Two Different Functions of Connexin43 Confer Two Different Bone Phenotypes in Zebrafish*

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Fish remain nearly the same shape as they grow, but there are two different modes of bone growth. Bones in the tail fin (fin ray segments) are added distally at the tips of the fins and do not elongate once produced. On the other hand, vertebrae enlarge in proportion to body growth. To elucidate how bone growth occurs, we investigated a zebrafish mutant, stp (stptl28dt22d). Vertebral bone growth was found to be shorter (59–81%) than vertebrae of wild type fish in adults. In contrast, the lengths of fin rays are only slightly shorter (~95%) than those of the wild type in both larvae and adults. Positional cloning revealed that stp encodes Connexin43 (Cx43), a connexin that functions as a gap junction and hemichannel. Interestingly, Cx43 was also identified as the gene causing the short-of-fin (sof) phenotype, in which the fin ray segments are shorter but the vertebrae are normal. To identify the cause of this difference between the alleles, we expressed Cx43 exogenously in Xenopus oocytes and performed electrophysiological analysis of the mutant proteins. Gap junction coupling induced by Cx43*95* or Cx43*95* was reduced compared with Cx43-WT. On the other hand, only Cx43*95* induced abnormally high (50× wild type) transmembrane currents through hemichannels. Our results suggest that Cx43 plays critical and diverse roles in zebrafish bone growth.

Vertebrate bones develop during embryonic stages and grow continuously as body size increases. Because bone shape and size determine body shape, the mechanisms that generate and maintain bone shape are of great interest to biologists. Recent studies have increased our knowledge of bone formation mechanisms. Interactions among three types of cells—osteoblasts, osteocytes, and osteoclasts—play major roles in bone formation and bone remodeling (1–8).

Zebrafish (Danio rerio) is a model organism for developmental and genetic studies, and several mutants related to bone formation have been identified and analyzed (9–13). Mutant bone formation phenotypes can be categorized into two types: bone development mutants and bone growth mutants. Genes corresponding to bone development mutants are usually expressed at early developmental stages of bone formation (type 1), and bone malformations are detected when the bones first appear in embryo (14–16). On the other hand there are several zebrafish bone growth mutants, in which bone development looks almost normal at early stages, but mutant phenotypes appear at late stages (type 2) (17–19).

In this study, we focused on the stp (stptl28ds) mutant fish (20) to study bone morphogenesis in adult stages. The stp mutant was originally isolated by a large scale screen of zebrafish mutants induced byENU (20). This mutant looks normal until ~35 days after fertilization (dpf); however, the vertebral bone growth of the mutant fish is shorter than those of wild type fish at later growth stages. The stp allele is dominant and homozygous lethal. We re-examined the stp phenotypes in detail using modern techniques, and we performed positional cloning to identify the stp gene. Surprisingly, we isolated a mutation in the connexin43 gene, which is known as the sof (short-of-fin) gene in zebrafish (21). sof is a well studied type 2 zebrafish mutant, in which fin segments are short in the caudal fin (21). Four sof mutant alleles were isolated by mutagenesis screening, and three single mutations that cause amino acid substitutions in Connexin43 protein were identified (22). In the sof*123 allele, no amino acid substitution was identified in the connexin43 gene, but Cx43 expression was reduced in the mutant fish (21, 23, 24).

Connexin proteins are four-pass transmembrane proteins that are subunits of gap junctions (25). Approximately 20 connexin genes are known from the mammalian genome, and ~36 connexin genes are predicted in the zebrafish genome (26, 27). Six connexin proteins make a hexamer called a connexon; docking of two connexons on adjacent cells creates a gap junction. Gap junctions allow movement of small molecules (~<1000 Da; e.g. ATP, inositol trisphosphate, ions, etc.) between neighboring cells; a connexon acts as a hemichannel on the cytoplasmic membrane (25). Connexin43 is one of the most studied connexin genes because it is linked to several human diseases (28–30). Oculodentodigital dysplasia is one Cx43-related human disease, which causes small eyes, underdeveloped teeth, and malformed fingers (31, 32). To identify the cause of the substan-

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2 The abbreviations used are: ENU, N-ethyl-N-nitrosourea; AP, anterior-posterior; dpf, days after fertilization; DV, dorsoventral; TALEN, transcription activator-like effector nucleases.
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TABLE 1
SNP Markers used in positional cloning
SNP Markers were developed from comparison of sequences between AB and stp. Locations of SNP Markers are on the Zv9 assembly. Both forward (F) and reverse (R) primers were used for sequencing. All SNPs were assessed by sequencing reads.

Experimental Procedures
Animal Care—All experiments in this study were conducted in accordance with the guidelines and approved protocols for animal care and use (approval number FBS-14-002-1) at Osaka University (Osaka, Japan). We used the Tu and AB strains as wild type zebrafish lines. stoepsel (stp**/**stptl28d**) mutant was obtained from the Tubingen Zebrafish Stock Center.

Clearing and Measuring of Vertebral Length and Height—Fish samples were fixed with 3.7% formaldehyde and treated with ethanol to remove lipids. Then they were transferred back to H2O. Next, samples were incubated in a saturated aqueous solution of sodium tetraborate with 3 mg/ml trypsin) at 48 °C to clear the samples. Transparent samples were dyed with bone dye solution (90 mM KOH, 50 μg/ml alizarin red S) for 1 day and then incubated in 90 mM KOH for 1 day. Finally, samples were immersed in glycerol with thymol and preserved.

CT Scanning—A LaThera LCT-100 (Hitachi Aloka Medical, Ltd.) x-ray micro-CT scanner at Osaka Bioscience Institute and an inspeXio SMX-100CT microfocus x-ray CT system at Osaka Bioscience Institute and the Institute for Advanced Research (Osaka University, Osaka, Japan) were used to examine the bone structure. Fish samples were fixed with 3.7% formaldehyde and stored in PBS. Samples were scanned in air.

Positional Cloning and Sequence Analyses—The stp**/**stptl28d**+/+ (TE background) mutant was crossed with the wild type AB strain. TE is a mixture of Tu and TL lines. Here, we used Tu instead of TE. F1 heterozygous fish were backcrossed with wild type parents, and F2 fish were used for genetic mapping. stp was mapped to chromosome 20 by checking simple sequence length polymorphism markers. Then stp was mapped between single nucleotide polymorphism markers listed in Table 1, which were identified within chromosome 20 (40.55–41.01 Mb). Gene predictions were derived from Ensemble (Sanger Institute). Candidate gene sequencing was performed using a 3130 Genetic Analyzer (Applied Biosystems).

Rescue of stp Homozygote Phenotype—A cx43 fragment was cloned from a bacterial artificial chromosome clone (DKEY-261A18) with a forward primer (5′-GCTCGAGTCTATGAGGGATGAG-3′) and a reverse primer (5′-GGGCGCGCCGCCATAGCCTCAGGTCTAGC TTC-3′) and ligated into a Tol2 plasmid (pT2AL200150G) (33). The cx43 sequence was injected into stp**/+** embryos to generate Tg(cx43pro-cx43)stp**/+** transgenic fish lines. Tg(cx43pro-cx43)stptl28d/tl28d** transgenic fish were made by crossing stp**/+** fish and Tg(cx43pro-cx43)stp**/+** fish.

cx43 Reporter Line—The cx43 promoter sequence was cloned from a bacterial artificial chromosome clone (DKEY-261A18) with a forward primer (5′-CCGGCTCGAGCTTAAAGGGTCA CAGAAACACC-3′) and a reverse primer (5′-GCCCAGATCTTGA GAGGTCTAGCTGAAAATA-3′) and ligated into a Tol2 plasmid (pT2AL200150G). A reporter construct of cx43 was made by inserting mCherry cDNA downstream of the cx43 promoter region.

TALEN-mediated cx43 Knock-out—Transcription activator-like effector nucleases (TALEN) pairs for cx43 knock-out were designed using the TALEN Targeter program (available at the Cornell University website). TAL repeats were assembled using a Golden Gate TALEN and TAL Effector kit 2.0 (34) with some modifications (35) and cloned into expression vectors pCS2TAL3DD or pCS2TAL3RR (36). The TALEN expression plasmids were linearized by NotI digestion, and then mRNAs were in vitro transcribed using an mMESSAGE mMACHINE SP6 transcription kit (Ambion). Synthetic TALEN mRNAs were injected into one-cell stage embryos (100 pg/embryo each). To test the efficacy of mutagenesis, TALEN mRNA-injected embryos were harvested 1 day after fertilization. The genomic DNA was extracted, and the loci containing the TALEN target sequence were amplified by PCR with a forward primer (5′-TTCAAGTGCTCACAAAGACTAGTCT-3′) and a reverse primer (5′-CTGCTGGGTATTGCACTTGA-3′). TALEN-induced mutations were detected by T7 endonuclease I assay. The most effective TALEN pair was used for cx43 knock-out. The target sequence is 5′-TGGGATGAG-3′ and is located downstream of Tg(cx43pro-cx43)stptl28d/tl28d**/++/+** transgene modules were amplified by PCR with a forward primer (5′-TGGGATGAG-3′) and a reverse primer (5′-TGGGATGAG-3′) and inserted into a Tol2 plasmid (pT2AL200150G). A reporter construct of cx43 was made by inserting mCherry cDNA downstream of the cx43 promoter region.

Measurement of mRNA Expression for cx43 Alleles—To precisely compare the expression levels of the wild type and mutated alleles, which differ at only one nucleotide, we generated cDNA clones from the heterozygous fish and then sequenced them. mRNAs were collected from tail fins of stp**/+** fish and stp**/+**/sof**/+** fish by RNeasy Protect mini kit (Qiagen), respectively. Then mRNAs were reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen). The cx43 sequence around the stp mutation site was amplified from the cDNAs, and the amplified fragments were cloned with plasmid vector. Then Escherichia coli colonies were picked up randomly, and DNA fragments in the plasmid were sequenced. The frequencies of DNA fragments derived from stp allele or wild type allele in stp**/+**/sof**/+** fish and DNA fragments from the stp allele and sof allele in stp**/+**/sof**/+** were compared.
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Preparation of Connexin mRNA and Injection into Xenopus Oocytes—Connexin cDNAs were amplified using PCR and cloned into pGEM-HeFx plasmid (37). The plasmids were linearized using restriction enzyme and then applied to the mRNA preparation kit (T7 mMessage mMachine; Ambion) according to the manufacturer’s protocol. An adult Xenopus laevis female was anesthetized with MS-222, and the ovarian lobes were collected using a surgical knife and forceps. The eggs were treated with collagenase solution (2 mg/ml collagenase I (Sigma) and 2 mg/ml hyaluronidase (Sigma) in OR2 buffer: 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES, adjusted to pH 7.5 using NaOH) at 18 °C for 2 h. Stage V and VI oocytes were collected manually and used for mRNA injection. Then mRNA (0.05 ng for gap junction recording; 5 ng for hemichannel recording) was co-injected with 10 ng of antisense oligonucleotide DNA for Xenopus cx38 into Xenopus oocytes. For the negative control, only antisense oligonucleotide was injected. Oocytes injected with mRNA were incubated overnight at 18 °C in 1/2× diluted L15 medium (Sigma) with 2 mM CaCl₂ (HEPES-buffered and adjusted to pH 7.5 with NaOH).

Voltage Clamp Recording—Vitelline membranes of mRNA-injected oocytes were removed manually using forceps in a hypertonic solution (200 mM aspartic acid, 1 mM MgCl₂, 10 mM EGTA, 20 mM KCl, and 10 mM HEPES, pH 7.5). The oocytes were manually paired, touching at the vegetal poles, while in ND96 (+) solution (93.5 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂, and 5 mM HEPES). The transjunctional current was measured using the whole cell dual whole cell voltage clamp technique. Current and voltage electrodes were prepared with a micropipette puller P-1000 (Sutter Instruments) to obtain a resistance of 0.5–1.0 MΩ. The pipette was filled with solution containing 3 M KCl, 10 mM EGTA, and 10 mM HEPES (pH 7.4). Voltage clamp experiments were performed using two Multi Electrode Clamp Amplifiers iTEV90 (HEKA). To measure the transjunctional current, both cells were initially clamped at −40 mV, and one cell was then subjected to alternating pulses of from −160 to +80 mV in 20-mV increments. Currents detected in the second oocyte were recorded, and junctional conductance was calculated using the current value at the end of each 3-s pulse (steady state). The conductance was obtained by G₀ = I₀/(V₁ − V₀), and normalized G values were compared.

Hemichannel Recording—Hemichannel currents were recorded from a single oocyte using a Multi Electrode Clamp Amplifier iTEV90. Modified ND96 (+) solution with/without 2 mM CaCl₂ was used as a bath solution. To obtain the hemichannel current, the cells were initially clamped at −40 mV and then subjected to 5-s voltage steps from −30 to +60 mV in 10-mV increments.

Western Blotting and Immunofluorescence Staining—Xenopus oocytes, in which the antisense oligonucleotide for Xenopus cx38 and mRNA for cx43, cx43Δsp, or cx43sp were injected, were harvested after overnight incubation. Then each oocyte was subjected to alternating pulses of from −60 mV in 10-mV increments.

Results

Stoepel (stp1226d) Phenotype—The stp mutation was isolated in a large scale screen of zebrafish mutants induced by ENU (20). In the original paper, stp was described as a mutant associated with abnormalities of the axial skeleton (Fig. 1, A and A’). stp homozygous (stp1226d/1226d) fish die from edema of the eye and abdomen at −10 dpf (Fig. 2, A–C). Because wild-type zebrafish start to form vertebrae along the notochord at −14 dpf, we could not observe the bone phenotype in stp1226d/1226d fish. Heterozygous mutants (stp1226d+/−) had similar body lengths to the wild type at early stages, but during later growth they gradually became shorter relative to the wild type along the anterior-posterior (AP) axis (Fig. 1, A and A’). The number of vertebrae appeared similar between the mutant and wild type fish. The body height along the dorsoventral (DV) axis was not affected by the stp mutation (Fig. 1, B and B’).

Next, we examined the bone phenotype in detail by microCT scanning (Figs. 1, B–F and B’–F’, and 3). AP length (Fig. 3A), DV height (Fig. 3B), and the proportions (Fig. 3C) of corresponding vertebrae (identification numbers from anterior to posterior) were compared between wild type and stp mutant fish. Vertebrae in stp1226d+/− fish were obviously shorter along the AP axis than those of wild type fish (Fig. 3A), whereas the head and fins did not differ notably (Fig. 1, B and B’).

Each vertebra is composed of a centrum and appendixes, which include a ventral arch (Fig. 1, C, C’, D, and D’, red arrowheads), hemal arch (Fig. 1, C, C’, D, and D’, green arrowheads), and complex beam structures (Fig. 1, C and C’, blue arrowheads). The centrum appears at −14 dpf. The appendixes form after centrum growth. In a wild type centrum, an hourglass-like structure was seen along the AP axis (Fig. 1F). On the other hand, in stp1226d+/− mutants, the hourglass-like structure was distorted and sometimes filled with clots (Fig. 1, D’ and E’). Although the centrum was malformed in the stp1226d+ mutant, the hemal arch (Fig. 1, C’ and D’, green arrowheads), neural arch (Fig. 1, C’ and D’, red arrowheads), and beam structures (Fig. 1C’, blue arrowhead) were approximately normal. These data indicate that the stp mutation mainly affects the formation of the centrum of the vertebrae.

Next, to reveal when vertebral malformation occurred, we investigated the time course of centrum growth (Fig. 1, G–I). We measured the AP length (Fig. 1G) and DV height (Fig. 1H) of the centrum of caudal (No. 1–5) vertebrae (Fig. 3). Graphs in Fig. 1 (G–I) show that early vertebral development (35 dpf) was not affected by the stp mutation; however, after 35 dpf, centrum growth was abnormally low along the AP axis (Fig. 1G), but not along the DV axis (Fig. 1H). This suggests that the stp mutation affects the mechanism that maintains appropriate vertebral shape during growth.

Cloning the Gene Affected by the stp Mutation—To identify the gene affected by the stp mutation, we next performed positional cloning and identified a critical region of 460 kb in chromosome 20 (Fig. 4A). This region contains eight genes, of which
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TALEN mRNA was injected into embryos of was designed to target the middle region of the first extracellular expression in vertebrae, we generated transgenic fish that were sequenced, and their amino acid sequences were

We also examined the lethality of the stp homozygote. If the lethality was caused by the loss of normal cx43 protein, survival of 

To confirm that the mutation in cx43 mRNAs were highly conserved in vertebrates (Fig. 4C). A BLAST search revealed that the region around the mutation was highly conserved in vertebrates (Fig. 4D). To confirm cx43 expression in vertebrae, we generated transgenic fish that expressed mCherry under the control of the cx43 promoter (Fig. 4, E, E’, F, and F’). This transgenic fish showed mCherry expression at the growing edges of the centrum, where the defect resulting in the stp phenotype was expected to occur (Fig. 4, E and E’), and at the junctions between fin bones (Fig. 4, F and F’).

FIGURE 1. stp vertebral phenotype. Panels show adult fish (A and A’), CT scans of their skeletons (B–F and B’–F’), and graphs of vertebral growth rate (G–I). B and B’, the numbers indicate vertebrae identity along the AP axis. The stp allele causes defects in fin bones. Segment lengths of both sof and sofb123/123/+/+ fish are normal in size and shape. These data indicated that a simple decrease of cx43 mRNA level did not affect vertebral growth. However, the vertebral of stp/+/+ fish are drastically shorter than those of stpallele causes defects in fin bones. Segment lengths of sof and sofb123/123/+/+ fish were 64 and 87% of wild type, respectively. This showed that fin ray length is sensitive to the amount of cx43. This showed that cx43 functions in vertebra and fin of zebrafish.

FIGURE 2. Phenotypes of stp and Tg(cx43pro-cx43) stp+/ht28d/ht28d fish. A, genotype ratio of stp/ht28d/ht28d and siblings at each time point. Larvae derived from crossing stp/ht28d/ht28d and stp/ht28d/ht28d were harvested at indicated day and genotyped. B and C, wild type and stp/ht28d/ht28d larvae at 13 dpf. D and E, wild type and Tg(cx43pro-cx43) stp/ht28d/ht28d fish at 56 dpf. Scale bars, 1000 μm in B and C and 5000 μm in D and E. stp/+, stp/ht28d/ht28d, stp/ht28d/ht28d, stp/ht28d/ht28d.
Cx43 protein. stp^{*28d} fish also had shorter fin ray segments than wild type fish, but the difference was only 5% (Fig. 5I, arrowhead 1). The difference of the fin ray segment length between stp/sof^{**123} and sof^{**123}/+ fish was 7% (Fig. 5I, arrowhead 2). Therefore, the stp allele also causes the short fin bone phenotype, but the severity is less than that of the sof alleles.

Functional Analysis of Cx43^{stp}—As described above, the influence of the stp allele is severe in vertebrae but mild in fin bones. On the other hand, the influence of the sof^{**123} allele is undetectable in vertebrae (Fig. 5H) and severe in fin bones. This apparent discrepancy suggests that the Cx43 protein has more than one distinct function. It is known that there are two distinct functions of connexins: formation of gap
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To examine gap junction activity of these Cx43 mutants, we performed voltage clamp current recording using Xenopus oocytes. In this study, we injected only 0.05 ng of mRNAs for Connexin proteins into each oocyte because we detected very large transjunctional currents when we injected 5 ng of mRNA for wild type Cx43 (22). Fig. 7A shows examples of junctional currents induced by wild type Cx43 (Cx43-WT) and Cx43W78L (Cx43\(^{W78L}\)). Cx43W78L (Cx43\(^{W78L}\)) showed reduced junctional currents between the oocytes compared with Cx43-WT (Fig. 7B). On the other hand Cx43P191S (Cx43\(^{P191S}\)) did not show significant junctional current in this experimental condition (0.05 ng of mRNA/oocyte).

We next examined the hemichannel activities of wild type and mutant Cx43s. Panels A and B of Fig. 8 show current traces of Cx43-WT and Cx43W78L (Cx43\(^{W78L}\)), and panels C–G summarizes the current-voltage relationship of these hemichannels. As reported previously, Cx43-WT and Cx43P191S (Cx43\(^{P191S}\)) did not increase current in comparison with the negative control (Fig. 8, C, E, and G). On the other hand Cx43W78L (Cx43\(^{W78L}\)) induced extraordinarily high hemichannel activity (Fig. 8D), which was more than 50-fold greater than that of Cx43-WT. We examined the hemichannel activity of Cx43W78L (Cx43\(^{W78L}\)) co-expressed with Cx43-WT, mimicking the situation in stp\(^{126d/1}\) fish in vivo. The hemichannel activity of Cx43W78L (Cx43\(^{W78L}\)) co-expressed with Cx43-WT was also very high (Fig. 8F). Assuming that wild type and mutant connexins interact heteromerically, these results suggest that heteromeric hemichannels of Cx43-WT and Cx43W78L (Cx43\(^{W78L}\)) in stp\(^{126d/1}\) fish would have increased activity. These results suggest that the hemichannel activity of Cx43P191S (Cx43\(^{P191S}\)) is similar to that of Cx43-WT, whereas that of Cx43W78L (Cx43\(^{W78L}\)) is abnormally increased.

To determine the protein expression and functional changes of connexin proteins in Xenopus oocytes, we performed Western blotting and immunofluorescence staining. The bracket in Fig. 9A indicated that Cx43 and its mutants were expressed in Xenopus oocyte, although several bands were detected in the Cx43W78L (Cx43\(^{W78L}\)) lane. The bands detected in Cx43W78L imply that the W78L mutation causes a protein modification defect in Cx43. Further study is needed to determine whether this modification of Cx43 is related to the stp phenotype. 50-kDa bands in Fig. 9A are due to nonspecific binding of the anti-Cx43 antibody used in this study. The arrow in Fig. 9A′ indicates the expression of β-tubulin (positive control). Protein localization of Cx43 and its mutants were also examined, and membrane localization was detected by immunofluorescence staining (Fig. 9, B–E and B′–E′). Compared with the wild type Cx43 (Fig. 9B), the signals of Cx43W78L (Cx43\(^{W78L}\)) (Fig. 9D) and Cx43P191S (Cx43\(^{P191S}\)) (Fig. 9E) detected at the cell membranes looked weak (22). For the negative controls (NC), only antisense oligonucleotide for Xenopus cx38 was injected into Xenopus oocytes (Fig. 9, A, A′, and C).

Discussion

In this paper we analyzed a zebrafish mutation that disrupts normal vertebrae formation. Homozygous (stp\(^{126d/1}\)) fish die; heterozygous (stp\(^{126d/1}\)) fish grow to adults but show a specific phenotype of shorter vertebrae along only the AP axis. Because
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A

B

C

D

E

F

G

H

I
this phenotype is not seen in young fish (~35 dpf) and becomes evident only after ~60 dpf, we deduced that the mutation caused some defect in the control of bone growth. To identify the gene altered in the stp mutant, we performed positional cloning and TALEN knock-out of the genomic sequence and found that a single base mutation in cx43 causes the phenotype.

Curiously, a previous paper reported that another mutation in cx43 is responsible for the sof phenotype, in which fins are short but vertebrae are normal. To determine why these alleles of the same gene differed in their effects, we investigated two short but vertebrae are normal. To determine why these alleles of the same gene differed in their effects, we investigated two different functions of connexins: as gap junctions and hemichannels. Both Cx43stp and Cx43sof showed reduced gap junction currents relative to wild type, which confirmed that the sof phenotype was caused by the defect in gap junction activity, as suggested by Hopetak-Solga et al. (22). On the other hand, Cx43stp hemichannels showed abnormally high (50-fold higher than wild type) conductance, whereas Cx43sof hemichannel conductance was almost the same as Cx43-WT. These results are consistent with the hypothesis that extremely high hemichannel activity causes the vertebral phenotype of the stp allele.

Although the mechanism by which gap junctions and hemichannels affect different bones is unknown, we assume that it is related to the difference in the bone forming process between vertebrae and fin bones. Fins are mechanically supported by fin rays arrayed in parallel. Each fin ray is composed of short segments of bone called lepidotrichia. Each segment is almost the same size along the AP axis (for tail fins). When the fin grows, a new fin ray segment is made at the tip of each fin ray. The newly made segments are nearly as long as the old segments along the AP axis and do not grow longer with time or with increasing fin length. On the other hand, vertebrae initially appear at ~14 days post fertilization and continuously enlarge during the growth of the fish. Therefore, we infer that Cx43 gap junctions are involved in initial bone formation, and Cx43 hemichannels are involved in later bone growth.

One proposed mechanism of mechanical stress sensing by osteocytes is as follows. When bones are exposed to mechanical stress, they deform slightly, causing movement of the fluid surrounding the osteocytes. This movement of fluid causes shear stress that induces effusion of prostaglandin E2 through hemichannels (38). Prostaglandin E2 promotes osteoclast differentiation by down-regulating Osteoprotegerin (39), a protein that blocks RANKL–RANK interaction and promotes osteoclast differentiation.

**FIGURE 6. cx43 mRNA expression ratios in mutant fish.** A, cx43 mRNA expression ratio in stp/stp/sof fish, normalized to WT fish. 39–48 colonies were sequenced for each fish (n = 6). B, cx43 mRNA expression ratio in stp/sof fish. 28–48 colonies were sequenced for each fish (n = 5). Error bars, S.E. ***, p < 0.01.

**FIGURE 7. Electrophysiological analysis of Cx43 gap junctions.** A, examples of junctional current traces for Cx43 (Cx43-WT), Cx43W78L (Cx43sof), and Cx43P1915 (Cx43sof) shown below voltage protocol (top). B, normalized junctional conductance of mutants compared with Cx43-WT. NC, negative control (only antisense oligonucleotide for Xenopus cx38 was injected). Error bars, S.E. ***, p < 0.01.
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We hypothesize that the *stp* allele reduces vertebral elongation by mimicking high mechanical stress. We found that *cx43* expression in zebrafish vertebral is highest in the growing edges (Fig. 4, E and E'). Because these parts of the vertebra contact the adjacent vertebra, we expect that mechanical stress is highest there. Therefore, one possible explanation for the effect of the *stp* mutation on vertebral shape is that it enhances hemichannel activity, allowing more prostaglandin E₂ effusion. This would mimic the effects of high mechanical stress and lead to increased osteoclast differentiation and, hence, increased rates of bone breakdown at the edges of the centrum.

The major finding of our study is that there are two distinct functions of Cx43 in bone formation, one of which affects early development only and one of which affects subsequent growth only. These two functions correlate with differences in the electrophysiological properties of gap junctions and hemichannels among *cx43* alleles. We hypothesize that the differences in gap junction and hemichannel function among alleles cause their differing effects on bone development and growth. Future studies are needed to test this hypothesis in vivo.

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