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Expression and Functional Analysis of *cofilin1-like* in Craniofacial Development in Zebrafish

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The authors declare no potential conflict of interest.

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Abstract

Pharyngeal pouches, a series of outgrowths of the pharyngeal endoderm, are a key epithelial structure governing facial skeleton development in vertebrates. Pouch formation is achieved through collective cell migration and rearrangement of pouch-forming cells controlled by actin cytoskeleton dynamics. While essential transcription factors and signaling molecules have been identified in pouch formation, regulators of actin cytoskeleton dynamics have not been reported yet in any vertebrates. Cofilin1-like (Cfl1l) is a fish-specific member of the Actin-depolymerizing factor (ADF)/Cofilin family, a critical regulator of actin cytoskeleton dynamics in eukaryotic cells. Here, we report the expression and function of *cfl1l* in pouch development in zebrafish. We first showed that fish *cfl1l* might be an ortholog of vertebrate *adf*, based on phylogenetic analysis of vertebrate *adf* and *cfl* genes. During pouch formation, *cfl1l* was expressed sequentially in the developing pouches but not in the posterior cell mass in which future pouch-forming cells are present. However, pouches, as well as facial cartilages whose development is dependent upon pouch formation, were unaffected by loss-of-function mutations in *cfl1l*. Although it could not be completely ruled out a possibility of a genetic redundancy of Cfl1l with other Cfls, our results suggest that the *cfl1l* expression in the developing pouches might be dispensable for regulating actin cytoskeleton dynamics in pouch-forming cells.

Keywords: *cofilin1-like*, Craniofacial development, Facial cartilages, Pharyngeal pouch, Zebrafish

INTRODUCTION

In vertebrates, facial skeletons arise in the cranial neural crest cells (cNCCs) that populate a series of pharyngeal arches. Besides cNCCs, the arches are composed of the pharyngeal endoderm (PE), head mesoderm, and pharyngeal ectoderm. Interactions among these tissues are crucial for craniofacial development, including the facial skeletons (Graham, 2008). In particular, PE is essential for cNCCs in the arches to form facial skeletal elements, as evidenced by the loss of facial skeletons in the absence of the endoderm of *sox32* mutants in zebrafish (Piotrowski & Nüsslein-Volhard, 2000; Dickmeis et al., 2001; Kikuchi et al., 2001). During craniofacial development, PE forms a series of pharyngeal pouches,

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Conceptualization: Choe CP.
Data curation: Jin S.
Validation: Jeon H.
Writing-original draft: Jin S.
Writing-review & editing: Jin S, Jeon H, Choe CP.

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

outpocketings of the PE, that segments the pharyngeal arches then provides signals, such as sonic Hedgehog and Jagged, for the cNCCs to differentiate into the facial skeletal elements (Miller et al., 2000; Piotrowski & Nüsslein-Volhard, 2000; Zuniga et al., 2010). In addition, the pouches continue to develop into endocrine glands in the head and neck, including the thymus and parathyroid (Grevellec & Tucker, 2010). Abnormal pouch formation has been associated with defects in facial skeletons, along with malformation or absence of the thymus and parathyroid in all vertebrates examined so far and is an etiology of DiGeorge syndrome (DGS), a congenital craniofacial disability, in human (Driscoll et al., 1992; Piotrowski & Nüsslein-Volhard, 2000; Lindsay et al., 2001; Tran et al., 2011). Despite the importance of pouches in craniofacial development, the genetic and cellular mechanisms underlying pouch formation are just being revealed. Tbx1 is a master regulator for developing pouches in fish to human, and *TBX1* haploinsufficiency is the genetic cause of DGS (Lindsay et al., 2001; Piotrowski et al., 2003; Tran et al., 2011). Pax1/9 and Foxi1/3 are required for pouch formation in fish and mice (Peters et al., 1998; Nissen et al., 2003; Solomon et al., 2003; Edlund et al., 2014; Okada et al., 2016; Jin et al., 2018; Liu et al., 2020). Signaling molecules such as Fgf3/8, Wnt11r/4a, EphrinB2/B3, and Bmp are also necessary for pouch development in either zebrafish or mice (Crump et al., 2004; Herzog et al., 2004; Agrawal et al., 2012; Choe et al., 2013; Choe & Crump, 2015; Lovely et al., 2016; Li et al., 2019). Tbx1 regulates pouch morphogenesis by controlling Wnt11r and Fgf8a expression in the head mesoderm in zebrafish (Choe & Crump, 2014). Pax1 promotes pouch formation by expressing *tbx1* and *fgf3* in the PE in medaka and zebrafish, with Foxi1 controlling pouch development through ectodermal Wnt4a expression in zebrafish (Okada et al., 2016; Jin et al., 2018; Liu et al., 2020). The cellular process of pouch formation has been studied in detail in zebrafish. Pouch formation begins with destabilizing the bi-layered epithelial structure of the PE that is promoted by the Tbx1-Wnt11r pathway (Choe et al., 2013; Choe & Crump, 2014). In the destabilized PE, a population of endodermal cells, called pouch-forming cells, migrates out collectively toward the head mesoderm located adjacent to the PE, with Fgf8a expression in the head mesoderm acting as a chemoattractant (Choe et al., 2013; Choe & Crump, 2014). The migrating pouch-forming cells are rearranged into a bi-layered structure followed by re-stabilization of the pouch epithelia to complete pouch formation, with Wnt4a and EphrinB2/B3 required for the rearrangement and re-stabilization, respectively (Choe et al., 2013; Choe & Crump, 2015). Thus, at the cellular level, the migration and rearrangement of pouch-forming cells that cytoskeleton dynamics might control are important driving forces for pouch formation. Indeed, inhibiting actin cytoskeleton dynamics in chick caused severe defects in pouch formation, indicating an essential role for actin cytoskeleton dynamics in pouch development (Quinlan et al., 2004). However, any regulators of actin cytoskeleton dynamics required for pouch formation have not been identified yet in any vertebrates. Here, we analyze the expression and function of *cofilin1-like* (*cfl1l*), a regulator of actin cytoskeleton dynamics, during pouch formation in zebrafish.

The Cofilin family is included in the Actin-depolymerizing factor (ADF)/Cofilin superfamily that contains the conserved ADF homology (ADF-H) domain from yeast to human (Lappalainen et al., 1998; Ono, 2007). Traditional Cofilins (Cfls) consist of non-muscle type Cfl1, muscle type Cfl2, and ADF (also named Destrin) in mammals (Ono, 2007; Ohashi, 2015). In vertebrate cells, Cfls remodel the actin cytoskeleton by promoting depolymerization or polymerization of actin filaments, with the activity of Cfls being regulated by the cellular microenvironments such as pH, phosphatidylinositols, protein kinases, and phosphatases (Ono, 2007; Shishkin et al., 2016). For example, in vertebrate cells, while LIM-kinases and testicular protein kinases phosphorylate and inactivate Cfl1, slingshot protein phosphatases, protein phosphatases 1 and 2A, and chronophin dephosphorylate and activate Cfl1 (Ono, 2007; Shishkin et al., 2016). Consequently, vertebrate

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cells change their shape, migrate, and are rearranged through reactions of the phosphorylation/dephosphorylation of Cfls, which are essential for developmental processes (Abe et al., 1996; Obinata et al., 1997). Indeed, *Cfl1* knockout mice were embryonic lethal, with significant defects in neural crest cell migration and neural tube closure (Gurniak et al., 2005). Muscle-specific *Cfl2* knockout mice were normal at birth. Still, they died at the postnatal stage due to the degeneration of myofibers, sarcomeric disruptions, and actin accumulation, indicating an essential role of Cfl2 in muscle maintenance (Agrawal et al., 2012). Cfl1 and Adf are also necessary for developing kidney and eye in mice. Kidney-specific deletion of *Cfl1* in *Adf* mutants resulted in complete loss of kidney, with kidney-specific deletion of *Cfl1* in the heterozygotic mutant for *Adf* resulting in the hypoplastic kidney (Kuure et al., 2010). A missense mutation in Adf caused defects in eyes, with the corneal epithelium abnormally thickening in mice (Ikeda et al., 2003). In zebrafish, *cfl1* mutants showed defects in the heart and kidney, indicating a role for Cfl1 in heart and kidney development (Ashworth et al., 2010; Fukuda et al., 2019). In addition, a morpholino-mediated knockdown of *cfl1* resulted in defects in morphogenic movements of the deep cell layer during gastrulation (Lin et al., 2010). Zebrafish contains three paralogs of the *cfl* gene, including *cfl1*, *cfl2*, and *cfl1l*, but no *adf*, in the genome, with the role of *cfl2* and *cfl1l* during embryonic development undetermined. Here, we report the expression of *cfl1l* in the pharyngeal pouches, which is dispensable for head development, including pouch formation in zebrafish.

MATERIALS AND METHODS

1. Zebrafish lines

According to the Animal Protection Act (2017) in Korea, zebrafish were grown and maintained. All zebrafish work was approved by Gyeongsang National University Institutional Animal Care and Use Committee. *cfl1l* mutant lines were generated by CRISPR/Cas9 system (Hwang et al., 2013). 150 pg of gRNA targeting the second exon of the *cfl1l* gene 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)*, a PE reporter transgene (Tallafuß & Bally-Cuif, 2003). Injected embryos were grown to adulthood and outbred to wild-type TU animals to identify zebrafish bearing in/del mutations in the *cfl1l* gene. Two *cfl1l* mutant lines ($cfl1l^{GNU38}$ and $cfl1l^{GNU39}$) were secured. For genotyping of $cfl1l^{GNU38}$ and $cfl1l^{GNU39}$, a PCR amplified *cfl1l* fragment with primers *cflf1*_GT_ F (5'-CCAAAGAGTGTCGCTACG-3') and *cfl1l*_ GT_R (5'-CCCATCTGACAACGCTAC-3'), was digested with Sau96I, with a wild-type fragment producing 185 and 63 bp and mutant fragment generating 248 bp.

2. Phylogenetic analysis

Phylogenetic analysis was carried out with the amino acid sequences of vertebrate homologs of Cfl and Adf proteins obtained from National Center for Biotechnology Information (NCBI): (Aa-Cfl1, *Anguilla anguilla* (European eel) XP_035266577; Om-Cfl1, *Oryzias melastigma* (Indian medaka) XP_024139063; Dr-Cfl1, *Danio rerio* (zebrafish) NP_998806; Xt-Cfl1, *Xenopus tropicalis* (tropical clawed frog) NP_998878; Mm-Cfl1, *Mus musculus* (house mouse) NP_031713; Hs-CFL1, *Homo sapiens* (human) NP_005498; Cm-Cfl2, *Callorhinchus milii* (elephant shark) XP_007891564; Aa-Cfl2, XP_035275350; Om-Cfl2, XP_024123733; Dr-Cfl2, NP_991263; Xt-Cfl2, NP_001011156; Mm-Cfl2, NP_031714; Hs-CFL2, NP_001230574; Aa-Cfl1l, XP_035255492; Om-Cfl1l, XP_024153813; Dr-Cfl1l, NP_998804; Cm-Cfl2l, NP_001280090; Xt-Adf, XP_002937490; Mm-Adf, NP_062745; Hs-ADF, NP_006861). Whole amino acid

sequences deduced from the coding sequence (CDS) of vertebrate *cfl* and *adf* genes were aligned by CLUSTAL Omega with default parameters (Sievers & Higgins, 2021). The RAxML (Randomized Axelerated Maximum Likelihood) method with default parameters was applied to construct a maximum likelihood tree (Stamatakis, 2006).

3. Staining

Fluorescent in situ hybridization in conjunction with GFP immunohistochemistry (Torrey Pines Biolabs, 1:1,000), immunohistochemistry for Alcama (Zebrafish International Resource Center, 1:400), and Alcian Blue staining were carried out as described previously (Crump et al., 2004; Zuniga et al., 2011). Partial cDNA fragments of *cfl1l* were amplified from mixed-stage embryos with primers *cfl1l*_rF (5'-CCTCAGGTGTAGCGATCA-3') and *cfl1l*_rR (5'-CCACCAAGTTTTTCCACA-3'). The PCR fragments were cloned into the pGEM®-T easy vector (Promega). Antisense riboprobes were transcribed with SP6 RNA polymerase (Roche Life Sciences) using digoxigenin (DIG)-labeled nucleotides (Roche) from sequence-verified plasmids as described previously (Jeon et al., 2019).

4. Imaging

Fluorescent images were taken on an Olympus FLUOVIEW FV3000 confocal microscope using FV31S-SW software (Olympus). Approximately 100 μm Z-stacks at 3.5-μm intervals were captured with an Olympus UPLXAPO 20X objective lens, then were stacked into a single image. Facial cartilages were dissected manually from Alcian Blue staining larvae and were flat-mounted for imaging with an Olympus BX50 upright microscope using mosaic V2.1 software (Tucsen Photonics). Any adjustments were applied to all panels using Adobe Photoshop (Adobe Systems).

RESULTS

1. Fish *cofilin-like* genes might be orthologs of vertebrate *adf* gene

To identify the orthologs of zebrafish *cfl1l* in vertebrates, we searched the NCBI database. Apparently, *cfl1l* genes were only found in the fish genomes, including medaka and European eel, with *cfl2l* genes identified in sharks. Interestingly, orthologs of the *adf* gene were not identified in the fish genomes bearing the *cfl1l* or *cfl2l* gene. Compared to the vertebrate *cfl* and *adf* genes, fish *cfl1l* and *cfl2l* genes shared most residues essential for interactions of yeast Cfl with actin (red in Fig. 1; Lappalainen et al., 1997) as well as a phosphorylation site at Serine-3 (highlighted in green in Fig. 1; Agnew et al., 1995; Moriyama et al., 1996). Still, they showed some variability in the region that was also important for actin interactions (blue in Fig. 1; Yonezawa et al., 1989) as well as in the nuclear localization signal (NLS) (highlighted in yellow in Fig. 1; Munsie et al., 2012). To verify vertebrate orthologs of zebrafish *cfl1l*, we carried out the phylogenetic analysis with the multiple sequence alignment for amino acids deduced from the CDS of vertebrate *cfl* and *adf* genes (Fig. 1). Interestingly, fish *cfl-like* (*cfl1l* and *cfl2l*) genes belonged to the vertebrate *adf* group with a 32% bootstrap value (red lines in Fig. 2). Fish *cfl1* and *cfl2* genes were also grouped with vertebrate *cfl1* and *cfl2* genes, respectively, with lower bootstrap values than the *adf* group (blue and green lines, respectively in Fig. 2). Although the bootstrap values are low, our preliminary phylogenetic analysis of vertebrate *cfl* and *adf* genes suggests that the fish *cfl-like* genes might be orthologs of the vertebrate *adf* gene.

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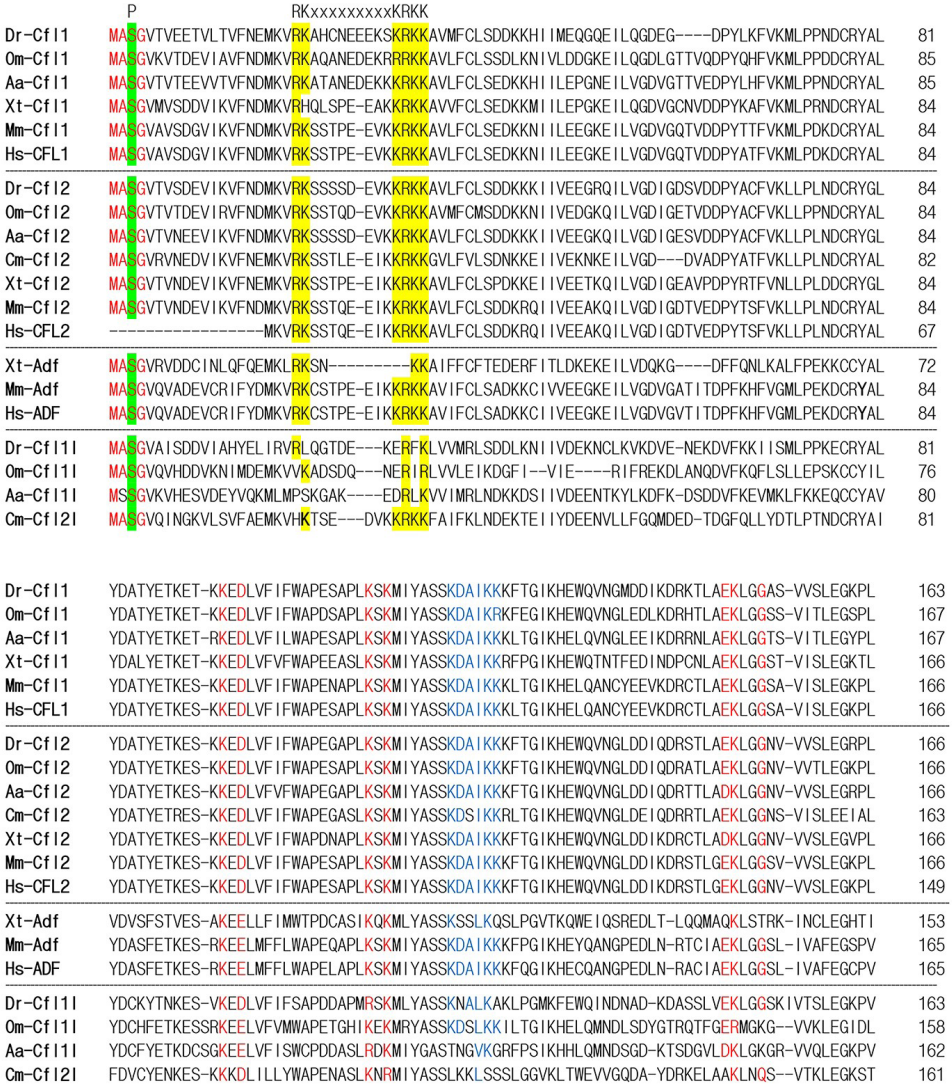

Fig. 1. Multiple sequence alignment of vertebrate ADF/Cofilin family members. Complete amino acids sequences deduced from the CDS of each *adf* and *cofilin* gene are aligned by Clustal Omega. The residues important for yeast Cfl to interact with actin are red, and the region required for actin interaction revealed by peptide inhibition studies is blue. Serine-3 that can be phosphorylated is highlighted in green and indicated by P above the sequences. The bipartite NLS sequence is highlighted in yellow, with the NLS consensus marked on the top of the sequences. Aa, *Anguilla anguilla* (European eel); Cm, *Callorhinchus milii* (elephant shark); Dr, *Danio rerio* (zebrafish); Hs, *Homo sapiens* (human); Mm, *Mus musculus* (house mouse); Om, *Oryzias melastigma* (Indian medaka); Xt, *Xenopus tropicalis* (tropical clawed frog). ADF, actin-depolymerizing factor; CDS, coding sequence; NLS, nuclear localization signal.

2. Zebrafish *cfl1l* is expressed in pharyngeal pouches during craniofacial development

In zebrafish, *cfl1* is required to develop the heart and kidney, with *cfl2* being implicated in heart development, whereas the physiological role of *cfl1l* has not been reported yet (Ashworth et al., 2010; Fukuda et al., 2019). Recently, a single-cell RNA sequencing in zebrafish revealed *cfl1l* expression in 24 hours-post-fertilization (hpf) old PE cells, implying a potential role of Cfl1l in craniofacial development (Wagner et al., 2018). To investigate the developmental role of *cfl1l* in zebrafish, we first analyzed the expression of *cfl1l* during the morphogenesis of pouches. To do so, we performed in situ hybridization for *cfl1l* in wild-type TU embryos carrying *Tg(her5:GFP)* transgene, a reporter of the PE and pouches (Tallafuß & Bally-Cuif, 2003). When the first two

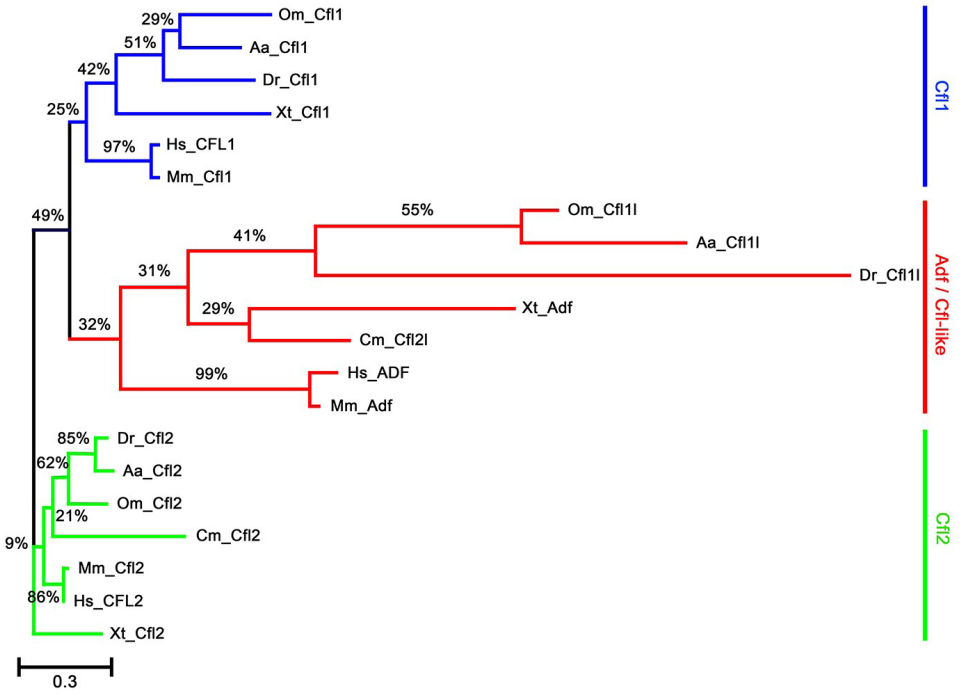

Fig. 2. Phylogenetic analysis of vertebrate ADF/Cofilin family members. This phylogenetic tree is constructed using the RAxML, based on the multiple sequence alignment presented in Fig. 1. Bootstrap values are indicated at branches. In this maximum likelihood tree, zebrafish Cfl1l is grouped with the fish Cfl1l with a 41% bootstrap value, with the fish Cfl1l group being included in the vertebrate Adf group with a 32% bootstrap value. Zebrafish Cfl1 and Cfl2 were grouped with other vertebrate Cfl1 and Cfl2 proteins, respectively. The Cfl1, Cfl2, and Adf/Cfl-like groups are marked with blue, green, and red lines, respectively. Aa, *Anguilla anguilla*; Cm, *Callorhinchus milii*; Dr, *Danio rerio*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Om, *Oryzias melastigma*; Xt, *Xenopus tropicalis*. ADF, actin-depolymerizing factor.

pouches formed at 18 hpf, *cfl1l* was expressed in the pouches, with its expression in the second pouch being intense (Fig. 3A). However, *cfl1l* expression in the posterior cell mass was not seen, in which future pouches formed (Fig. 3A). As the third pouch developed at 24 hpf, *cfl1l* expression in the pouches was evident, whereas the posterior cell mass still did not express *cfl1l* (Fig. 3B). At 30 hpf, when posterior pouches, including the fourth pouch, were grown out, *cfl1l* was expressed in the pouches, with the *cfl1l* expression in the first pouch being abolished. Still, *cfl1l* was not expressed in the posterior cell mass (Fig. 3C). Besides, *cfl1l* expression was observed in other tissues adjacent to the pouches at 30 hpf (asterisks in Fig. 3C). We verified *cfl1l* expression in the pouches with a high magnification single section image showing colocalization of *cfl1l* transcripts with GFP expressed in *her5*-positive pouches (Fig. 3D). In summary, *cfl1l* was expressed sequentially in the pouches during pouch formation but was not expressed in the posterior cell mass, in which pouch-forming cells had not formed pouches yet.

3. Loss-of-function mutations in the *cfl1l* gene are generated

Given the essential role of pharyngeal pouches in craniofacial development (Piotrowski & Nüsslein-Volhard, 2000; Lindsay et al., 2001; Tran et al., 2011), *cfl1l* expression in the pouches could be necessary to develop pouch itself and/or facial skeletons. To analyze the function of *cfl1l* in craniofacial development, we generated loss-of-function mutations in the *cfl1l* gene with CRISPR/Cas9 system. We designed a gRNA targeting the second exon among four exons of the *cfl1l* gene with ZiFIT (Fig. 4A; Baker, 2014). We established two mutant $cfl1l^{GNU38}$ and $cfl1l^{GNU39}$ alleles in

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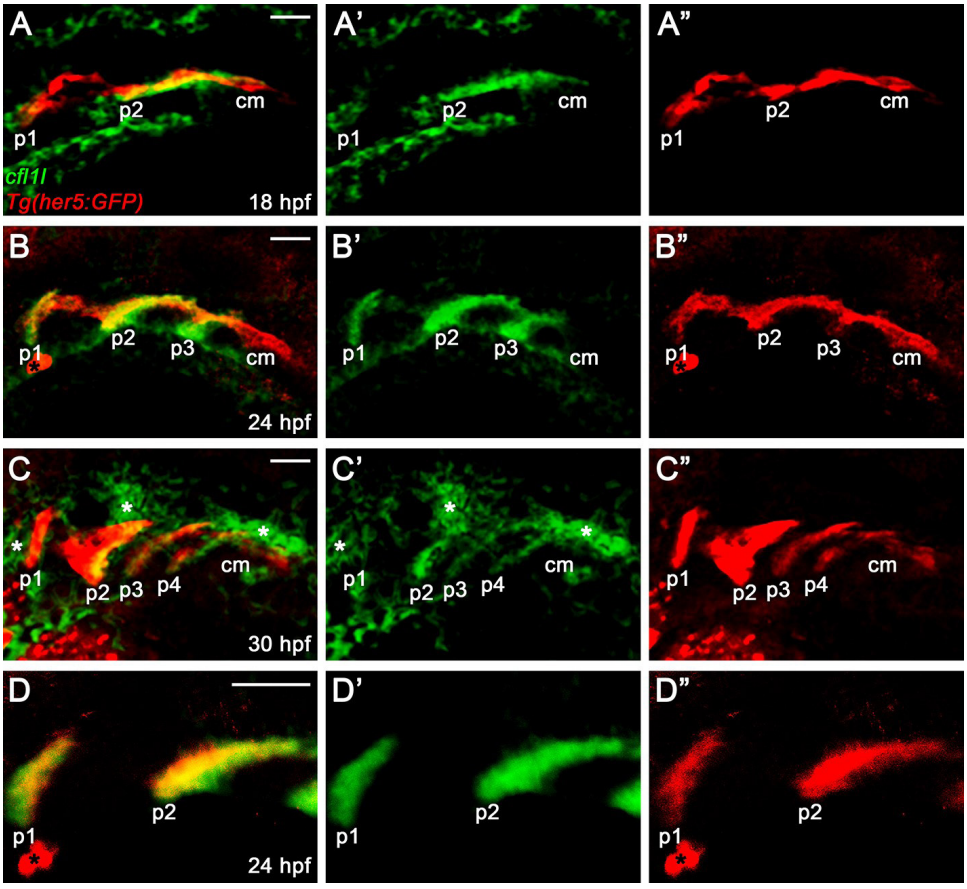

Fig. 3. Expression of *cfl1l* during pouch development in zebrafish. [A–D] *In situ* hybridization of *cfl1l* (green) in conjunction with the GFP immunohistochemistry (red) in wild-type *Tg(her5:GFP)* animals. Anterior is to the left. (A) At 18 hpf, *cfl1l* is expressed in the *her5*-positive first (p1) and second (p2) pouches but not in the posterior cell mass (cm). (B) At 24 hpf, *cfl1l* expression continues in the pouches, including the newly formed third pouch (p3), with no expression seen in the posterior cell mass. (C) At 30 hpf, *cfl1l* is expressed in the pouches (p2–p4), with the *cfl1l* expression in the first pouch (p1) not seen. In the posterior cell mass, *cfl1l* expression is not observed. New *cfl1l* expression appears in the regions adjacent to the pouches and posterior cell mass, and is indicated with asterisks. (D) A single section of a confocal image showing coexpression of *cfl1l* and GFP in the pouches at 24 hpf. [B,D] *her5*-positive oral ectoderm is marked with black asterisks. [A'–D'] Green channel only. [A"–D"] Red channel only. Scale bars: 40 μm.

which five and four nucleotides were deleted in the target region, respectively (Fig. 4B). While the wild-type *cfl1l* gene was expected to encode 163 amino acids bearing six α-helixes and seven β-sheets in the secondary structure of Cfl1l, both *cfl1l*^{GNU38} and *cfl1l*^{GNU39} alleles were predicted to encode 101 and 106 amino acids, respectively, with the resulting mutant Cfl1l^{GNU38} and Cfl1l^{GNU39} proteins being truncated at the sixth β-sheet due to the misframed amino acids and an early termination codon (Fig. 4C). Considering the importance of the sixth β-sheet and the following two α-helixes as a central part in the ADF-H domain of ADF/Cofilin superfamily proteins (Lappalainen et al., 1998; Ono, 2007), Cfl1l^{GNU38} and Cfl1l^{GNU39} mutant proteins lacking the sixth β-sheet and the following two α-helixes, were expected to be less functional than wild-type Cfl1l protein. Thus, we suggest that *cfl1l*^{GNU38} and *cfl1l*^{GNU39} are hypomorphic alleles.

4. *cfl1l* is not necessary for the development of pharyngeal pouches and facial skeletons

To access the function of *cfl1l* in craniofacial development, we first examined the pouches in single mutants of *cfl1l*^{GNU38} and *cfl1l*^{GNU39} harboring *Tg(her5:GFP)* transgene that allowed us to

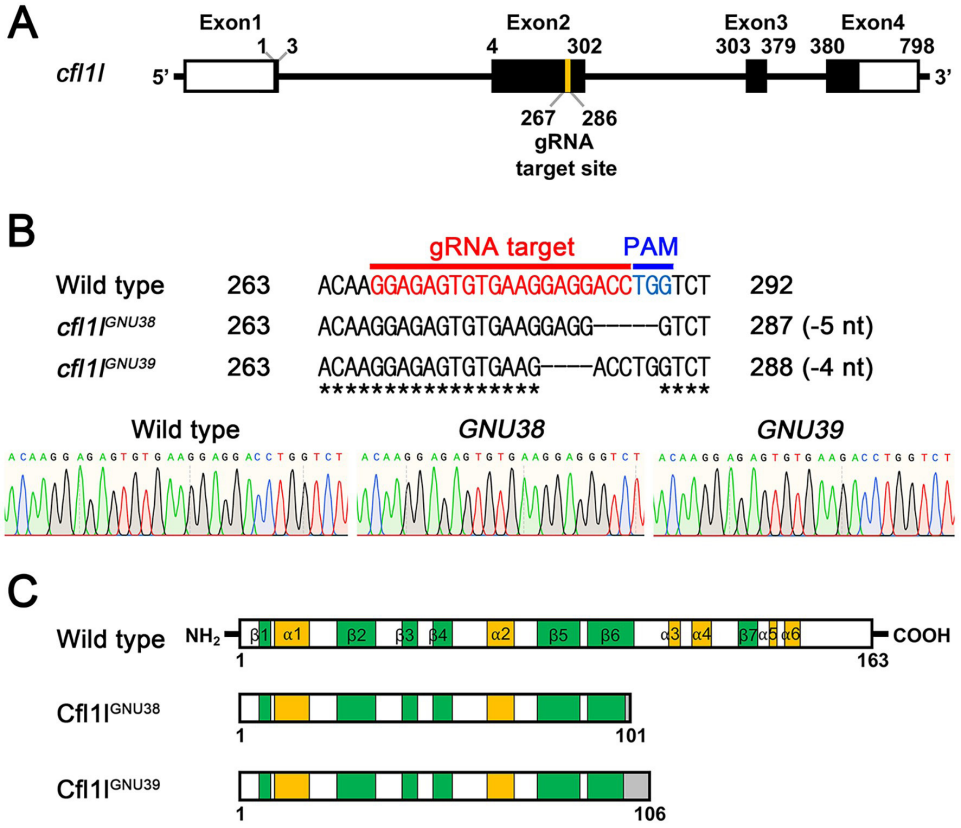

Fig. 4. Generation of loss-of-function mutations in *cfl1l* gene. (A) Structure of *cfl1l* gene. *cfl1l* gene consists of four exons bearing sequences for the protein-coding region (black box) and the 5' and 3' untranslated regions (open box). The gRNA target site is marked in yellow. (B) Loss-of-function alleles of *cfl1l* gene. The deletion mutation of each mutant allele is shown in the multiple sequence alignment, with the gRNA target and the PAM sites being red and blue, respectively, in the wild-type *cfl1l* sequence. The electrophoretograms verify the lesion in each *cfl1l* mutant allele. [A,B] The number indicates the nucleotide number of the CDS. (C) Schematic of the Cfl1l proteins encoded by the wild-type and mutant alleles. The regions forming α-helixes and β-sheets in the ADF-H domain are presented by yellow and green boxes, respectively, with grey boxes indicating miss-translated amino acids in the mutant Cfl1l proteins. Mutant Cfl1l proteins are truncated at the sixth beta-sheet region. The number indicates the amino acid number of the Cfl1l protein. CDS, coding sequence; ADF-H, actin-depolymerizing facto-homology.

visualize the pouches during the morphogenesis of pouches directly. In wild-type siblings, pouch formation was completed at 32 hpf, with five pouches seen by the *Tg(her5:GFP)* transgene (Fig. 5A). Like wild types, five pouches were observed in *cfl1l* mutants by the *Tg(her5:GFP)* transgene (Fig. 5B). We have analyzed pouch formation in 64 *cfl1l* mutant animals, with none showing defects in pouches at 32 hpf, which suggests that *cfl1l* is not essential for pouch development. Since genes expressed in the pouches could be required for facial skeleton development rather than pouch formation (Miller et al., 2000; Zuniga et al., 2010), we next analyzed facial cartilages in *cfl1l* mutants at 5 dpf. We examined facial cartilages in 286 wild-type siblings and 93 *cfl1l* mutants. In wild-type siblings and *cfl1l* mutants, all elements of facial cartilages driven from the pharyngeal arches were normal, including the hyosymplectic (hs) and ceratobranchial (cb) cartilages whose formation was dependent upon appropriate pouch development (Fig. 5C,D). Even though *cfl1l*^{GNU38} and *cfl1l*^{GNU39} are likely hypomorphic rather than null alleles, normal development of the pouches and facial cartilages seen in *cfl1l* mutants suggests that *cfl1l* expression in the pouches might not be essential for craniofacial development, such as the pouches and facial skeletons, in zebrafish.

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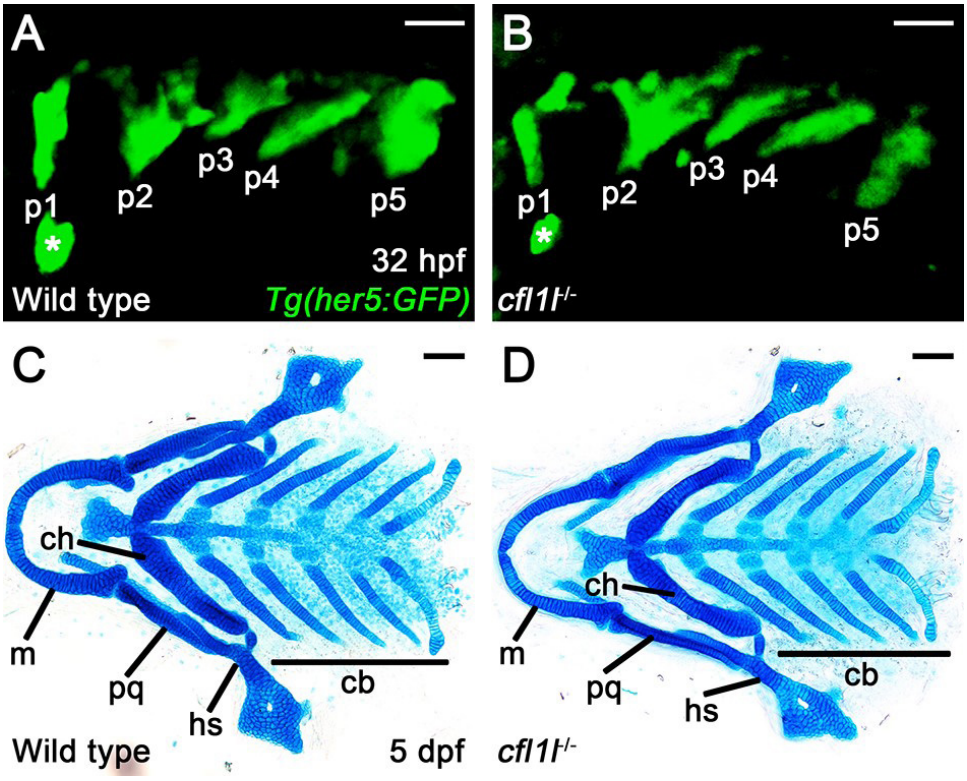

Fig. 5. Normal development of pharyngeal pouches and facial cartilages in *cfl1l* mutant fish. Anterior is to the left. [A,B] *Tg(her5:GFP)* transgene (green) labels five normal pouches (p1–p5) in wild-type siblings and *cfl1l*^{GNU39} mutant animals at 32 hpf. The oral ectoderm is marked with asterisks. Scale bars: 40 μm. [C,D] Ventral of dissected facial cartilages, with each element of facial cartilages being indicated. m, Meckel's cartilage; pq, palatoquadrate cartilage; hs, hyosymplectic cartilage; ch, ceratohyal cartilage; cb, ceratobranchial cartilage. All elements of the facial cartilages, including the bilateral set of hs and five cb cartilages, are normal in wild-type and *cfl1l*^{GNU39} mutant animals at 5 dpf. Scale bars: 100 μm.

5. *cfl1* and *cfl2* are not expressed in pharyngeal pouches during craniofacial development

The normal pouches and facial cartilages observed in *cfl1l* mutants could be a consequence of the genetic redundancy of Cfl1l with Cfl1 or Cfl2 for craniofacial development. If this is the case, *cfl1l* expression would overlap with *cfl1* or *cfl2* in the pouches during craniofacial development. To examine a potential redundancy among *cfl* genes for pouch formation, we analyzed the expression of *cfl1* and *cfl2* in the pharyngeal regions. At 30 hpf, *cfl1* was expressed in the pharyngeal arches rather than *sox17*-positive pouches (numbers in Fig. 6A). Similar to *cfl1*, *cfl2* was expressed in the ventral domains of the arches, with its expression in *sox17*-positive pouches being barely seen (numbers in Fig. 6B). Expression of *cfl1* and *cfl2* in the arches adjacent to the pouches at 30 hpf, suggests that Cfl1l is unlikely to be redundant with Cfl1 or Cfl2 for pouch formation.

DISCUSSION

In this study, we analyzed the expression and function of *cfl1l*, which might be an ortholog of vertebrate *adf*, in craniofacial development in zebrafish. While *cfl1l* was expressed in the pouches during the morphogenesis of pouches, loss-of-function mutations in *cfl1l* did not affect the development of pouches and facial cartilages. Given the importance of ADF/Cfl family proteins

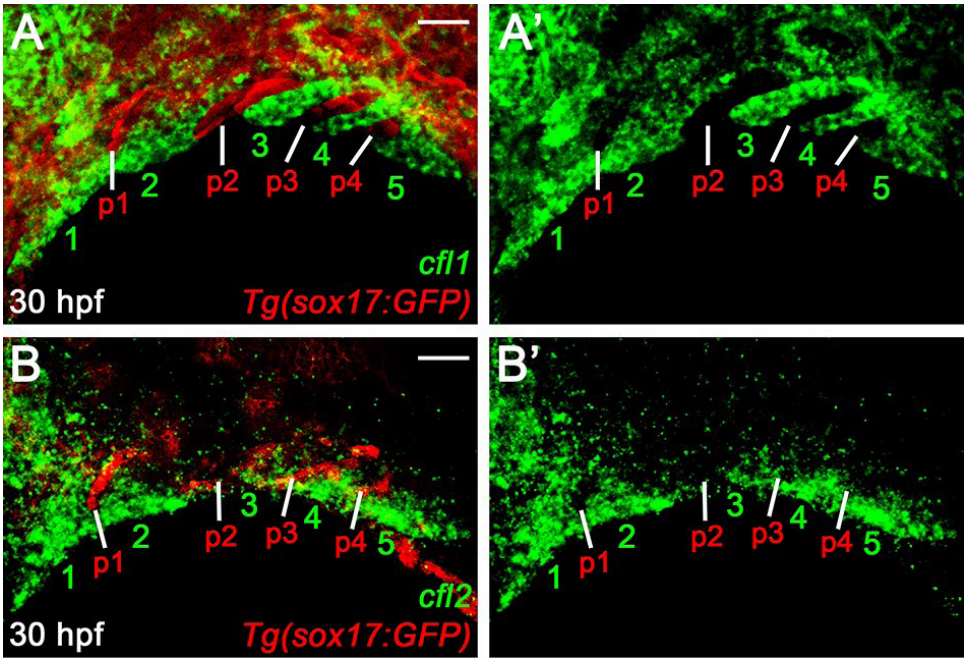

Fig. 6. Expression of *cfl1* and *cfl2* during pouch development. *In situ* hybridization of *cfl1* and *cfl2* (green) in conjunction with the GFP immunohistochemistry (red) in wild-type *Tg(sox17:GFP)* animals. Anterior is to the left. [A,B] At 30 hpf, *cfl1* and *cfl2* are expressed complementary in the arches adjacent to *sox17*-positive pouches (p1–p4). Arches expressing *cfl1* and *cfl2* are marked with numbers, with the pharyngeal pouches (p1–p4) being indicated by lines. In the pouches, expression of *cfl1* and *cfl2* is barely seen. [A'–B'] Green channel only. Scale bars: 40 μm.

in actin cytoskeleton dynamics, together with the essential role of actin cytoskeleton dynamics in pouch formation (Quinlan et al., 2004; Ono, 2007; Shishkin et al., 2016), the normal pouches seen in *cfl1l* mutants were unexpected. The normal pouches could be due to the genetic redundancy of Cfl1l with Cfl1 or Cfl2. Indeed, in mice, Adf and Cfl1 show redundant requirements for kidney development, with kidney-specific *Cfl1* knockout mice showing normal kidney (Kuure et al., 2010). Although the function of Cfl1 and Cfl2 in heart development remains undetermined, Cfl1 and Cfl2 are expressed together in mammalian cardiomyocytes, implying a possibility of genetic redundancy of Cfl1 and Cfl2 in heart development (Kremneva et al., 2014). However, our analysis of the expression of *cfl1* and *cfl2* during craniofacial development suggests that the genetic redundancy among Cfls is unlikely in pouch formation. The normal pouches observed in *cfl1l* mutant fish might be due to the independence of actin cytoskeleton dynamics from Cfl1l in pouch-forming cells. In vertebrate cells, actin cytoskeleton dynamics are achieved by not only ADF/Cfl family proteins but also other actin regulators, including the actin regulator Actin related protein 2/3 (Arp2/3) complex and neural Wiskott-Aldrich syndrome protein (N-WASP, currently renamed as WASP like actin nucleation promoting factor [WASL]). In *Xenopus* extracts, Cdc42 regulates actin cytoskeleton dynamics through the Arp2/3 complex activated by WASL (Rohatgi et al., 1999). In zebrafish, the Arp2/3 is necessary for proper lamellipodia-like protrusion formation in the migrating posterior lateral primordium through actin dynamics (Olson & Nechiporuk, 2021). Moreover, Wasl acting downstream of Cdc42 is involved in the assembly of endothelial filopodia through actin remodeling in zebrafish (Wakayama et al., 2015). In zebrafish pouch formation, Cdc42 is required to rearrange pouch-forming cells (Choe et al., 2013). Interestingly, in the Zebrafish Model Organism Database (ZFIN), we have found that *actin related protein 2/3 complex*

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(*arpc*) genes composing the Arp2/3 complex are expressed ubiquitously in most embryonic tissues or the pharyngeal regions, with *wasl-b* being expressed in the pharyngeal regions, during pouch formation. Determination of the specific expression domains of the *arpc* and *wasl-b* genes in the pharyngeal regions, followed by the genetic analysis of these genes with *cfl1l* in pouch formation, will provide a better insight into the function of *cfl1l* in craniofacial development.

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