OUKA
The University of Osaka
Institutional Knowledge Archive

Title	Compromised actin dynamics underlie the orofacial cleft in Baraitser-Winter Cerebrofrontofacial syndrome with a variant in ACTB
Author(s)	Tsujimoto, Takayuki; Ou, Yushi; Suzuki, Makoto et al.
Citation	Human Molecular Genetics. 2024, 33(22), p. 1975-1985
Version Type	AM
URL	https://hdl.handle.net/11094/103591
rights	
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

The University of Osaka

Compromised actin dynamics underlie the orofacial cleft in Baraitser-Winter Cerebrofrontofacial Syndrome with a variant in *ACTB*

Takayuki Tsujimoto[1], Yushi Ou[2], Makoto Suzuki[2], Yuka Murata[1], Toshihiro Inubushi[1], Miho Nagata[3], Yasuki Ishihara[3], Ayumi Yonei[4], Yohei Miyashita[3], Yoshihiro Asano[3], Norio Sakai[4,5,6], Yasushi Sakata[3], Hajime Ogino[2], Takashi Yamashiro[1] and Hiroshi Kurosaka[1]*

1. Department of Orthodontics and Dentofacial Orthopedics, Osaka University Graduate School of Dentistry, Suita, Japan.

2. Amphibian Research Center, Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-hiroshima, Japan.

3. Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan.

4. Department of Genetic Counseling, Osaka University Hospital, Osaka, Japan.

5. Department of Pediatrics, Osaka University Graduate School of Medicine, Suita, Japan.

6. Department of Health Science, Child Healthcare and Genetic Science, Osaka University Graduate School of Medicine, Suita, Japan.

Corresponding Author: Hiroshi Kurosaka, Department of Orthodontics and Dentofacial Orthopedics,

Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan.

phone number: +81-6-6879-2958

FAX: +81-6-6879-2960

E-mail: kurosaka.hiroshi.dent@osaka-u.ac.jp

Abstract

Craniofacial anomalies encompassing the orofacial cleft are associated with >30% of systemic congenital malformations. Baraitser-Winter Cerebrofrontofacial syndrome (BWCFF) is a rare genetic disorder attributed to variants in the actin beta (*ACTB*) or actin gamma genes that are correlated with a range of craniofacial abnormalities, including cleft lip and/or palate. The underlying pathological mechanism of BWCFF remains elusive, and it is necessary to investigate the etiology of orofacial clefts in patients with BWCFF.

In this study, we identified a missense variant (c.1043C>T: p.S348L) in the *ACTB* gene of a patient with BWCFF and concomitant cleft lip and palate. Furthermore, we performed functional assessments of this variant using various disease models such as the MDCK cell line and *Xenopus laevis*. These models revealed a compromised capacity of mutated ACTB to localize to the epithelial junction, consequently affecting the behavior of epithelial cells. Additionally, we discovered that the mutated ACTB exhibited an impaired ability to bind PROFILIN1, a critical factor in actin polymerization. This defective ability may contribute to the molecular etiology of aberrant epithelial cell adhesion and migration, resulting in orofacial cleft formation in BWCFF.

Introduction

Baraitser-Winter Cerebrofrontofacial syndrome (BWCFF) is a congenital disorder characterized by diverse phenotypes, including short stature, ptosis, lissencephaly, and characteristic craniofacial features, such as hypertelorism and occasional orofacial cleft(1, 2). Actin beta (ACTB), a known causative gene of BWCFF, encodes an actin protein that forms actin filaments that comprise the cytoskeleton(3). Based on this background, the phenotypes of various organs in BWCFF are thought to be caused by malfunctioning actin complexes.

Actin performs a diverse range of functions by binding to and forming complexes with several proteins(4). Typical functions include cell migration and adhesion through homeostatic behaviors such as actin polymerization and depolymerization of actin-associated proteins(5, 6). Cell migration commences with adhesion to the extracellular matrix at the tips of the extended filopodia and lamellipodia, which serve as fulcrums for repeated traction and detachment of the cell body in the direction of movement(7). Actin filaments also localize at intercellular junctions and play critical roles in cell adhesion by forming adherence junctions via different classes of Cadherin proteins(5).

A distinctive craniofacial phenotype of BWCFF is an orofacial cleft that occurs in approximately 20% of cases(1). Orofacial cleft is the most common congenital disorder of the oral and maxillofacial regions, and developmental defects at any step of palatogenesis play a major role in its pathogenesis. In humans, secondary palate formation begins with the elongation of secondary palatal processes at approximately six weeks of gestation. The right and left secondary palatine processes, which originate

from the maxillary process, grow vertically on the lateral surface of the tongue, move upward as the processes extend, and eventually come into contact with each other at the midline to initiate fusion. At this stage, the midline edge epithelial seam (MES), an epithelial layer derived from the palatine processes, remains in the area of the palatine process fusion(8). One of the critical steps in the fusion of facial processes is the removal of the MES to achieve mesenchymal continuity(8). The mechanisms by which MES disappear have been extensively studied, including epithelial cell migration(9), apoptosis(10), epithelial-mesenchymal transformation (EMT)(11), and cell protrusion(12). Several studies have revealed the importance of polymerized actin (F-actin), which manifests as an orofacial cleft phenotype when actin activity is inhibited.

Given the genetic etiology and orofacial cleft phenotype in BWCFF, there is a compelling indication that aberrations in the actin complex during embryonic development result in an orofacial cleft. In recent years, new variants have been reported in BWCFF; however, detailed functional analyses of each variant have not yet been performed. The mechanism by which *ACTB* variants cause orofacial cleft formation remains largely unknown. Here, we identified a pathological missense variant of *ACTB* (c.1043C > T: p.S348L) in a patient with BWCFF and cleft lip and palate. We further utilized multiple disease models to elucidate the molecular and cellular etiologies of orofacial clefts in conjunction with malfunctioning actin complexes.

Results

Missense variant in actin beta *(ACTB)* (c.1043C > T:p.S348L) results in Baraitser-Winter Cerebrofrontofacial syndrome (BWCFF) with multiple craniofacial defects

A 9-year-old boy presented with systemic symptoms such as bilateral cleft lip (Figure1A) and complete cleft palate (Figure1B), thin calvarial bone (Figure1C), ptosis, hearing impairment, otitis media, hypertelorism, ventricular septal defect, atrial septal defect, small intestinal stenosis, cryptorchidism, and intellectual disability. The patient remained undiagnosed and underwent trio exome analysis along with his unaffected parents.

Subsequently, we identified a missense variant in the final exon of the *ACTB* gene (c.1043C>T: p.S348L), which led to the genetic diagnosis of BWCFF (Figure 1A–D). This variant is located in exon 5 and has been previously reported to cause BWCFF (13, 14) (Figure 1E). The most commonly reported variants of BWCFF in *ACTB* are heterozygous missense mutations, such as the present mutation (Figure 1E). This variant was not found in databases, such as gnomAD (https://gnomad.broadinstitute.org/) or ToMMo (54KJPN) (https://jmorp.megabank.tohoku.ac.jp/downloads/tommo-54kjpn-20230626-gf_snvindelall), indicating that it is extremely rare. Multiple in silico algorithms indicated high pathogenicity, with a CADD score of 33 and a PROVEAN score of -3.31.

Actin beta *(ACTB)* expression in embryonic frontonasal process and fusing secondary palatal

shelves

In situ hybridization of E9.5 mouse embryo demonstrated widespread expression of *ACTB* mRNA throughout the body, with certain tissues exhibiting higher expression, such as the frontonasal process and first branchial arch (Figure 2A). Notably, as embryonic development progressed, an increase in the contrast of expression was observed, with greater intensity in the frontonasal processes, branchial arches, and limb buds in the E10.5 embryo (Figure 2B). Notably, a strong signal of *Actb* was detected in the epithelium of the growing palatal shelves and midline edge epithelial seams (MES) at the fused secondary palatal shelves (Figure 2C and E). Furthermore, immunohistochemistry for ACTB together with the epithelial marker E-cadherin revealed a strong ACTB signal in the epithelial seam of the secondary palate (Figure 2G-J, Supplemental Figure 1). However, it is essential to note that the signal was ubiquitously detected throughout various tissues, including oral epithelial and mesenchymal cells. This is attributable to its function as a housekeeping gene across diverse tissues, with its expression level being subject to context-dependent alterations. No negative controls for *in situ* hybridization or immunohistochemistry showed detectable signals (Figures 2D, F, and K). It is also important to note that the negative control, in both *in situ* hybridization and immunohistochemistry, did not show any detectable signal of *Actb* (Supplemental Figure 2).

Overexpression of mutant ACTB (p.S348L) in MDCK cells affects cell migration

One of the major mechanisms preventing secondary palate fusion is retarded palatal shelf conversion and MES cell migration(9). Therefore, we used MDCK cells overexpressing GFP-labeled ACTB (p.S348L) for the migration assays. We observed that the mutant ACTB (p.S348L)-overexpressing cells displayed slower migration over time than the wild-type ACTB-overexpressing cells (Figure 3A–D). Furthermore, the area of cell migration was measured over time by repeating the experiment thrice, revealing that the population of mutant ACTB (p.S348L)-overexpressing cells had predominantly smaller areas filled with cell migration 7 h after the beginning of cell culture, which eventually closed the wound (Figure 3M). These findings indicate that overexpression of mutant ACTB (p.S348L) adversely affects epithelial cell migration and conversion of the palatal shelf.

Mutant ACTB (p.S348L) fails to localize at epithelial cell junctions

From a detailed observation of MDCK cells overexpressing ACTB in a previous experiment, we noticed that mutant ACTB (p.S348L) in MDCK cells showed a different localization than wild-type ACTB. Wild-type ACTB showed a strong signal at the cell junction, which overlapped with the phalloidin staining and detected F-actin (Figure 3E and G). In contrast, the mutant ACTB (p.S348L) did not show an intense signal at the cell junction but rather showed uniform expression in the cytoplasm (Figure 3F and H). In both experiments, the luminance value of GFP at the cell junction was significantly lower in cells overexpressing the mutant ACTB (p.S348L) (Figure 3N).

To confirm that this phenomenon was also true *in vivo*, we used animal cap cells of developing *Xenopus leavis* embryos, which were overexpressed with mutant ACTB (p.S348L). Wild-type ACTB showed a strong signal at the epithelial cell junction, similar to that observed in MDCK cells (Figure 3I and K). Interestingly, the mutant ACTB (p.S348L) in *Xenopus leavis* animal cap cells failed to localize to the cell-cell junction (Figure 3J and L). These results indicate that the variant detected in the present BWCFF case inhibited ACTB from localizing to the epithelial cell-cell junction.

Effect of mutated ACTB (p.S348L) on actin dynamics

The dynamic behavior of actin molecules, encompassing both polymerization and depolymerization, plays a pivotal role in modulating the homeostatic equilibrium of the actin complex, which is critical for various cellular activities(15). For example, actin proteins assemble into fibrous actin filaments that are crucial for cell adhesion and migration(5, 6). Latrunculin A compound inhibits actin polymerization through its binding affinity to the ATP-binding domain of actin proteins(3, 16). In this study, we observed cell surface shrinkage and fragmentation of GFP-labeled wild-type ACTB at the edges of MDCK cells after treatment with Latrunculin A (Figure 4A and B). Cells treated with mutant ACTB (p.S348L) also showed shrinkage after treatment with Latrunculin A whereas GFP-labeled ACTB did not exhibit a fragmented appearance (Figure 4D and E). The fixed cells were stained with phalloidin to detect F-actin. Multiple fragmented yellow dots with overlapping phalloidin (red) and

GFP-labeled wild-type ACTB (green) were observed (Figure 4C). In contrast, MDCK cells overexpressing mutant ACTB (p.S348L) did not show fragmented GFP at the cell junction, whereas the endogenous actin cable, which was marked by phalloidin, exhibited fragmentation similar to that of cells overexpressing wild-type ACTB (Figure 4F). These results indicate that the overexpressed mutant ACTB (p.S348L) was not incorporated into endogenous F-actin, which is marked by phalloidin, and thus did not change its behavior upon treatment with the actin polymerization inhibitor Latrinculin A.

Mutant ACTB (p.S348L) reduces its binding affinity with PROFILIN1 (PFN1)

The dynamics of the actin complexes are supported by various actin-associated proteins. In the protein database (RCSB.org)(https://www.rcsb.org/structure/2btf)(PDB ID:2BTF), the ACTB variant (p.S348L) was located adjacent to the PFN1 binding site(17) (Figure 4G). Therefore, we investigated the role of PFN1, an actin-binding protein that binds to the ACTB monomer and facilitates ADP-ACTB nucleotide exchange, leading to ATP-ACTB storage and induction of actin filament polymerization. Immunoprecipitation with an antibody against GFP was performed using cellular extracts of both wild-type and mutant ACTB (p.S348L)-overexpressing MDCK cells. Immunoprecipitated GFP-labelled ACTB was incubated with exogenous PFN1 and subjected to western blot analysis. We detected similar expression levels between the mutant (p.S348L) and wild-

type ACTB, while the signal of PFN1 was noticeably reduced in the sample with mutant ACTB (p.S348L) (Figure 4H). These results suggest that the variant compromises the affinity of ACTB for PFN1, which in turn could lead to the inhibition of actin polymerization. To test this hypothesis, we overexpressed RFP-labeled PFN1 in conjunction with mutant ACTB (p.S348L) to evaluate its behavior. As previously mentioned, overexpressed wild-type ACTB was present at the cell junction with phalloidin (Figure 4I and J), whereas the mutant ACTB (p.S348L) lacked this ability (Figure 4K and L). Interestingly, PFN1 overexpression partially restored the localization of mutated ACTB (p.S348L) to the cell junction (Figure 4M and N). These findings imply that the presence of mutated ACTB (p.S348L) reduces its binding affinity for PFN1, resulting in aberrant behavior.

Genome editing of *ACTB* in *Xenopus* embryos resulted in a craniofacial phenotype

To further investigate the influence of mutant ACTB on craniofacial development, genome editing using CRISPR/Cas9 was performed on *X. laevis* embryos. The sequence of the guide RNA to two *ACTB* homologs, *actb.L* and *actb.S* is shown in green, which could make a double-strand break (DSB, yellow arrowhead) at approximately S348, as indicated in red (Figure 5A and B). Sequencing of the targeted region was performed in F0 crispant embryos, and the results showed that more than 90% of the cells exhibited indel mutations (Supplemental Figure 2). Compared with the linear shape of the mouth opening in the uninjected (Figure 5C, F, and I) and *tyr* F0 mutants (Figure 5D, G, and J), the

experimental *actbL/S* crispants exhibited a substantial increase in embryos manifesting craniofacial deformities, including facial clefts, which are shown by a small mouth opening and an obvious notch at the upper jaw (Figure 5E, H, and K). Additionally, intercanthal distance and mouth width were measured in each group (Figure 5L). We detected a significant reduction in both lengths in the *actb.L/S* crispant group, whereas no significant difference was observed between the uninjected and *tyr.L/S* crispant groups (Figures 5M and N). Interestingly, supplementation with wild-type ACTB ameliorated the intercanthal distance and mouth width phenotypes of crispants, whereas mutated ACTB (p.S348L) did not (Figures 5O, P, and Q). These results strongly indicated the impact of mutated *ACTB* during embryonic craniofacial development *in vivo*.

Discussion

The spectrum of diseases associated with mutations in the actin family is defined as actinopathies, which are characterized by a wide range of systemic phenotypes depending on the class of actin that harbors a variant(1). ACTB and actin gamma are non-muscle actin proteins that are the major components of the cytoskeleton. Thus, these variants, including BWCFF, have a wide range of effects throughout the body (18). The case of BWCFF presented here exhibited a *de novo* missense variant in Exon5 of *ACTB* (p.S348L) with multiple craniofacial phenotypes, including cleft lip and palate.

ACTB is a housekeeping protein ubiquitously expressed in most cellular entities within an organism.

Notably, based on our expression analysis, *ACTB* exhibited widespread distribution, with certain tissues demonstrating heightened expression in the mouse embryo, specifically in the maxillary complex at E10.5, or the epithelial seam during secondary palate fusion at E14.5 (Figure 1). ACTB functions as a major component of stress fibers in cell-cell junctions and peripheral edges of cells, depending on the cellular subtype and state(19). These results suggest that different cells require ACTB at varying levels, as certain cells display greater intensity than others. This divergence in tissue-specific and status-dependent ACTB levels may underlie the mechanism governing the disparate phenotypic characteristics observed in BWCFF across distinct tissues.

Based on these results and the significance of epithelial cell behavior in palatal fusion, we investigated the biological importance of ACTB (p.S348L) in an epithelial cell line (MDCK). MDCK cells are commonly employed to model epithelial cell behavior, including migration, during wound healing, and are occasionally used to simulate palatal fusion(20). Interestingly, the group of cells overexpressing ACTB (p.S348L) showed slower wound closure than cells that overexpressed wild-type ACTB (Figure 3A-D). The epithelial tissue between the fused facial prominences must be removed via several mechanisms. Among these, epithelial cell migration governed by actomyosin contractility is critical for palatal fusion, and its retardation results in facial clefts(9, 12). Additionally, epithelial cells have been shown to require proper actin polymerization and lamellipodia formation for migration(21). These results suggest that the overexpression of mutant ACTB (p.S348L) slows down

epithelial cell migration, which could influence the behavior of the embryonic palatal epithelium. Moreover, we observed a diminished signal for the overexpressed mutant ACTB (p.S348L), in contrast to the wild-type ACTB, at the intercellular junctions of MDCK cells. Remarkably, this phenomenon was reproduced in the animal cap cells of *Xenopus* embryos (Figure 3I-L). The animal cap of Xenopus left *in situ* eventually develops into ectodermal tissue and is thus used to investigate the role of exogenous proteins in ectodermal cells. In particular, a combination of animal caps and MDCK cells has been used to model embryonic epithelial behavior and molecular dynamics at cell-cell junctions (22). In this set of experiments, we revealed that ACTB (p.S348L) loses its ability to localize to the epithelial cell-cell junction both *in vitro* and *in vivo*. Actin polymerization is also critical for the proper localization of actin bundles in cells, such as stress fibers and cell-cell junctions(23). During the growth and fusion of the embryonic facial prominence, the surface ectoderm cells must contact the opposing palatal shelf, which requires epithelial adhesion. Notably, variants of the E-cadherin–P120 catenin complex, a vital constituent of adherens junctions that are crucial for epithelial adhesion, have been associated with familial facial clefts in humans(24). Furthermore, the polymerized actin complex plays a critical role in epithelial adherens junctions by interacting with various classes of cadherins and catenin proteins(25). The limited ability of ACTB(p.S348L) to localize to cell-cell junctions in epithelial cells observed in this study may reflect a defect in actin polymerization, consequently impairing surface epithelial cell adhesion and palatal shelf convergence in the affected individual and

contributing to the orofacial clefting phenotype.

To further investigate the mechanism underlying the effect of retarded cell migration and mislocalization, we focused on PFN1, a protein critical for actin polymerization that has been shown to bind to the adjacent area of ACTB (p.S348L)(15, 26). Immunoprecipitation showed that wild-type ACTB effectively precipitated PFN1, whereas the quantity was noticeably diminished by mutant ACTB (p.S348L) in MDCK cells. Notably, the mutant ACTB (p.S348L) regained its ability to localize at the cell-cell junction when PFN1 was simultaneously overexpressed (Figure 4H-N). These results indicated that the reduced ability of mutant ACTB(p.S348L) to bind PFN1 underlies the mechanism of the malfunctioning protein. A missense variant could be associated with protein malfunction due to misfolding, altering the affinity with associated proteins, in this case, possibly PFN1.

Inhibition of actin polymerization using latrinculin-A in ACTB overexpressed cells also revealed noticeable actin fragmentation with wild-type ACTB but not with mutant ACTB (p.S348L) (Figure 4A-F). These results indicated the effect of p.S348L on the behavior of ACTB. First, it is possible that actin polymerization is retarded because actin polymerization is required for proper F-actin localization, such as in cell-cell junctions. Second, the p.S348L variant may render F-actin resistant to disassembly. Based on these results, the mutant ACTB (p.S348L) showed a reduced ability to bind to PFN1, supporting the first theory. However, further studies are essential to confirm the effect of p.S348L on actin polymerization.

We conducted a functional assay employing CRISPR/Cas9 to perturb the *Actb* gene, with the guide RNA strategically inducing a double-stranded break around serine (S) at the 348th position of *actb* in developing *Xenopus* embryos, resulting in craniofacial defects (Figure 5). This outcome strongly implies that ACTB (p.S348L) exerted a considerable influence on the etiology of the craniofacial defects in the present case of BWCFF.

However, the F0 crispants used in this study mostly carried indel mutations around ACTB (p.S348), instead of the exact missense mutation found in the patient (ACTB (p.S348L)). Investigating how PFN1 modulation can alter the phenotype of ACTB (p.S348L) *in vivo* will be a future direction for the development of novel therapeutic methods for BWCFF. It is also important to note that there is a substantial difference in embryonic craniofacial development between humans and Xenopus, particularly in terms of the growth and fusion of different facial prominences, between humans and *Xenopus*. Therefore, different animal models, such as mice whose craniofacial development closely resembles that of humans, are required for further functional validation of this mutation.

Previous studies investigated the role of ACTB (p.S348L)(13, 14). Several common human phenotypes involving craniofacial anomalies have been observed in these studies. However, other studies did not explicitly describe the presence of any form of orofacial cleft in these patients. There are several possible explanations for the phenotypic variation within the same variant in patients with *BWCFF*. First, subtle manifestations of orofacial cleft may have been disregarded. In particular,

microclefts or submucous clefts are sometimes challenging to detect in newborns, and some patients remain undiagnosed. Second, the prevalence of orofacial cleft could be influenced by genetic background; the East Asian population exhibits a higher incidence of orofacial cleft than other populations, which influences the phenotypic variation among BWCFF patients(1, 3, 27). There is speculation regarding East Asian specificity in exhibiting certain genetic factors as modifiers that increase the incidence of orofacial clefts; however, this requires further confirmation. Investigating the relationship between race-specific genetic influences and ACTB in future studies would be intriguing.

Functional assays involving different variants of *ACTB* and employing various models have been proposed, with certain variants demonstrating a gain-of-function in specific cell types, including lymphoblasts and yeast cells(3, 13, 28). To the best of our knowledge, this is the first study to assess the biological implications of *ACTB* variants associated with BWCFF in epithelial tissue and orofacial cleft development. Additionally, there have been proposals positing a defect in cranial neural crest cell development due to an *ACTB* variant based on both the craniofacial phenotype of BWCFF and animal studies(1, 29). *Actb* null embryos exhibit increased cell death in pre-migratory neural crest cells, which could be one of the underlying mechanisms of the broad craniofacial phenotype(29). Therefore, it is reasonable to surmise that ACTB (p.S348L) profoundly influences cranial neural crest cell development, synergistically, leading to a myriad of craniofacial defects.

Materials and Methods

Molecular analysis

In collaboration with the Initiative on Rare and Undiagnosed Diseases, we conducted a trio-exome analysis of a family presenting with a patient exhibiting systemic phenotypes, including bilateral cleft lip and palate. Informed consent was obtained from the patients and their parents, in accordance with the institutional review board. Whole-exome sequencing was performed on genomic DNA extracted from the peripheral lymphocytes of patients and their families using the SureSelect Human All Exon Kit V6 (Agilent Technologies, Santa Clara, CA, USA) and sequenced on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA). We checked the quality of the FASTQ files using FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and removed low-quality reads using trimmomatic-0.36 (http://www.usadellab.org/cms/?page=trimmomatic). Quality-checked reads were aligned to GRCh37 using the Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/), and variants were called using the GATK HaplotypeCaller. These genes were annotated using ANNOVAR (http://annovar.openbioinformatics.org/en/latest/). In silico analyses of the variants were performed using CADD (http://cadd.gs.washington.edu/) and PROVEAN (http://provean.jcvi.org/seq_submit.php). High-frequency (minor allele frequency > 5% in the Japanese population) synonymous and intergenic variants were filtered manually.

***In situ* hybridization, immunohistochemistry and immunocytochemistry**

Pregnant wild-type ICR mice were purchased from CLEA Japan, Inc.. Dissected embryos were fixed in 4% Paraformaldehyde overnight at 4 ℃. Immunohistochemistry was performed using the M.O.M. Immunodetection Kit (VECTOR) in accordance with the manufacturer's protocol. Briefly, 12-μm thick frozen sections were incubated with selected primary antibodies that recognize ACTB (Proteintech) and E-cadherin (Cell Signaling Technology) after heat-induced antigen retrieval. The samples were subsequently incubated with appropriate secondary antibodies for 1 h at 37 ℃ and counterstained with DAPI (Sigma-Aldrich). The cultured cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.1% Triton X-100 for 5 min. The samples were counterstained with Alexa Fluor 594 or 350 phalloidin (Invitrogen; 1:250) for 1 h and DAPI (Sigma-Aldrich) (1:1,000) for 20 min to visualize F-actin and the nuclei. Negative control of immunohistochemistry was performed without the primary antibody. The samples were mounted with a fluorescent mounting medium (DakoCytomation) and visualized using either a Leica TCS SP8 (Leica) or a BZ-X710 All-in-one Fluorescence Microscope (Keyence). *In situ* hybridization was performed as previously described(30). The primer sets used to produce the antisense oligo probe of mouse *Actb* were as follows: sense 5-CACACCTTCTACAATGAGCTGC -3 and antisense 5-GGCATAGAGGTCTTTACGGATG-3 according to the Allen Brain Atlas (https://atlas.brain-map.org/). A negative control was prepared using

the sense probe. Following *in situ* hybridization, heads were embedded in Tissue-Tek (OCT compound, Sakura), and cut into 20-μm-thick frozen sections for histological observation.

Plasmid construction

The nucleotide sequences encoding *ACTB*, its mutated form (MANE Select *ACTB*(NM_001101.5):c.1043C>T (p.Ser348Leu) GRCh37 Chr7:5567464) and *PFN1* were amplified and cloned into the pcDNA3-GFP vector to produce a fusion protein of ACTB and PFN1 with EGFP and RFP, respectively, under the control of the CMV promoter. This was performed using the In-Fusion HD Cloning Plus Kit (Takara Bio, Shiga, Japan), following the manufacturer's instructions. For *Xenopus*, GFP-fused Actb WT and S348L in pcDNA3-GFP were amplified using KOD One (TOYOBO, Osaka, Japan) and cloned into pCSf107mT(31) using the In-Fusion HD Cloning Kit (Takara, Shiga, Japan) resulting in pCSf107mT-GFP-Actb WT and S348L. mCherry was amplified and cloned into the pCS2p vector to generate pCS2p-mCherry. The sequences of these plasmids were confirmed by Sanger Sequencing. pCS2-mRFP-CAXX (membrane-targeted mRFP) has been previously described (32).

mRNA preparation

Capped mRNAs for microinjection into *Xenopus* embryos were synthesized using the mMessage

mMachine SP6 Transcription Kit (AM1340; Thermo Fisher Scientific, MA, USA) and purified by phenol/chloroform extraction using the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands).

Guide RNA and Cas9 protein preparation

A crRNA (5'-CATCTGCTGGAAGGTGGACA-3') was designed to target the exon 6 of *X. laevis actb.L* and *actb.S* genes with *Xenopus* genome database (http://viewer.shigen.info/xenopus/) and CHOPCHOP(33). crRNA, tracrRNA (#1072533), and Cas9 (#1081059) were purchased from IDT (Coralville, MD, USA). The crRNA-tracrRNA duplex was formed as described previously (34). The background effect of genome editing was assessed by designing a guide RNA for two tyrosinase homologs, *tyr.L* and *tyr.S* as previously described(35, 36). The Cas9 protein and gRNAs were incubated at 37 ℃ for 5 min in a buffer containing 150 mM KCl and 20 mM HEPES (pH 7.5) to form Cas9-gRNA RNP complexes, then kept at room temperature until microinjection.

Embryo manipulation and microinjection of *Xenopus*

Wild-type *X. laevis* adults were purchased from Watanabe Zoushoku (Hyogo, Japan) and kept in a recirculating aquarium system with a water temperature of 20 ℃. *In vitro* fertilization, dejellying, and embryo staging were performed as previously described(37, 38).

For mRNA overexpression, 200 pg GFP-Actb WT or S348L mRNA and 250 pg RFP-CAAX mRNA

were injected into the animal pole of two-cell stage embryos in 3% Ficoll/0.3 × Marc's Modified Ringer's medium (MMR). The injected embryos were cultured in the same medium until they reached stage 9.

For the Cas9-RNP injection, 20 nL of the injection mixture containing 300 pg gRNA, 4 ng Cas9 protein, and 100 pg mCherry mRNA was injected radially (10 nL × 2) into the marginal zone of one-cell-stage embryos in 3% Ficoll/0.3 × MMR within 40 min of fertilization. The injected embryos were kept in the same medium at 22 ℃ until the following day, then cultured in 0.3 × MMR until the appropriate stages at 22 ℃.

Genotyping

Tailbud stage embryos with uniform mCherry fluorescence were treated at 95 ℃ for 5 min in the lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, and 0.1% Nonidet P-40), then digested with Proteinase K (Takara) at 55 ℃ overnight. Heat-inactivated supernatants were collected after the centrifugation of crude genomic DNA samples. The 324-bp *actb.L* target region was amplified from crude DNA samples using KOD FX Neo (TOYOBO). The amplified PCR products were purified using the EnzSAP PCR Clean-Up Reagent Kit (Edge BioSystems, CA, USA) and analyzed by direct sequencing. The sequences of PCR primers used are as follows: forward primer, 5'-GCACCATGAAAATCAAGGTATG-3'; reverse primer, 5'-AAGAGTAAAGCCATGCCAATGT-3'.

The efficiency of indel formation was analyzed using the CRISPR Edits tool(39).

Imaging of *Xenopus* embryos

Stage 9–10 embryos were mounted in 1% low-melting agarose/0.3 × MMR on a glass-based dish (3910-035; AGC, Shizuoka, Japan). Confocal fluorescence images were acquired using FV1000-D (Olympus, Tokyo, Japan) with a 40× (UPLSAPO 40x NA0.90; Olympus) objective lens at a room temperature adjusted to 18–20 ℃. Bright-field images were acquired using an Axio Zoom.V16 zoom microscope (Zeiss, Oberkochen, Germany) and analyzed using ZEN (Zeiss) and Fiji software. The violin plot was created using the ggplot package in RStudio based on measurements of the intercanthal distance and mouth width, as described previously (40).

Cell lines and cell culture

MDCK cells (JCRB cell bank: IFO50071) were cultured in αMEM with nucleosides (Gibco) and 10% FBS (Gibco) under standard conditions. For electroporation, MDCK cells were transfected with overexpression vectors containing either wild-type or mutant (c.1043C > T) ACTB in Opti-MEM (Gibco) using a NEPA21 electroporator (Nepagene). Five square pulses of 50-ms duration with 50-ms intervals at 20 V were applied.

For migration assays, MDCK cells transfected with either construct seeded onto fibronectin-coated

35 mm glass bottom dishes (Matsunami Glass Ind., Ltd) in a two-well silicone insert with a defined cell-free gap (Ibidi). After the cells reached confluence at 24 h, the culture insert was removed, and the area of cell movement was quantified 10 h after removing the silicone insert.

MDCK cells transfected with either construct were treated with Latrunculin A (AdipoGen) at a concentration of 100 uM. Live imaging was performed using a BZ-X710 All-in-one Fluorescence Microscope (Keyence, Osaka, Japan).

Immunoprecipitation and Western Blot

Sample protein was extracted from MDCK cells which was transfected with GFP-tagged wild-type ACTB and mutant ACTB (p.S348L) which was mixed with the recombinant PFN1 to let them bind to extracted protein with a concentration of 2 μM. The GFP-Trap agarose kit (Chromotek) was used for immunoprecipitation according to the manufacturer's protocol.

The pulled-down proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). Membranes were then incubated with anti-ACTB (Proteintech) and anti-PFN1 (Santa Cruz Biotechnology) antibodies. The bound antibodies were detected using horseradish peroxidase-conjugated antibodies (Cell Signaling Technology) and an ECL detection kit (Bio-Rad Laboratories), according to the manufacturer's protocol.

Statistics

The Mann-Whitney test with Bonferroni adjustment is shown in Figure 5. Two-tailed Student's *t*-tests were performed for data presented in Supplementary Figure 3. P was set at $p < 0.05$ in all experiments.

Data availability

The data that support the findings of this study are not publicly available but are available from the corresponding author upon reasonable request.

Ethics declaration

Written informed consent was obtained from patients for the publication of their photographs. This study was reviewed and approved by the Committee on the Ethics of Animal Experiments of Osaka University Graduate School of Dentistry and the Ethics Committee of Osaka University.

Acknowledgements

We thank the National BioResource Project (Clawed Frogs/Newts) of MEXT at the Hiroshima University Amphibian Research Center (RRID: SCR_019015) for their technical assistance. We thank Dr. Shoichiro Ono for providing valuable insights in conducting this study. We wish to express our

sincere appreciation to the patients and their families for their consent to participate in this study.

Funding

This study was supported in part by grants from the Initiative on Rare and Undiagnosed Diseases project of the Japanese Agency for Medical and Development (JP23bk0304002), Ministry of Education, Science, Sports, and Culture of Japan (no. 19H03858 and 23K27803 to HK, 24K20055 to TT), NOVARTIS Foundation (Japan) for the Promotion of Science, and Takeda Science Foundation.

Conflict of interest statement

The authors declare that no conflicts of interest exist.

REFERENCES

1 Verloes, A., Di Donato, N., Masliah-Planchon, J., Jongmans, M., Abdul-Raman, O.A., Albrecht, B., Allanson, J., Brunner, H., Bertola, D., Chassaing, N. *et al.* (2015) Baraitser-Winter cerebrofrontofacial syndrome: delineation of the spectrum in 42 cases. *Eur. J. Hum. Genet.* , **23**, 292-301.

2 Baraitser, M. and Winter, R.M. (1988) Iris coloboma, ptosis, hypertelorism, and mental retardation: a new syndrome. *J. Med. Genet.* , **25**, 41-43.

3 Rivière, J.B., van Bon, B.W., Hoischen, A., Kholmanskikh, S.S., O'Roak, B.J., Gilissen, C., Gijsen, S., Sullivan, C.T., Christian, S.L., Abdul-Rahman, O.A. *et al.* (2012) De novo mutations in the actin genes ACTB and ACTG1 cause Baraitser-Winter syndrome. *Nat. Genet.* , **44**, 440-444, S441-442.

4 Dominguez, R. and Holmes, K.C. (2011) Actin structure and function. *Annu. Rev. Biophys.* , **40**, 169-186.

5 Braga, V. (2016) Spatial integration of E-cadherin adhesion, signalling and the epithelial

cytoskeleton. *Curr. Opin. Cell. Biol.* , **42**, 138-145.

6 Vaezi, A., Bauer, C., Vasioukhin, V. and Fuchs, E. (2002) Actin cable dynamics and Rho/Rock orchestrate a polarized cytoskeletal architecture in the early steps of assembling a stratified epithelium. *Dev. Cell.* , **3**, 367-381.

7 Trepat, X., Chen, Z. and Jacobson, K. (2012) Cell migration. *Compr. Physiol.* , **2**, 2369-2392.

8 Bush, J.O. and Jiang, R. (2012) Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development. *Development*, **139**, 231-243.

9 Aoyama, G., Kurosaka, H., Oka, A., Nakatsugawa, K., Yamamoto, S., Sarper, S.E., Usami, Y., Toyosawa, S., Inubushi, T., Isogai, Y. *et al.* (2019) Observation of Dynamic Cellular Migration of the Medial Edge Epithelium of the Palatal Shelf. *Front. Physiol.* , **10**, 698.

10 Cuervo, R., Valencia, C., Chandraratna, R.A. and Covarrubias, L. (2002) Programmed cell death is required for palate shelf fusion and is regulated by retinoic acid. *Dev. Biol.* , **245**, 145-156.

11 Shuler, C.F., Halpern, D.E., Guo, Y. and Sank, A.C. (1992) Medial edge epithelium fate traced by cell lineage analysis during epithelial-mesenchymal transformation in vivo. *Dev. Biol.* , **154**, 318-330.

12 Kim, S., Lewis, A.E., Singh, V., Ma, X., Adelstein, R. and Bush, J.O. (2015) Convergence and extrusion are required for normal fusion of the mammalian secondary palate. *PLoS Biol.* , **13**, e1002122.

13 Sibbin, K., Yap, P., Nyaga, D., Heller, R., Evans, S., Strachan, K., Alburaiky, S., Nguyen, H.M.A., Hermann-Le Denmat, S., Ganley, A.R.D. *et al.* (2022) A de novo ACTB gene pathogenic variant in identical twins with phenotypic variation for hydrops and jejunal atresia. *Am. J. Med. Genet. A*, **188**, 1299-1306.

14 Fakhro, K.A., Robay, A., Rodrigues-Flores, J.L., Mezey, J.G., Al-Shakaki, A.A., Chidiac, O., Stadler, D., Malek, J.A., Imam, A.B., Sheikh, A. *et al.* (2019) Point of Care Exome Sequencing Reveals Allelic and Phenotypic Heterogeneity Underlying Mendelian disease in Qatar. *Hum. Mol. Genet.* , **28**, 3970-3981.

15 Ono, S. (2013) The role of cyclase-associated protein in regulating actin filament dynamics - more than a monomer-sequestration factor. *J. Cell. Sci.* , **126**, 3249-3258.

16 Morton, W.M., Ayscough, K.R. and McLaughlin, P.J. (2000) Latrunculin alters the actin-monomer subunit interface to prevent polymerization. *Nat. Cell. Biol.* , **2**, 376-378.

17 Schutt, C.E., Myslik, J.C., Rozycki, M.D., Goonesekere, N.C. and Lindberg, U. (1993) The structure of crystalline profilin-beta-actin. *Nature*, **365**, 810-816.

18 Greve, J.N., Schwäbe, F.V., Pokrant, T., Faix, J., Di Donato, N., Taft, M.H. and Manstein, D.J. (2022) Frameshift mutation S368fs in the gene encoding cytoskeletal β-actin leads to ACTB-associated syndromic thrombocytopenia by impairing actin dynamics. *Eur. J. Cell. Biol.* , **101**, 151216.

19 Dugina, V., Zwaenepoel, I., Gabbiani, G., Clément, S. and Chaponnier, C. (2009) Beta and

gamma-cytoplasmic actins display distinct distribution and functional diversity. *J. Cell Sci.* , **122**, 2980-2988.

20 Yu, W., Zhang, Y., Ruest, L.B. and Svoboda, K.K. (2013) Analysis of Snail1 function and regulation by Twist1 in palatal fusion. *Front. Physiol.* , **4**, 12.

21 Ponti, A., Machacek, M., Gupton, S.L., Waterman-Storer, C.M. and Danuser, G. (2004) Two distinct actin networks drive the protrusion of migrating cells. *Science*, **305**, 1782-1786.

22 Haigo, S.L., Hildebrand, J.D., Harland, R.M. and Wallingford, J.B. (2003) Shroom induces apical constriction and is required for hingepoint formation during neural tube closure. *Curr. Biol.* , **13**, 2125-2137.

23 Vasioukhin, V., Bauer, C., Yin, M. and Fuchs, E. (2000) Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell*, **100**, 209-219.

24 Cox, L.L., Cox, T.C., Moreno Uribe, L.M., Zhu, Y., Richter, C.T., Nidey, N., Standley, J.M., Deng, M., Blue, E., Chong, J.X. *et al.* (2018) Mutations in the Epithelial Cadherin-p120-Catenin Complex Cause Mendelian Non-Syndromic Cleft Lip with or without Cleft Palate. *Am. J. Hum. Genet.* , **102**, 1143-1157.

25 Tsukita, S., Nagafuchi, A. and Yonemura, S. (1992) Molecular linkage between cadherins and actin filaments in cell-cell adherens junctions. *Curr. Opin. Cell. Biol.* , **4**, 834-839.

26 Korenbaum, E., Nordberg, P., Björkegren-Sjögren, C., Schutt, C.E., Lindberg, U. and Karlsson, R. (1998) The role of profilin in actin polymerization and nucleotide exchange. *Biochemistry*, **37**, 9274-9283.

27 Beaty, T.H., Marazita, M.L. and Leslie, E.J. (2016) Genetic factors influencing risk to orofacial clefts: today's challenges and tomorrow's opportunities. *F1000Res.* , **5**, 2800.

28 Procaccio, V., Salazar, G., Ono, S., Styers, M.L., Gearing, M., Davila, A., Jimenez, R., Juncos, J., Gutekunst, C.A., Meroni, G. *et al.* (2006) A mutation of beta -actin that alters depolymerization dynamics is associated with autosomal dominant developmental malformations, deafness, and dystonia. *Am. J. Hum. Genet.* , **78**, 947-960.

29 Tondeleir, D., Noelanders, R., Bakkali, K. and Ampe, C. (2014) Beta-actin is required for proper mouse neural crest ontogeny. *PLoS One*, **9**, e85608.

30 Kurosaka, H., Iulianella, A., Williams, T. and Trainor, P.A. (2014) Disrupting hedgehog and WNT signaling interactions promotes cleft lip pathogenesis. *J. Clin. Invest.* , **124**, 1660-1671.

31 Mii, Y. and Taira, M. (2009) Secreted Frizzled-related proteins enhance the diffusion of Wnt ligands and expand their signalling range. *Development*, **136**, 4083-4088.

32 Suzuki, M., Sato, M., Koyama, H., Hara, Y., Hayashi, K., Yasue, N., Imamura, H., Fujimori, T., Nagai, T., Campbell, R.E. *et al.* (2017) Distinct intracellular Ca2+ dynamics regulate apical constriction and differentially contribute to neural tube closure. *Development*, **144**, 1307-1316.

33 Labun, K., Montague, T.G., Krause, M., Torres Cleuren, Y.N., Tjeldnes, H. and Valen, E.

(2019) CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Res.* , **47**, W171-W174.

34 Hoshijima, K., Jurynec, M.J., Klatt Shaw, D., Jacobi, A.M., Behlke, M.A. and Grunwald, D.J. (2019) Highly Efficient CRISPR-Cas9-Based Methods for Generating Deletion Mutations and F0 Embryos that Lack Gene Function in Zebrafish. *Dev. Cell*, **51**, 645-657.e644.

35 Tanouchi, M., Igawa, T., Suzuki, N., Suzuki, M., Hossain, N., Ochi, H. and Ogino, H. (2022) Optimization of CRISPR/Cas9-mediated gene disruption in Xenopus laevis using a phenotypic image analysis technique. *Dev. Growth Differ.* , **64**, 219-225.

36 Wang, F., Shi, Z., Cui, Y., Guo, X., Shi, Y.B. and Chen, Y. (2015) Targeted gene disruption in Xenopus laevis using CRISPR/Cas9. *Cell Biosci.* , **5**, 15.

37 Nieuwkoop, P.D.F., J. (1967) *Normal Table of Xenopus laevis (Daudin)*. North-Holland Publ. Co., Amsterdam.

38 Sive, H.L., Grainger, R. M. & Harland, R. M. (2000) *Early Development of Xenopus Laevis: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

39 Conant, D., Hsiau, T., Rossi, N., Oki, J., Maures, T., Waite, K., Yang, J., Joshi, S., Kelso, R., Holden, K. *et al.* (2022) Inference of CRISPR Edits from Sanger Trace Data. *CRISPR J.* , **5**, 123-130.

40 Wyatt, B.H., Raymond, T.O., Lansdon, L.A., Darbro, B.W., Murray, J.C., Manak, J.R. and Dickinson, A.J.G. (2021) Using an aquatic model, Xenopus laevis, to uncover the role of chromodomain 1 in craniofacial disorders. *Genesis*, **59**, e23394.

Legends to Figures

Figure 1. Craniofacial appearance and Identification of the missense variant in the actin beta (*ACTB)*

(A) Facial, (B) intraoral, and (C) Computed Tomography images of the patient at the age of 6. (D) The pedigree of the present case with chromatogram and major phenotypes. (E) Summary of reported variants of *ACTB* in Baraitser-Winter Cerebrofrontofacial syndrome (BWCFF). A missense variant detected in the present study is shown in red at the final exon of the *ACTB* gene (c.1043C > T: p.S348L).

Figure 2. Expression pattern of actin beta (*Actb*) in embryonic craniofacial development

(A–D) *Actb* expression at different stages and in different tissues during embryonic development. Whole-mount *in situ* hybridization of *Actb* using E9.5 (A) and E10.5 (B) mouse embryos. (C) Ventral view of the dissected maxilla at E14.5. (D) The samples were stained with a sense probe as a negative control. (E) Frontal section of the E14.5, maxilla at the location of the black line shown in C and (D) frontal section of the negative control. Black arrowheads indicate midline edge epithelial seams (MES). (G-J) Immunohistochemistry of ACTB (red) and E-cadherin (green) in the frontal section of the embryonic head of E14.5. (H and J) Magnified images of white squares in panels G and I. (K) Negative control for immunohistochemical staining without primary antibodies against *ACTB*. FNP, frontal nasal process. Flb, forelimb bud. HIb, hind limb bud. PS, palatal shelf. Scale bar, 1 mm (A–D), 50 μm (E, F, G, I, and K), and 20 μm (H and J).

Figure 3. Functional assays of MDCK cells with overexpressed ACTB

(A–D) Time course of the wound healing assay using MDCK cells overexpressing GFP-labeled ACTB.

(A) Immediately after beginning the assay with the wild-type and (B) mutant ACTB (p.S348L). (C)

Ten hours after beginning the assay with the wild-type and (D) mutant ACTB (p.S348L). The yellow dashed line indicates the leading edge of migrating MDCK cells. Scale bar, 50 μm. (E and G) MDCK cells overexpressing either GFP-labeled wild-type ACTB or (F and H) mutant ACTB (p.S348L). White arrowheads indicate cell-cell junctions. Scale bar, 10 μm. (I and K) Images of *Xenopus* animal cap cells injected with either wild-type ACTB or (J and L) mutant ACTB mRNA with RFP-CAAX mRNA to label cell-cell junctions. Yellow arrowheads indicate epithelial cell junctions. Scale bar, 50 μm. (M) The cell migration area depicted in A, measured over time in three replicates. Blue and orange lines represent populations of wild-type and mutant ACTB (p.S348L)-overexpressing cells, respectively. (N) Luminance values of GFP at cell-cell junctions for both wild-type and mutant ACTB (p.S348L), presented in E-H. *$p<0.05$.

Figure 4. The effect of mutant actin beta (ACTB) (p.S348L) on actin dynamics

(A, B, D, and E) Live imaging of MDCK cells which were transfected with GFP labeled wild type ACTB (A) and mutant ACTB (p.S348L) (D) which was immediately treated with Latrunclin A. (B and E) Same cells with A and D after 30 min of Latrunculin A treatment. (C) Fixed MDCK cells transfected with wild-type ACTB and (D) mutant ACTB (p.S348L) treated with Latrunculin A. White arrowheads indicate cell surface shrinkage as well as fragmentation of GFP-labeled ACTB at the cell-cell junction. Scale bar, 10 μm.

(G) Three-dimensional structure of ACTB-PFN1 complex image from the RCSB PDB

(https://www.rcsb.org/structure/2btf). The red circle indicates the position of ACTB (p.S348L). (H) Immunoprecipitation of GFP-labeled ACTB from the mixture of exogeneous PFN1 and cellular extract in MDCK cells transfected with wild type and mutant ACTB (p.S348L). MDCK cells transfected with wild type ACTB (I and J) and mutant ACTB (p.S348L) (K and L). Co-transfection of mutant ACTB (p.S348L) and PFN1 recovered the localization of mutant ACTB (p.S348L) into cell-cell junction (M and N). Scale bar, 10 μm.

Figure 5. Craniofacial defects induced by disrupting *actb* in *Xenopus*

(A) Schematic drawing of the locus of *actb* S348 (red), guide RNA (green), and the predicted double-strand break (yellow). The sequences of *actb.L* and *actb.S* in this region are identical. (B) 1-cell stage embryos were selected for injecting gRNA/Cas9 and facial morphology was analyzed at Stage45/46. Frontal view of *Xenopus* face of (C, F, and I) uninjected and (D, G, and J) *tyr.L/S* crispants and (E, H, and K) *actb.L/S* crispants. (L) Schematic drawing showing the measurements for intercanthal distance and mouth width. Violin plots of intercanthal distance (M) and mouth width (N) across three groups. (O) Schematic drawing of the timing of injection of gRNA/Cas9 and WT/mutant *actb* mRNA. (P and Q) Violin plot of intercanthal distance and mouth width of each experimental group which were injected with different materials shown at the bottom of the plot. An asterisk indicates statistical significance (***P<0.001, **P<0.01). Scale bars, 500 um in C-H, 250um in I-K.

Abbreviations

BWCFF: Baraitser-Winter Cerebrofrontofacial syndrome

ACTB: actin beta

MDCK cell: Madin-Darby canine kidney cell

MES: midline edge epithelial seam

EMT: epithelial-mesenchymal transformation

GFP: green fluorescent protein

RFP: red fluorescent protein

PEN1: PROFILIN1

ADP: Adenosine diphosphate

ATP: Adenosine triphosphate