (Aggarwal et al., 2006). Interestingly, pharyngeal endoderm-specific depletion of *Fgfr1* and *Fgfr2* displayed more severe pouch defects than mutants for *Fgf3* or *Fgf8* did, implying that additional *Fgf* genes might be involved in pouch formation (Jackson et al., 2014). Furthermore, epistatic analysis of *Tbx1*, *Fgf3*, and *Fgf8* genes suggested potential functional redundancy with additional Fgfs in the development of the pharyngeal pouches via Tbx1 (Aggarwal et al., 2006). Indeed, *Fgf4* and *Fgf16* are also expressed in pharyngeal endoderm, with their roles in pouch formation not being determined (Niswander & Martin, 1992; Wright et al., 2003). In zebrafish, redundant essential functions of *fgf3* and *fgf8a* are also implicated in pouch formation; knockdown of *fgf3* in *fgf8a* mutants caused more severe defects in pouch formation than *fgf8a* mutants did (Crump et al., 2004). Also, it was suggested that *fgfr1a*, *fgfr1b*, and *fgfr2* acted redundantly to regulate pouch development (Leerberg et al., 2019). A high degree of redundancy in Fgf signaling implicated in pouch formation suggests additional Fgf genes in the development of the pharyngeal apparatus in mice and zebrafish. However, besides *fgf3* and *fgf8a*, the requirement for other *fgf* genes in pouch formation has not yet been analyzed in zebrafish.

In order to identify additional *fgf* genes acting in pouch formation in zebrafish, we looked at individual *fgf* genes that are expressed in the pharyngeal endoderm. We found that three *fgf* genes, *fgf4*, *fgf24*, and *fgf17*, were expressed in distinct regions of the pharyngeal endoderm in the morphogenesis of pouches. Furthermore, we observed that they acted downstream of Tbx1 or Fgf8a in pouch formation; their expression was strongly downregulated in *tbx1* or *fgf8a* mutant embryos, with their expression being barely affected in *fgf3* mutants. However, single, all double mutant combinations, and triple mutant for *fgf4*, *fgf24*, and *fgf17* showed normal pouches and facial skeletons. Our results suggest that *fgf4*, *fgf24*, and *fgf17* could be involved in pouch formation, with a high degree of redundancy.

MATERIALS AND METHODS

1. Zebrafish lines

Zebrafish were grown according to the Animal Protection Act (2017) in Korea. All zebrafish

https://doi.org/10.12717/DR.2024.28.2.55

work was approved by Gyeongsang National University Institutional Animal Care and Use Committee. Published lines include *tbx1*^{tu285} (Piotrowski et al., 2003), *fgf3*^{t24152} (Herzog et al., 2004), *fgf8*^{ati282a} (Reifers et al., 1998), *fgf24*^{tm127c} (van Eeden et al., 1996), *Tg(~3.4her5:EGFP)* (Tallafuss & Bally-Cuif, 2003), and *Tg(nkx2.3:Gal4VP16)* (Choe et al., 2013). *fgf4* and *fgf17* mutant lines were generated with CRISPR/Cas9 system (Hwang et al., 2013). 150 pg of gRNA and 150 pg of mRNA encoding a nuclear-localized Cas9 were injected together into one-cell stage wild-type Tübingen (TU) embryos harboring *Tg(~3.4her5:EGFP)*. Injected embryos were grown and outbred to wild-type TU animals to identify zebrafish bearing in/del mutations in *fgf4* and *fgf17* genes. Three mutant lines for *fgf4* (*fgf4*^{GNU6}, *fgf4*^{GNU8}, and *fgf4*^{GNU9}) and three for *fgf17* (*fgf17*^{GNU1}, *fgf17*^{GNU4}, and *fgf17*^{GNU5}) were secured. *fgf4*^{GNU8} and *fgf17*^{GNU1} were used for this study. For genotyping of *fgf4*^{GNU8}, a PCR amplified *fgf4* fragment with primers *fgf4*_GT_ F and *fgf4*_GT_R, was digested with AccI, with a wild-type fragment producing 358 bp and mutant fragment generating 176 and 178 bp. For genotyping of *fgf17*^{GNU1}, primers *fgf17*_GT_ F and *fgf17*_ GT_R converted *fgf17*^{GNU1} into a codominant polymorphism, with a wild-type product of 147, 108 and 90 bp and mutant products of 245 and 90 bp after TauI digestion. *Tg(UAS:DN-Fgfr1)* transgenic construct was generated using the Gateway (Invitrogen, Carlsbad, CA, USA) Tol2kit (Kwan et al., 2007). For pME-DN-Fgfr1, a dominant-negative form of Fgfr1 that is missing its intracellular kinase domain was produced by PCR using primers DN-Fgfr1-B1F and DN-Fgfr1-B2R from multi-stage zebrafish cDNA (Fürthauer et al., 2004). 30 ng of UAS:DN-Fgfr1 transgenic construct and 35 ng of DNA construct bearing Tol2 transposase gene were injected into one-cell stage wild-type TU embryos. Five independent transgenic lines for *Tg(UAS:DN-Fgfr1:pA)* were established based on *α -crystallin*:Cerulean eye fluorescence. Primers are listed in Table 1.

2. Staining

Immunohistochemistry for Alcama (Zebrafish International Resource Center, Eugene, OR, USA, 1:400), fluorescent in situ hybridization in conjunction with GFP immunohistochemistry (Torrey Pines Biolabs, 1:1,000), and Alcian blue and alizarin red staining were performed as described previously (Crump et al., 2004; Zuniga et al., 2011). Partial cDNA fragments of *fgf4*, *fgf24*, and *fgf17* were amplified from mixed-stage zebrafish cDNA. The PCR fragments were cloned into the pGEM®-T easy vector (Promega, Madison, WI, USA), and Digoxigenin (DIG)-labeled antisense riboprobes were transcribed with T7/SP6 RNA polymerase (Roche Life Sciences, Basel, Switzerland) from linearized plasmids. Primers used for riboprobes are listed in Table 2.

3. Imaging

Fluorescent images for immunohistochemistry and in situ hybridization were captured with an Olympus FLUOVIEW FV3000 confocal microscope using FV31S-SW software

Table 1. List of primers used for genotyping and to create transgenic lines

Primer	Sequences (5' to 3')
*fgf4*_GT_ F	TGTTTTGTGCGTCTTTTTGG
*fgf4*_GT_R	TGTGTACGCCGGTGATTTTA
*fgf17*_GT_ F	AGCAGGAGGGCGAATAATTT
*fgf17*_GT_ R	TCAATGACGCAATCAATCATC
DN-Fgfr1-B1F	GGGGACAAGTTTGTACAAAAAAGCAGGCT CCACCATGATAATGAAGACCACGCTG
DN-Fgfr1-B2R	GGGGACCACTTTGTACAAGAAAGCTGGGT CTAAGAGCTGTGCATTTTGGC

Table 2. List of primers used to create in situ probes

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
fgf4	TTGCCAATCCTGGTCTTA	GAAAGTGCATCGTCGTTC
fgf24	TTATATCGCGTGCTGGAT	CCACAAGCAGAGCGTACT
fgf17	GAGAACGCCAGACATGAG	AGGCGTTGAAGATGAACA

(Olympus, Tokyo, Japan). We took approximately 100 μm Z-stacks at 3.5-μm intervals with an Olympus UPLXAPO 20× objective lens and projected them into a single image. Flat-mounted facial cartilages and bones dissected manually from Alcian Blue and alizarin red staining larvae were imaged with an Olympus BX50 upright microscope using mosaic V2.1 software (Tucsen Photonics, Fuzhou, China). Any adjustments were applied to all panels using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

RESULTS

1. Fgf signaling is required in the pharyngeal endoderm to develop all pharyngeal pouches

In wild type, a total of six pouches form at 34 hours post-fertilization (hpf), with the sixth pouch hardly seen (Fig. 1A). Although *fgf3* and *fgf8a* are essential for pouch formation, the loss of *fgf3* and *fgf8a* resulted in mild defects in pouches in that the anterior two pouches were less affected, with the posterior four pouches being malformed or absent (Fig. 1B and C). Consistently, the development of hyomandibular (hm) cartilage that is controlled by the first pouch was less affected, whereas that of ceratobranchial (cb) cartilage that is regulated by the posterior pouches

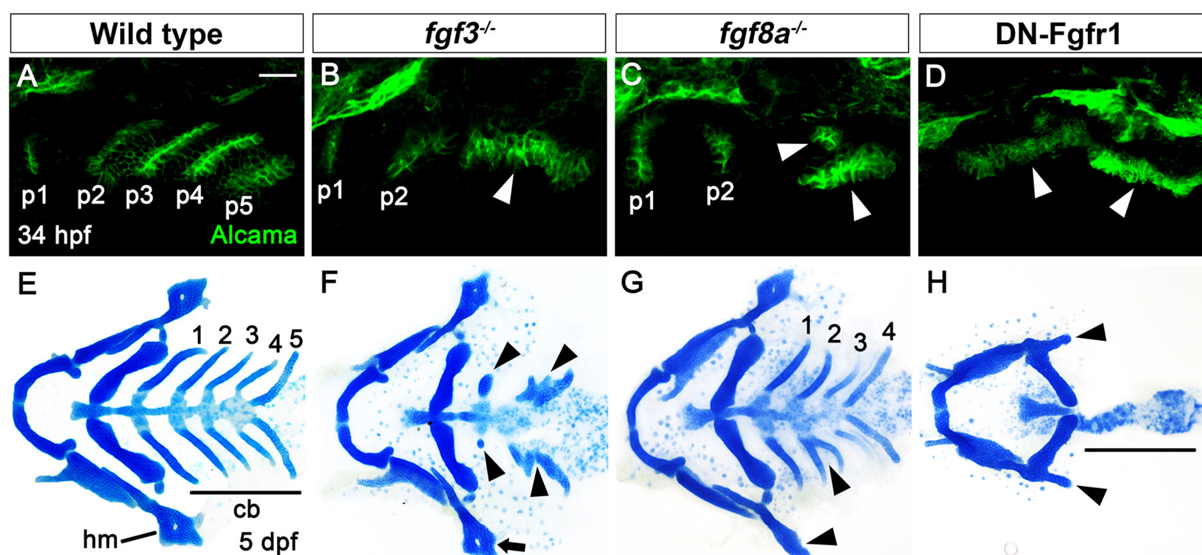

Fig. 1. Essential role of Fgf signaling to develop all pharyngeal pouches. (A–D) Alcama staining labels to visualize pouches. Pouches are numbered, with a malformed pouch or pharyngeal endoderm that does not form pouches being marked with arrowheads. Five bi-layered pouches are seen in wild types at 34 hpf (A, n=41), whereas two pouches are observed in the single mutants for *fgf3* (B, n=26) and *fgf8a* (C, n=23), with other pouches being malformed or not formed. Overexpression of DN-Fgfr1 in *nkx2.3*-positive endoderm shows a loss of all pouches (D, n=39). (E–H) Ventral of dissected facial cartilages. Normal ceratobranchial (cb) cartilages are numbered, with malformed hyomandibular (hm) and cb cartilages being marked with arrowheads. While the wild type displays the bilateral set of five cb cartilages and triangular hm (E, n=45), the *fgf3* mutant shows missing, malformed, or fused cb cartilages (F, n=19), Normal hm is marked with an arrow. In the *fgf8a* mutant, cb cartilages are missing or fused, and hm is also defective (G, n=25). Overexpression of DN-Fgfr1 in *nkx2.3*-positive endoderm shows complete loss of cb cartilages (underlined in H, n=34) and defects in hm (H, n=34). Scale bar: 40 μm. n, numbers of animals analyzed. Anterior is to the left.

https://doi.org/10.12717/DR.2024.28.2.55

was severely defective in single mutant for *fgf3* and *fgf8a* at 5 days post-fertilization (dpf) (Fig. 1E–G). If Fgf signaling is critical for the development of all six pouches, the absence of Fgf signaling in the pharyngeal endoderm would be able to cause defects in all pouches, and hm and cb cartilages whose development relied on all six pouches (Crump et al., 2004). We utilized a dominant-negative approach to test this hypothesis. To do so, we generated *Tg(UAS:DN-Fgfr1a)* lines harboring a dominant-negative mutant allele of *fgfr1a*. Utilizing the pharyngeal endoderm driver *Tg(nkx2.3:Gal4VP16)* lines that are able to express target genes in pouch-forming endoderm, including the anterior pouches (Choe et al., 2013), we expressed DN-Fgfr1a specifically in the endoderm during pouch formation. Pharyngeal endoderm-specific expression of the DN-Fgfr1a blocking Fgf signaling in the endoderm resulted in the absence of all pouches at 34 hpf as well as defects in hm and cb cartilages at 5 dpf (Fig. 1D and H). This result suggests that Fgf signaling, which is required in the endoderm, is essential for the formation of all pouches.

2. Zebrafish *fgf4*, *fgf24*, and *fgf17* are expressed in pharyngeal endoderm forming pharyngeal pouches

The phenotypic difference in pouches among *fgf3* mutant, *fgf8a* mutant, and endoderm-specific block of Fgf signaling suggests that additional *fgf* genes might be necessary for the development of pouches and associated facial cartilages, in addition to *fgf3* and *fgf8a*. To identify additional *fgf* genes necessary for pouch formation, we first examined the expression of *fgf* genes in the pharyngeal endoderm at 30 hpf, in which four pouches form and the fifth pouch is just being established. To do so, we performed in situ hybridization for candidate *fgf* genes in wild-type embryos harboring *Tg(her5:GFP)* transgene, a pharyngeal endoderm and pouches reporter (Tallafuss & Bally-Cuif, 2003). Consequently, we found that *fgf4*, *fgf24*, and *fgf17* were expressed in the distinct regions of pharyngeal pouches at 30 hpf. *fgf4* was expressed locally in the basal region of the fourth pouch, with its expression not seen in the anterior three pouches and the forming fifth pouch (arrowheads in Fig. 2A and A'). It was also expressed in the adjacent mesoderm to the fourth pouch (asterisks in Fig. 2A and A'). Transcripts for *fgf24* were observed in the second to fourth pouches, with those in

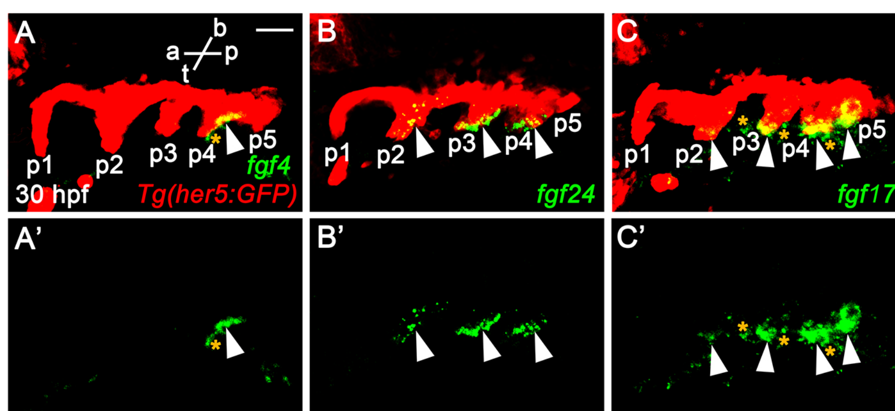

Fig. 2. Expression of *fgf4*, *fgf24*, and *fgf17* in pharyngeal endoderm and pouches. (A–C) Fluorescence in situ hybridization of *fgf* genes (green) in conjunction with immunohisto-chemistry of GFP (red) in wild-type *Tg(her5:GFP)* reporter lines marking the pharyngeal endoderm and pouches. Pouches are numbered. (A) Arrowhead indicates *fgf4* expression observed in the basal region of the fourth (p4) pouch at 30 hpf. Mesodermal expression of *fgf4* in the adjacent region to the fourth pouch is marked with an asterisk. (B) *fgf24* expression in pouches at 30 hpf is marked with arrowheads. (C) Arrowheads indicate *fgf17* expression in pouches at 30 hpf, with asterisks marking mesodermal expression of *fgf17*. (A'–C') Green channel only. Scale bar: 40 μm. a, anterior; p, posterior; b, basal; t, tip.

the second pouch being faded compared to those in the third and fourth pouches (arrowheads in Fig. 2B and B'). They were also restricted in the posterior layer of the bi-layered pouches. However, they were not seen in the first and forming fifth pouches (Fig. 2B and B'). Apparently, *fgf24* was not expressed in the adjacent mesoderm to the pharyngeal endoderm at 30 hpf. Next, we found that *fgf17* was expressed at the tip of the second to fifth pouches, with its expression being faded in the second pouch at 30 hpf (arrowheads in Fig. 2C and C'). At the tip of pouches, *fgf17* was expressed in both anterior and posterior layers. It was also expressed in the mesoderm adjacent to the pharyngeal endoderm (asterisks in Fig. 2C and C'). Expression of *fgf4*, *fgf24*, and *fgf17* in the distinct regions of pharyngeal pouches suggests that they could play a role in pouch formation during craniofacial development.

3. Endodermal expression of *fgf4*, *fgf24*, and *fgf17* requires Tbx1 or Fgf8a in pouch formation

Expression of *fgf4*, *fgf24*, and *fgf17* in developing pouches at 30 hpf implies their potential roles in pouch formation and facial cartilage development. Since *tbx1*, *fgf3*, and *fgf8a* are key genes controlling pouch formation, we next analyzed whether the three *fgf* genes acted downstream of the key genes for pouch formation. Compared to the wild type, the endodermal expression of *fgf4*, *fgf24*, and *fgf17* in the pouches was significantly reduced in *tbx1* mutants in which most pouches did not form (Fig. 3A, B, E, F, I, and J). The mesodermal expression of *fgf4* and *fgf17* also being abolished in *tbx1* mutants at 30 hpf (Fig. 3B and J). Since Tbx1 acts in both pharyngeal endoderm and adjacent mesoderm for pouch formation (Choe & Crump, 2014), the endodermal

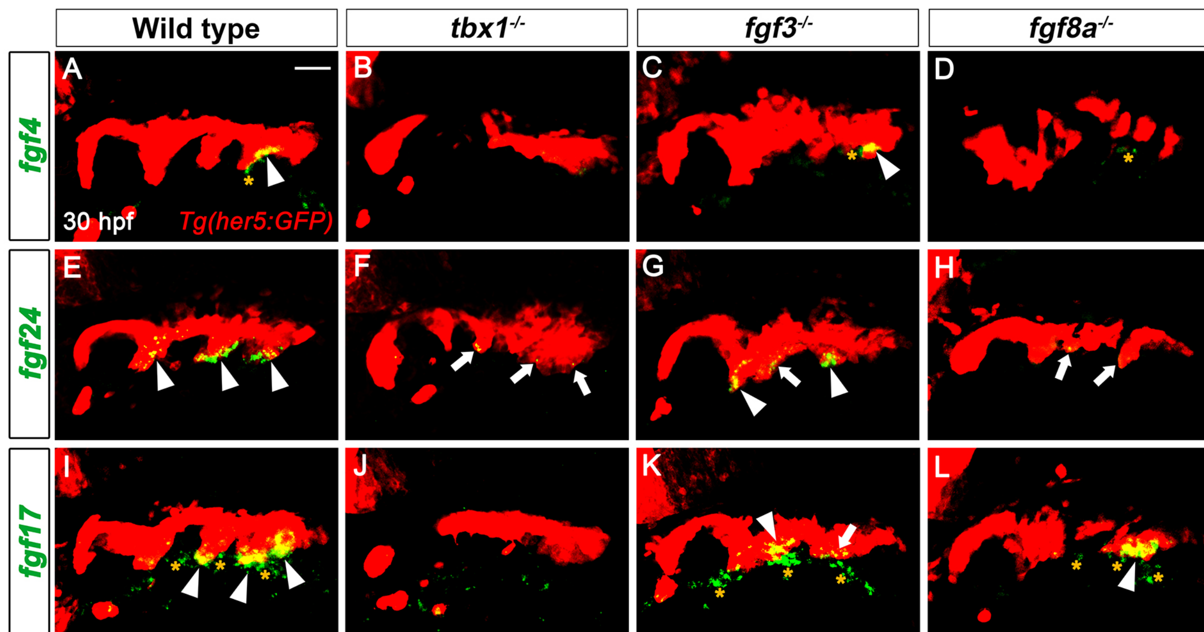

Fig. 3. Regulation of *fgf4*, *fgf24*, and *fgf17* expression by Tbx1 or Fgf8a in pouch formation. (A–L) Fluorescence in situ hybridization of *fgf* genes (green) in conjunction with immunohisto-chemistry of GFP (red) in wild type or mutants bearing *Tg(her5:GFP)* transgene. (A–D) While arrowhead indicates *fgf4* expression in the *her5*+ endoderm in wild type (A, n=23) and *fgf3* mutant (C, n=21), *fgf4* expression in the *her5*+ endoderm in single mutants for *tbx1* (B, n=15) and *fgf8a* (D, n=19) is not seen. Mesodermal expression of *fgf4* in wild type (A) and *fgf8a* mutant (D) is marked with an asterisk. (E–H) Arrowheads indicate *fgf17* expression in the *her5*+ endoderm in wild type (E, n=27) and *fgf3* mutant (G, n=25), with arrows marking significantly reduced *fgf17* expression in the *her5*+ endoderm in single mutants for *tbx1* (F, n=20), *fgf3* (G, n=25), and *fgf8a* (H, n=16). (I–L) Endodermal expression of *fgf17* seen in wild type (I, n=14), *fgf3* mutant (K, n=22), and *fgf8a* mutant (L, n=29) is marked with arrowheads, with the reduced expression in *fgf3* mutant being indicated with an arrow (K, n=22). (I, K, L) Asterisks mark the mesodermal expression of *fgf17*, with the mesodermal expression in *fgf8a* mutant being reduced. (J, n=19) Both endodermal and mesodermal expression of *fgf17* is barely seen in *tbx1* mutant. Scale bar: 40 μm. n, numbers of animals analyzed. Anterior is to the left.

https://doi.org/10.12717/DR.2024.28.2.55

and mesodermal expression of the three *fgf* genes require Tbx1 activity. In contrast to Tbx1, Fgf3 appears to be dispensable for the expression of the three *fgf* genes in the pharyngeal regions at 30 hpf. In *fgf3* mutants where only the anterior one or two pouches form, *fgf4* expression in the endoderm was normal (arrowhead in Fig. 3C). Although the endodermal expression of *fgf24* and *fgf17* was slightly reduced in some potential pouch regions (arrows in Fig. 3G and K), their expression was fairly normal in the endoderm of *fgf3* mutants (arrowheads in Fig. 3G and K). Also, the mesodermal expression of *fgf4* and *fgf17* was seen in *fgf3* mutants at 30 hpf (asterisks in Fig. 3C and K). The regulation of *fgf4* and *fgf24* by Fgf8a was similar to that by Tbx1 in that the endodermal expression of *fgf4* and *fgf24* were abolished or significantly reduced in *fgf8a* mutants (Fig. 3D and H). However, in *fgf8a* mutants, the endodermal expression of *fgf17* was not affected (Fig. 3L). In *fgf8a* mutants, the mesodermal expression of *fgf4* was unaffected, whereas that of *fgf17* was reduced (Fig. 3D and L). Thus, in the pharyngeal regions, the genetic regulation of the three *fgf* genes by Fgf8a is not necessarily identical to that by Tbx1. Misregulation of *fgf4*, *fgf24*, and *fgf17* in single mutants for *tbx1* and *fgf8a* implies that Tbx1 and Fgf8a could control pouch and facial cartilage formation through these *fgf* genes during craniofacial development.

4. Generation of loss-of-function mutations in *fgf4* and *fgf17* genes

If *fgf4*, *fgf24*, and *fgf17* are required for the development of pouches and associated facial cartilages as downstream targets of Tbx1 and Fgf8a, loss-of-function mutations in these *fgf* genes would result in defects in pouches and facial cartilages. In order to access a role for *fgf4*, *fgf24*, and *fgf17* in the development of pouches, we generated loss-of-function mutations in *fgf4* and *fgf17* genes with CRISPR/Cas9 system and secured a *fgf24* mutant available in the zebrafish community (van Eeden et al., 1996). For *fgf4* mutants, we established a four-nucleotide deletion allele in the *fgf4* gene (*fgf4^{GNU8}*), compared to wild-type allele (Fig. 4A). While wild-type allele produces 191 amino acids including 20 amino acids of a signal peptide, the mutant allele was predicted to produce only 34 normal amino acids including 20 amino acids of a signal peptide and an extra 9 amino acids (Fig. 4A). Thus, *fgf4^{GNU8}* seems to be a loss-of-function allele. For *fgf17* mutants, we secured a ten-nucleotide deletion allele in the *fgf17* gene (*fgf17^{GNU1}*), which was predicted to produce 48 normal

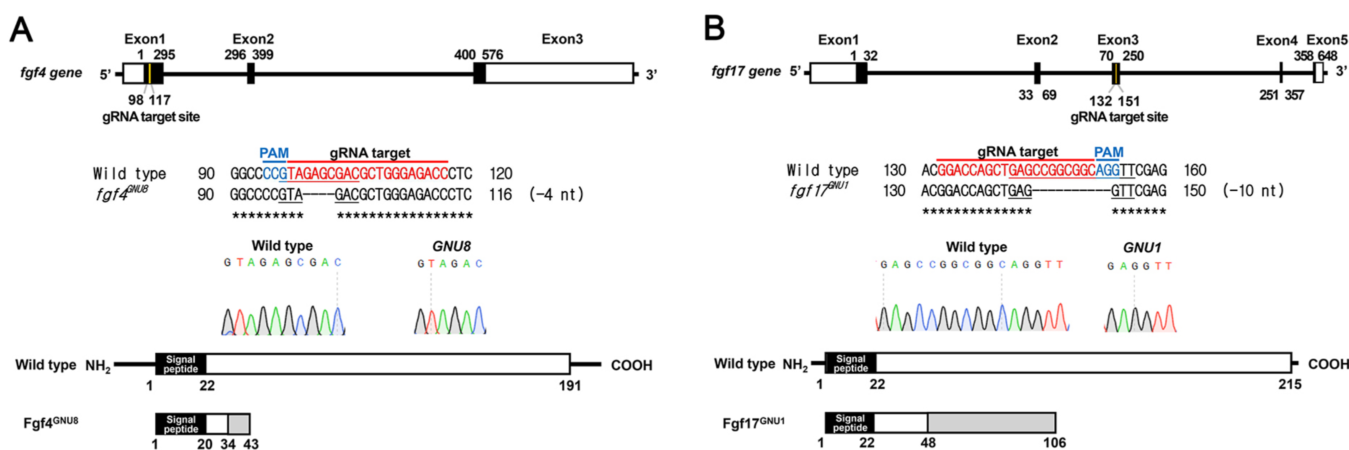

Fig. 4. Generation of loss-of-function mutations in *fgf4* and *fgf17* genes. (A, B) *fgf4* and *fgf17* genes consist of three and five exons, respectively, bearing sequences for the protein-coding region (black box) and the 5' and 3' untranslated regions (open box). The gRNA target site is marked in yellow. The deletion mutation of each mutant allele is shown in the multiple sequence alignments, with the gRNA target and the PAM sites being marked in red and blue, respectively, in the wild-type sequence. The electrophoretograms show the lesion in each mutant allele that is underlined in the multiple sequence alignments. Schematics of the Fgf4 and Fgf17 proteins encoded by the wild-type and mutant alleles show early truncation in the mutant Fgf4 and Fgf17 proteins (open box), with an extra non-specific region (grey box). The signal peptide is marked with a black box.

amino acids and an extra 58 amino acids that were completely different from those of wild-type Fgf17 (Fig. 4B). Thus, it is likely that *fgf17*^{GNU1} is also a loss-of-function allele.

5. Loss of *fgf4*, *fgf24*, and *fgf17* does not influence the development of pouches and facial cartilages

Expression of *fgf4*, *fgf24*, and *fgf17* in the distinct regions of pouches may suggest distinct roles for these genes in pouch formation. However, pouch formation was normal in single mutants for *fgf4*, *fgf24*, and *fgf17* at 34 hpf, with the pouches being almost indistinguishable from those in wild types (Fig. 5A–D). Consistently, the development of hm and cb cartilages whose development relied on pouches was also normal in single mutants for *fgf4*, *fgf24*, and *fgf17* at 5 dpf (Fig. 5I–L). To analyze a functional redundancy among the *fgf* genes in pouch formation, we examined all double mutant combinations for the *fgf* genes. However, pouches and facial cartilages, including

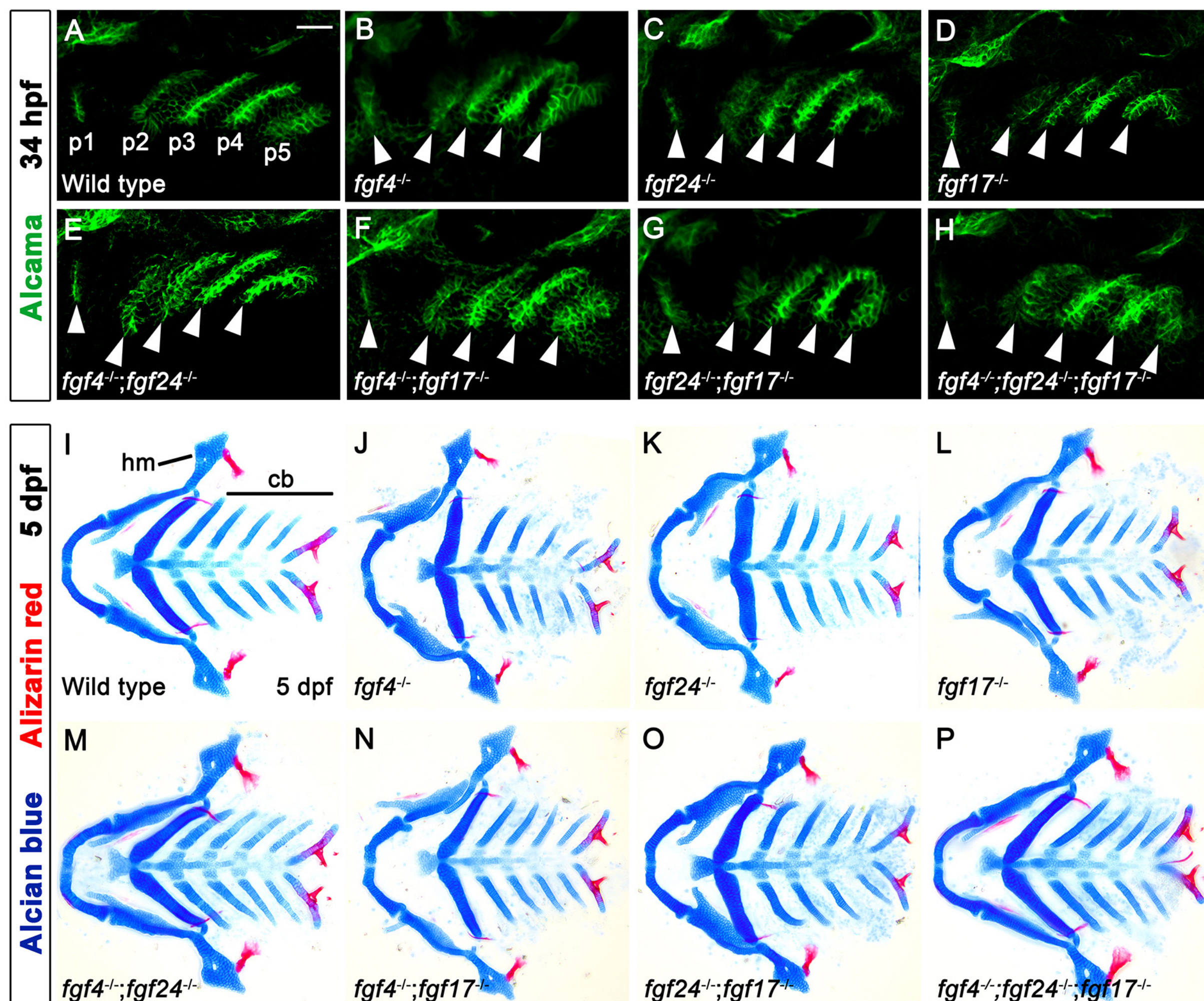

Fig. 5. Development of pharyngeal pouches and facial skeletons in single mutant, double mutant combinations, and triple mutant for *fgf4*, *fgf24*, and *fgf17*. (A–H) Alcama staining visualizes five bi-layered pouches (p1–p5) in wild type at 34 hpf. Five pouches are seen in single mutant, double mutant combinations, and triple mutant at 34 hpf, and they are almost identical to those seen in wild type. (I–P) Ventral of dissected facial cartilages (blue) and bones (red) at 5 dpf. Wild-type and fgf mutant zebrafish invariantly form a triangular hyomandibular (hm) and five ceratobranchial (cb) cartilages on each side. Facial bones in wild-type and *fgf* mutant animals are indistinguishable. Number of pouches and facial skeletons examined for each genotype: wild type (33, 36), *fgf4*$^{-/-}$ (27, 24), *fgf24*$^{-/-}$ (31, 36), *fgf17*$^{-/-}$ (29, 33), *fgf4*$^{-/-}$;*fgf24*$^{-/-}$ (20, 25), *fgf4*$^{-/-}$;*fgf17*$^{-/-}$ (23, 20), *fgf24*$^{-/-}$;*fgf17*$^{-/-}$ (17, 22), *fgf4*$^{-/-}$;*fgf24*$^{-/-}$;*fgf17*$^{-/-}$ (9, 13). Scale bar: 40 µm. Anterior is to the left.

https://doi.org/10.12717/DR.2024.28.2.55

hm and cb, were completely normal in all double mutant combinations (Fig. 5E–G, M–O), and they were also unaffected by the loss of all three *fgf* genes (Fig. 5H and P). We also examined facial bones in single, double, and triple mutants for the *fgf* genes, but they were indistinguishable from those in wild-type animals (red staining in Fig. 5I–P). Our result suggests that *fgf4*, *fgf24*, and *fgf17* that are expressed in the pharyngeal endoderm in pouch formation, are highly redundant with other *fgf* genes in the development of pouches and facial skeletons.

DISCUSSION

In this study, we identified the expression of *fgf4*, *fgf24*, and *fgf17* genes in the endoderm, with their regulation by Tbx1 and Fgf8a, in pouch formation. Their expression and regulation suggested that they might be necessary for pouch formation. However, functional analysis of the *fgf* genes did not support an essential role for these genes in pouch formation and facial skeletal development. It has been suggested that genetic redundancy in the Fgf signaling components generates a robust developmental system in zebrafish (Leerberg et al., 2019; Draper et al., 2003). Double mutants for *fgf8a* and *fgf24* lead to loss of posterior mesodermal derivatives, whereas single mutants for *fgf8a* and *fgf24* have normal mesoderm development (Draper et al., 2003). Similarly, while all single mutants for *fgfr1a*, *fgfr1b*, and *fgfr2* genes are viable with no embryonic phenotypes, certain double and triple mutant combinations have defects in the brain, facial cartilages, pectoral fin, and posterior mesoderm (Leerberg et al., 2019). Considering the fairly normal expression of *fgf4*, *fgf24*, and *fgf17* in *fgf3* mutants, normal development of pouches and facial skeletons, even in the triple mutant for the *fgf* genes, could be due to their redundancy with *fgf3* or other unidentified *fgfs* acting downstream of *fgf3*. To test this hypothesis, we need to analyze double and triple mutant combinations for *fgf3* and *fgf4*, *fgf24*, and *fgf17*, as well as a quadruple mutant for the *fgf* genes. We predict that the defects in pouches and facial skeletons seen in those mutants would be similar to those seen in the animals blocking Fgf signaling in the pharyngeal endoderm. Alternatively, the triple mutant for *fgf4*, *fgf24*, and *fgf17* shows normal development of pouches and facial skeletons due to a genetic compensation by other *fgf* genes, including *fgf3* and unidentified *fgf*, expressed in pharyngeal endoderm. It has been shown that indel mutation generated by CRISPR/Cas9 system can lead to phenotypes that are weaker than either point mutation or morpholino-mediated knockdown due to a genetic compensation; in mutants, expression of a gene(s) related to the mutated gene is upregulated and functionally compensates for the mutated gene (Rossi et al., 2015; El-Brolosy et al., 2019). Although it is preliminary to identify novel *fgf* genes in pouch formation, our study still implies a potential role for *fgf4*, *fgf24*, and *fgf17* in pouch formation. Currently, we are developing genetic tools to reveal the genetic mechanism for the high degree of redundancy of Fgf signaling in pouch formation.

REFERENCES

Aggarwal VS, Liao J, Bondarev A, Schimmang T, Lewandoski M, Locker J, Shanske A, Campione M, Morrow BE (2006) Dissection of Tbx1 and Fgf interactions in mouse models of 22q11DS suggests functional redundancy. Hum Mol Genet 15:3219-3228.

Brito JM, Teillet MA, Le Douarin NM (2006) An early role for sonic hedgehog from foregut endoderm in jaw development: Ensuring neural crest cell survival. Proc Natl Acad Sci USA 103:11607-11612.

Choe CP (2023) A feasible role of neuropilin signaling in pharyngeal pouch formation in zebrafish. Dev Reprod 27:137-147.

Choe CP, Collazo A, Trinh LA, Pan L, Moens CB, Crump JG (2013) Wnt-dependent epithelial transitions drive pharyngeal pouch formation. Dev Cell 24:296-309.

Choe CP, Crump JG (2014) Tbx1 controls the morphogenesis of pharyngeal pouch epithelia through mesodermal Wnt11r and Fgf8a. Development 141:3583-3593.

Crump JG, Maves L, Lawson ND, Weinstein BM, Kimmel CB (2004) An essential role for Fgfs in endodermal pouch formation influences later craniofacial skeletal patterning. Development 131:5703-5716.

Dickmeis T, Mourrain P, Saint-Etienne L, Fischer N, Aanstad P, Clark M, Strähle U, Rosa F (2001) A crucial component of the endoderm formation pathway, CASANOVA, is encoded by a novel sox-related gene. Genes Dev 15:1487-1492.

Draper BW, Stock DW, Kimmel CB (2003) Zebrafish *fgf24* functions with fgf8 to promote posterior mesodermal development. Development 130:4639-4654.

El-Brolosy MA, Kontarakis Z, Rossi A, Kuenne C, Günther S, Fukuda N, Kikhi K, Boezio GLM, Takacs CM, Lai SL, Fukuda R, Gerri C, Giraldez AJ, Stainier DYR (2019) Genetic compensation triggered by mutant mRNA degradation. Nature 568:193-197.

Fürthauer M, Van Celst J, Thisse C, Thisse B (2004) Fgf signalling controls the dorsoventral patterning of the zebrafish embryo. Development 131:2853-2864.

Grevellec A, Tucker AS (2010) The pharyngeal pouches and clefts: Development, evolution, structure and derivatives. Semin Cell Dev Biol 21:325-332.

Herzog W, Sonntag C, von der Hardt S, Roehl HH, Varga ZM, Hammerschmidt M (2004) *Fgf3* signaling from the ventral diencephalon is required for early specification and subsequent survival of the zebrafish adenohypophysis. Development 131:3681-3692.

Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31:227-229.

Jackson A, Kasah S, Mansour SL, Morrow B, Basson MA (2014) Endoderm-specific deletion of Tbx1 reveals an FGF-independent role for Tbx1 in pharyngeal apparatus morphogenesis. Dev Dyn 243:1143-1151.

Jin S, O J, Stellabotte F, Choe CP (2018) Foxi1 promotes late-stage pharyngeal pouch morphogenesis through ectodermal Wnt4a activation. Dev Biol 441:12-18.

Kikuchi Y, Agathon A, Alexander J, Thisse C, Waldron S, Yelon D, Thisse B, Stainier DY (2001) casanova encodes a novel Sox-related protein necessary and sufficient for early endoderm formation in zebrafish. Genes Dev 15:1493-1505.

Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien CB (2007) The Tol2kit: A multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. Dev Dyn 236:3088-3099.

Leerberg DM, Hopton RE, Draper BW (2019) Fibroblast growth factor receptors function redundantly during zebrafish embryonic development. Genetics 212:1301-1319.

Lindsay EA, Vitelli F, Su H, Morishima M, Huynh T, Pramparo T, Jurecic V, Ogunrinu G, Sutherland HF, Scambler PJ, Bradley A, Baldini A (2001) Tbx1 haploinsufficieny in the DiGeorge syndrome region causes aortic arch defects in mice. Nature 410:97-101.

Liu YH, Lin TC, Hwang SL (2020) Zebrafish Pax1a and Pax1b are required for pharyngeal pouch morphogenesis and ceratobranchial cartilage development. Mech Dev 161:103598.

Nissen RM, Yan J, Amsterdam A, Hopkins N, Burgess SM (2003) Zebrafish foxi one modulates cellular responses to Fgf signaling required for the integrity of ear and jaw patterning. Development 130:2543-2554.

Niswander L, Martin GR (1992) Fgf-4 expression during gastrulation, myogenesis, limb and tooth

 https://doi.org/10.12717/DR.2024.28.2.55

development in the mouse. Development 114:755-768.

Ober EA, Olofsson B, Mäkinen T, Jin SW, Shoji W, Koh GY, Alitalo K, Stainier DY (2004) Vegfc is required for vascular development and endoderm morphogenesis in zebrafish. EMBO Rep 5:78-84.

Okada K, Inohaya K, Mise T, Kudo A, Takada S, Wada H (2016) Reiterative expression of pax1 directs pharyngeal pouch segmentation in medaka. Development 143:1800-1810.

Piotrowski T, Ahn DG, Schilling TF, Nair S, Ruvinsky I, Geisler R, Rauch GJ, Haffter P, Zon LI, Zhou Y, Foott H, Dawid IB, Ho RK (2003) The zebrafish van gogh mutation disrupts *tbx1*, which is involved in the DiGeorge deletion syndrome in humans. Development 130:5043-5052.

Piotrowski T, Nüsslein-Volhard C (2000) The endoderm plays an important role in patterning the segmented pharyngeal region in zebrafish (*Danio rerio*). Dev Biol 225:339-356.

Reifers F, Böhli H, Walsh EC, Crossley PH, Stainier DY, Brand M (1998) *Fgf8* is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development 125:2381-2395.

Rossi A, Kontarakis Z, Gerri C, Nolte H, Hölper S, Krüger M, Stainier DY (2015) Genetic compensation induced by deleterious mutations but not gene knockdowns. Nature 524:230-233.

Tallafuss A, Bally-Cuif L (2003) Tracing of her5 progeny in zebrafish transgenics reveals the dynamics of midbrain-hindbrain neurogenesis and maintenance. Development 130:4307-4323.

Tran HT, Delvaeye M, Verschuere V, Descamps E, Crabbe E, Van Hoorebeke L, McCrea P, Adriaens D, Van Roy F, Vleminckx K (2011) ARVCF depletion cooperates with Tbx1 deficiency in the development of 22q11.2DS-like phenotypes in Xenopus. Dev Dyn 240:2680-2687.

van Eeden FJ, Granato M, Schach U, Brand M, Furutani-Seiki M, Haffter P, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Warga RM, Nüsslein-Volhard C (1996) Genetic analysis of fin formation in the zebrafish, Danio rerio. Development 123:255-262.

Wright TJ, Hatch EP, Karabagli H, Karabagli P, Schoenwolf GC, Mansour SL (2003) Expression of mouse fibroblast growth factor and fibroblast growth factor receptor genes during early inner ear development. Dev Dyn 228:267-272.

Zuniga E, Rippen M, Alexander C, Schilling TF, Crump JG (2011) Gremlin 2 regulates distinct roles of BMP and Endothelin 1 signaling in dorsoventral patterning of the facial skeleton. Development 138:5147-5156.