

Title: Biochemical characterization of medaka (*Oryzias latipes*) orthologue for mammalian Factor XIII and establishment of gene-edited mutant

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Running title: medaka orthologue for Factor XIII

Abbreviations:

BPA, 5-(biotinamido)pentylamine; CBB, Coomassie Brilliant Blue; DAB, 3, 3'-diaminobenzidine; FXIII, Factor XIII; H&E, hematoxylin and eosin; HMA, heteroduplex mobility assay; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TGase, transglutaminase.

Conflict of interest

The authors state no conflict of interest.

Keywords: medaka, transglutaminase, gene-editing, coagulation

Abstract

At the final process of blood coagulation, fibrin molecules are stabilized via a catalytic reaction by Factor XIIIa (FXIIIa), one of the family members of transglutaminase (TGase) that catalyzes protein cross-linking reactions. In this study, we characterized the orthologue of this enzyme in medaka (*Oryzias latipes*), an established model fish in which a coagulation system is also preserved. The recombinant protein of this orthologue enzyme was produced in baculovirus-infected insect cells and used for biochemical property analysis including activation by thrombin-proteolysis and calcium-dependence of the TGase enzymatic activity. Immunostaining and immunoblotting revealed that medaka FXIIIa is expressed in the kidney, bone and esophagus in addition to blood cells. Furthermore, the gene-mutant fish was established using the CRISPR/Cas9 system. The loss of FXIIIa expression was validated in the mutants, and phenotypes, such as absence of fibrin cross-linking, were investigated in the established fish.

Introduction

Blood coagulation is an elaborate system constituting an essential event during blood vessel injury. This process involves the alteration of various functional molecules and several enzymatic reactions in a cascade-manner. In this pathway, thrombin, as a key factor, proteolyzes fibrinogen to fibrin resulting in its assembly for clot formation [1, 2]. In the final step, fibrin is cross-linked via covalent bonds and further stabilized. The resulting fibrin network is resistant to degradation and protected from the fibrinolytic enzyme. The cross-linking reaction is catalyzed by Factor XIII A subunit (FXIII_A), a member of the transglutaminase (TGase: EC. 2. 3. 2. 11) family [3, 4].

TGase is an enzyme that catalyzes the formation of covalent isopeptide bonds between glutamine- and lysine- residues in a calcium ion-dependent manner [5-7]. This enzyme also catalyzes the incorporation of a primary amine instead of lysine residue into the glutamine residue. In addition, a water molecule can be a substrate in the substitution of a glutamine residue into a glutamic acid residue, resulting in the addition of negative charges to substrates. These post-translational modifications are essential for various biological phenomena in a wide variety of organisms from mammals to microorganism. In mammals, TGase enzyme family consists of eight isozymes: FXIII_A and TG1-TG7.

FXIII_A isozyme is synthesized as a dimer in platelets, and forms a tetramer with a Factor XIII subunit B (FXIII_B) dimer in the blood stream; the association is necessary to stabilize FXIII_A. Upon clot formation,

FXIIIa dissociates from FXIIIb and is activated by thrombin via limited proteolysis, resulting in the cross-linking of fibrin molecules. Hence, the loss of FXIIIa activity by genetic mutation causes several diseases mainly related to the clotting deficiencies [8, 9]. In addition, this enzyme expressing in osteoblasts and chondrocytes is also involved in bone formation [10-12]. Although the biological functions of the enzyme in blood clotting have been extensively investigated and specific inhibitors have been developed [13, 14], pathological and physiological significance of FXIIIa activity are not completely clarified.

Medaka (*Oryzias latipes*), a model organism similar to zebrafish (*Danio rerio*), has been recently used for pathological and pharmaceutical studies including observation of symptoms, drug screening, and for disease initiation mechanism [15-17]. This fish model presents several advantages for these studies by its high fertility, easy maintenance, and short generation time [18-20]. The model is also amenable to genetic modifications: gene-editing techniques such as TALEN (Transcription Activation-Like Effector Nuclease) [18] and CRISPR/Cas9 system are currently available [21-23]. These recent successes in establishing gene-deficient fish will further contribute to our basic scientific knowledge and stimulate application in pre-clinical science such as drug screening.

Recently, we identified all the medaka orthologues for mammalian TGases in the genome database and have characterized some enzymes biochemically [24, 25]. In this study, we present the biochemical analyses, the enzymatic properties, and tissue distribution of the orthologue (medaka

FXIII A) for human FXIII A. Moreover, we successfully established two different genetic allelic mutants carrying catalytically inactive medaka FXIII A and investigated their phenotypes.

Results

Sequence of FXIII A and its deduced primary structure

As shown in Fig 1A, the aligned amino acid sequences of human FXIII (HsFXIII) and medaka FXIII A (OIFXIII A), medaka orthologue, demonstrate an identity of 48% and a similarity of 83%. These values show significant homology between two genes, which is supported by phylogenetic tree analysis (Fig 1B). FXIII A encodes 739 amino acids with a possible cleavage site by thrombin as shown in Fig. 1 and 2B. The tertiary structure of major mammalian TGases analyzed consists of four domains (β -sandwich, catalytic core, β -barrels 1 and β -barrels 2). Based on the sequence homology, medaka FXIII A could adopt a similar structure. Additionally, possible calcium binding sites, which are preserved among the mammalian TG isozymes, are found mostly (11 out of 14 residues) in the medaka FXIII A sequence.

We searched also medaka orthologue of FXIII B subunit, which stabilize FXIII A subunit. Based on the cDNA sequence for mouse FXIIIB (NM_031164), similar search for the orthologues was carried out using cDNA database (NBRP) and also genome database (Ensemble genome browser <http://www.ensembl.org/index.html>), but we found no meaningful orthologue in the medaka gene.

Biochemical analyses of the recombinant protein for FXIIIa

To characterize the biochemical properties of medaka FXIIIa, different from those of the other orthologues we produced a recombinant protein in a baculovirus-insect cell system since the bacterial system resulted in the expression as insoluble protein probably due to incorrect folding (data not shown). The protein obtained using the baculovirus system was purified to homogeneity by metal ion affinity chromatography and size separation (Fig. 2A). Upon size separation, recombinant FXIIIa was eluted as peak fraction approximately at the molecular size of 160 kDa, suggesting that medaka FXIIIa forms a dimer as in the case of human FXIIIa (data not shown).

Because mammalian FXIIIa is activated by limited proteolysis with thrombin, we examined whether the purified recombinant FXIIIa was cleaved in a similar manner. Digestion with human thrombin produced a proteolyzed form of the medaka orthologue at the molecular size deduced from the predicted cleavage site (Fig. 2B and 2C). Then, we confirmed the TGase enzymatic activity by incorporation of BPA (biotin-labeled pentylamine), a glutamine donor substrate, into β -casein as lysine-donor substrate. When the non-treated and the thrombin proteolyzed forms of FXIIIa were compared, the enzymatic activity was apparently enhanced with thrombin digestion (Fig. 2D). The enzymatic reaction for the incorporation of BPA was time- and calcium-dependency (Fig. 3A). In the three different amounts of the activated enzyme, the reaction showed substrate concentration dependency (Fig. 3B). These results suggest that the proteolyzed form of FXIIIa becomes an active TGase in a calcium-ion

dependent manner, through thrombin-digestion similar to mammalian FXIIIa.

Tissue distribution of FXIIIa analyzed by immunoblotting and immunostaining

Using an affinity-purified polyclonal antibody, the expression pattern of FXIIIa was analyzed by immunoblotting. No significant cross-reactivity with other medaka TGases was observed using each recombinant protein (data not shown).

Tissue extracts from wild-type fish were subjected to immunoblotting. As shown in Fig. 4, expression of FXIIIa was observed in several tissues including gill, kidney, bone, and heart as well as in plasma clot. The molecular size was similar to that of its recombinant protein as a positive control at the precursor size. These results suggested that FXIIIa is expressed in the hematopoietic tissues, bone, and in plasma mainly in the precursor form.

Furthermore, whole tissue sections prepared in adult fish were reacted with the antibody in parallel with negative control and H&E staining (Fig. 5A). Three areas showed significant signals: the pharyngeal cavity, vertebra, and the distal pterygiophore (Fig. 5B). In the pharyngeal cavity, several upper layer cells were stained. Expression in vertebra, and distal pterygiophore, as bone and cartilage, showed higher reactive signal pattern than pharyngeal cavity.

Establishment of the medaka mutants for the FXIII^A gene by CRISPR/Cas9 system

To obtain mutant fish harboring aberrant FXIII^A activity, we applied CRISPR/Cas9 gene-editing technologies and generated gRNA targeting either exon 1 or exon 3 of the gene, both of which are upstream of the DNA region encoding the active site residue (Cys) of the enzyme. For this approach, two appropriate target sites were found on chromosome 18 on which the FXIII^A gene is located, and were designed to introduce Cas9 nuclease attack.

The embryos were injected with gRNA along Cas9 mRNA by standard procedure and these F0 founders were raised to adulthood. We outcrossed the F0 fish with wild-type fish, and analyzed their progeny (F1) by HMA (heteroduplex mobility assay) and sequence analyses around the target site. Among F1 progeny, two different alleles were selected based on the resulting deletion and frame-shift in primary structure. These heterozygotes were in-crossed and we successfully obtained homozygotes for each mutant, designated as FXIII^{Adel7} and FXIII^{Adel8} encoding 218 and 28 amino acid residues of FXIII^A, respectively (Fig. 6A). In their primary sequences, after their respective positions at 28 and 8 amino acid residues from the C-terminus, proteins were incorrectly translated by the frame-shift. Both mutated FXIII^A proteins lacked the amino acid residues for the catalytic triad that located after the early stop codon.

To test the expression level of FXIII^A in the gene-deficient fish, immunoblotting was carried out on tissue extracts from both mutant fish,

FXIIIAdel7 and FXIIIAdel8. Fig 6B shows expression of FXIII A in kidney, bone, clot, and heart of the wild-type, whereas no expression was observed as a complete form in the case of the both mutants (Fig. 6B).

Furthermore, possible thrombocytes of medaka were stained for anti-medaka FXIII A using blood from wild type and mutant medaka. Medaka blood cells, mostly erythrocytes, were stained by May-Giemsa staining as a standard method for mammalian cells. Specific immunostaining was obtained in the wild-type possible thrombocytes though we currently have specific medaka marker proteins in the cells. However, both mutants (FXIIIAdel7 and FXIIIAdel8) did not show any significant signal in the preparations. In Fig. 7, the representative areas were shown among the treated cells.

Analysis of the phenotype of the mutant medaka

To know the pattern of fibrin as substrate for FXIII A upon coagulation, the clot was analyzed by SDS-PAGE and immunoblotting. From the blood in wild-type and mutants, extract from the clot was prepared and subjected to pattern analysis. We attempted the immunological specific detection of medaka γ -fibrin using polyclonal antibody against human fibrin (Fig. 8). In the wild-type, the dimerized γ -fibrin by cross-linking was observed with a similar position to the dimerized human fibrin, but not as a monomer form. In contrast, fibrin remained as a monomer form in the case of both FXIII A mutants, although similar pattern was observed in the plasma. These results suggest that the FXIII A-mutant fish carries an inactive coagulation enzyme

resulting in absent fibrin-cross-linking.

Discussion

The activated form of FXIIIa plays an important role in the fibrin clot formation via cross-linking reactions, an essential step for initiating wound healing. FXIII, initially identified as blood coagulation factor, has possibly other functional roles and hence, unexpected diseases might be induced upon its aberrant activity. This enzyme is expressed also in bone and contributes to bone maturation via stabilization of extracellular matrix. Thus, with regard to the expression of FXIIIa in the platelets and regulation of fibrin stabilization, several issues still remain to be resolved.

In this study, we used medaka as a model animal to investigate the physiological functions of FXIIIa. Medaka has been established as a suitable model particularly for drug screening and pathological analysis upon gene mutation or pharmaceutical treatment. After biochemical analyses of the recombinant protein of medaka FXIIIa, the orthologue for human FXIIIa, we established a gene-mutant fish and analyzed their phenotype.

Structural and biochemical properties of medaka FXIIIa appeared quite similar to human FXIIIa in this study, in addition to the phylogenetic tree analyzed for all the medaka orthologues and mammalian enzymes (Fig. 1B, [24]). First, the primary sequence around the catalytic triad and calcium binding region are highly homologous with mammalian FXIII. Based on sequence conservation, FXIIIa might consist of four domains commonly

observed in all mammalian TGases. Second, FXIIIa is synthesized as a zymogen form and limitedly proteolyzed for activation by thrombin. In this experiment, human thrombin was used for digestion, but we consider similar proteolytic reaction may be responsible because a possible medaka orthologue to thrombin was found in the cDNA database (with 25% homology in the deduced primary sequence). Third, the transamidation enzymatic activity is present in a calcium-ion dependent manner (Fig. 3).

Expression analyses indicate that FXIIIa is apparently expressed in platelets and in the kidney. In the gill and heart, lower expression was observed probably due to remains of the clot. Expression in bone and esophagus was additionally revealed by the immunohistochemical analysis. Studies in mammals reported that FXIII is expressed in osteoblasts, chondrocyte, and preadipocyte cells [10-12], which are consistent with our present data. Our results support that FXIIIa plays multiple roles at several tissues including bone. While, recent report described that FXIIIa is not functionally expressed in bone [28]. Identification of substrates in these tissues and cells, by direct mass-spectrometry analysis of the cross-linking reaction products using labeled primary amine, could provide new clues.

We established medaka mutants with aberrant FXIIIa enzymatic activity using gene-editing method, CRISPR/Cas9. Two target sites in the genome were successfully mutated, disrupting the translation of a functional enzyme. From a series of analysis, it was apparent that both mutants did not express functional FXIIIa. FXIII deficiency frequently leads to abortion upon pregnancy in mammals even if bleeding symptom is not heavy [8]. In

our fish model, however, we observed normal shape and viability upon propagation. In addition, the heterozygotes produced mutant offspring at Mendelian ratio.

Although medaka fibrin has not been characterized, we validated them upon clot formation using polyclonal antibody against human γ -fibrin. Expectedly, aberrant fibrin polymerization was observed in both mutants in the clot. As another finding, abnormal excrescences (osteophyte-like) structures were observed in the spine in both mutant fish as reproducible observation (Fig. 9). Currently we do not clarify the cause of this phenotype, but it suggests that expression of FXIIIa in bone is necessary for spine development.

Aberrant expression levels of FXIIIa causes impairment in wound healing, cardiac pathology, obesity and arthritis [29-33]. Conversely, reduction of FXIIIa have been observed in several medical conditions such as pulmonary embolism, stroke, and leukemia [8]. Based on these studies for human diseases, analysis on other phenotypes in mutant fish is ongoing.

In this study, by biochemical and immunochemical data, we identified the orthologue for human FXIIIa. We also showed that this orthologue displays similar proteolytic activation, and established gene-deficient fish. Further phenotypic analyses of our mutants could contribute to a better understanding of FXIII-related diseases and drug screening.

Experimental procedures

Ethics

Fish care and maintenance were carried out according to the Regulations for Animal Experiments in Nagoya University. Animal experiments in this study were approved by the Animal Care and Use Committee of Nagoya University.

Sequence analysis of medaka FXIII A, orthologue for human FXIII A

The vector plasmid harboring FXIII A cDNA was obtained from NBRP (National BioResources Project Medaka, Okazaki, Japan): clone ID, olsp35n14. The nucleotide sequence for the open reading frame of FXIII A was analyzed and the data were deposited to DDBJ (DNA DataBank of Japan) by accession No. LC068825 [24]. Alignment, calculation of identity and similarity, and depiction of the primary sequence were performed by the DNA analysis software Genetyx (Osaka, Japan).

The name of the gene was firstly designated as OITGB [24, 25], however, to avoid confusion with orthologue of FXIII B subunit, we changed it to medaka FXIII A.

Expression and purification of recombinant protein

Initially, the FXIII A cDNA was inserted into pET24dHis vector for expression in bacteria as described previously [24]. This expression was enabled to produce recombinant protein attaching with hexahistidine-tag at the N-terminus. The constructed plasmid (pET24dHisOIFXIII A) was used for transformation of BL21(DE3)pLysS and the inducible expression by isopropyl β -D-1-thiogalactopyranoside was performed.

Since the expression in bacteria resulted in insoluble expression, baculovirus-insect cells system was used to obtain soluble protein. The FXIIIa cDNA attached with the hexahistidine-tag coding sequence was amplified from the pET24dHisOIFXIIIa and inserted into the baculovirus expression vector plasmid, pFastBacTM1 (Bac-to-Bac Baculovirus expression system, Thermo Fisher Scientific, Waltham, MA, USA). For recombination to produce packaged virus, the constructed plasmid was introduced into DH10Bac. The insect cells, Sf9, were propagated for infection and the obtained recombinant virus was infected at the multiplicity of infection of 1-10.

For purification of the recombinant protein, total cellular proteins were extracted from the infected Sf9 cells by the lysis with hypotonic buffer: 10 mM Tris-HCl pH 8.0, 1 mM β -mercaptoethanol, and protease inhibitor cocktail (Merck Millipore, Darmstadt, Germany). Then, after sonication and addition of NaCl to final concentration of 150 mM, the lysate was applied to the TALON gel (Clontech-TAKARA Bio, Kyoto, Japan) and eluted by TBS buffer containing 150 mM imidazole. For further purification, the eluate fraction was size-fractionated by Superdex-200 increase (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Proteolytic activation and measurement of the enzymatic activity

The enzymatic activity of the recombinant protein was examined after limited digestion with human thrombin (R&D SYSTEMS, Minneapolis, MN, USA) that triggers mammalian FXIIIa zymogen for activation. One

micro gram of the recombinant medaka FXIIIa was digested by thrombin for 20 min at 37°C at the final concentration of 40 ng/μl. Limited proteolysis was confirmed by 7.5% SDS-PAGE and then the products were subjected to the enzymatic assay.

For evaluation of the enzymatic activity, a microtiter plate assay was performed as described previously [24]. The β-casein was coated onto the 96 well and blocked with skim milk to avoid non-specific binding. For the proteolyzed FXIIIa followed by treatment with thrombin-inhibitor, the biotin-pentylamine (BPA) (Pierce, IL, USA) was mixed in a reaction buffer containing 40 mM Tris-HCl (pH 8.0), 5 mM CaCl₂, 1 mM β-mercaptoethanol, and protease inhibitor. In the case of the reaction containing the chelator for calcium ion, EDTA was included at the final concentration of 15 mM. After washing, streptavidin-peroxidase solution (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) was added to detect the biotin-conjugated complex. Then, color development was carried out using peroxidase substrate 3, 3', 5, 5'-tetramethylbenzidine (Rockland Immunochemicals Inc.).

Immunochemical analyses for tissue distribution of FXIIIa

Antibody was prepared using the purified recombinant protein for FXIIIa. Nine hundred μg protein was used for immunization to rabbit and its serum was obtained. The serum was subjected to affinity-purification using recombinant FXIIIa-immobilized gel. For immobilization, N-hydroxysuccinimide gel was mixed with the purified FXIIIa according

to the standard method. The cross-reactivity of the affinity-purified polyclonal antibody was examined with the recombinant proteins for other medaka orthologues, prior to use for immunoblotting and immunohistochemical analyses (data not shown).

For immunoblotting, the protein extracts were prepared from several tissues. Briefly, tissue was excised, and homogenized in a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM β -mercaptoethanol, and protein inhibitor cocktail. For preparation of extract of clot, blood was coagulated at 25°C for 1.5 hr. The extract of clot was obtained by homogenization in a lysis buffer followed by sonication. Each supernatant after centrifugation (12,000 *g* x 5 min) was subjected to 7.5% SDS-PAGE and transferred onto the polyvinylidene difluoride membrane (Merck Millipore). The membrane, after being blocked by skim milk, was reacted with the antibody at the concentration of 0.075 μ g/ml overnight at 4°C. The membrane was subjected to peroxidase-conjugated secondary antibody, and then developed using chemiluminescent reagent (Thermo Fisher Scientific).

For immunohistochemical analysis, fish was paraffin-embedded after fixation with Davidson solution (acetic acid, formaldehyde, and ethanol). Then, the 10 μ m tissue serial sections of the fish were prepared according to the standard method. The sections were de-paraffined and heat-treated. Immunohistochemical reaction was carried out using Vectastain ABC kit (Vector Laboratories; Burlingame, CA, USA) according to the manufacture's manual. After blocking by a buffer containing

non-immunized goat serum, the sections were reacted with the primary antibody against FXIIIa at 4°C overnight, and then with avidin and biotin-labeled secondary antibody in the kit. All the sections were finally treated by 3, 3'-diaminobenzidine (DAB) for color development. Hematoxylin and eosin (H&E) staining was paralleled after fixation by the standard method. The sections were observed under the fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

Blood cells were harvested and fixed on the slide glass. May-Giemsa stainings were simultaneously performed according to the standard method. For immunostaining, cells were fixed by 95 % ethanol and subjected to primary antibody solution. The secondary antibody was reacted with cells followed by DAB staining.

Plasmids expressing Cas9 mRNA and gRNAs

For design of the gRNAs, E-CRISP (<http://www.e-crisp.org/E-CRISP/designcrispr.html>) was used as a software tool to predict unique target sites throughout the medaka genome. Moreover, to find possible off-target sites, pattern match tool software (<http://viewer.shigen.info/medakavw/crisprtool/>) was used to avoid the false-attack by gRNA. The target sequence from two areas in exon 1 (LG18932206-LG18932226; actccctctaaggggaacagg) and exon 3 (LG18937465-LG18937485; ccagacaaaagccgagagatg) were selected as candidates as the final selected sequences to produce the efficient gRNAs (Fig. 6A). Then, two sets of the oligonucleotides were designed: 5'-TAG

GACTCTCTCGGCTTTTGTC-3' (sense) and
5'-AAACGACAAAAGCCGAGAGATG-3' (antisense) for exon 3;
5'-TAGGACTCCCTCTAAGGGGAAC-3' (sense) and
5'-AAACGTTCCCCTTAGAGGGAGT-3' (antisense) for exon 1. They
were annealed and inserted into BsmBI-digested pT7-gRNA vector
(Addgene plasmid #46759) [26].

For RNA synthesis, the two constructed vector plasmids as pT7-gRNA containing gRNA sequences were digested by BamHI and purified. The linearized plasmid was used as a template for *in vitro* transcription with T7 RNA polymerase (Promega, Fitchburg, WI, USA) to obtain each gRNA. For Cas9 mRNA production, pCS2hSpCas9 vector (Addgene plasmid #51815) [23] was linearized by Not I digestion and subjected as a template to capped RNA synthesis with mMessage mMachine SP6 Transcription Kit (Thermo Fisher Scientific). After digestion by DNase I, the reaction products were purified by phenol-chloroform extraction and ethanol precipitation.

Establishment of gene-mutated medaka by CRISPR/Cas9 system

The solution (approximately 0.5 nl) containing gRNA (50 ng/μl) and Cas9 mRNA (150 ng/μl) was injected into one to four cell stage embryos (OK-Cab strain) by manipulator (Narishige, Tokyo, Japan). Genome DNA was extracted from randomly selected embryos for analysis of the efficiency in gene-editing by heteroduplex mobility assay (HMA) as described below and also for DNA sequencing.

F0 fish, grown from the injected egg, was interbred with wild-type fish (OK-Cab strain) to produce F1 heterozygotes. Among the F1 fish, males and females that carry an identical mutation mated (in-crossed) to obtain homozygous progeny and/or a mutant family line.

For detection of mutation in the genome, HMA was carried out as a standard method [26]. Briefly, genomic DNA was prepared from the embryo or an excised fin by dissolving in a solution containing 0.2 mM EDTA and 25 mM NaOH followed by neutralization with 40 mM Tris-HCl (pH 8.0). Using the solutions as a template, PCR was carried out using KOD-Plus NEO (TOYOBO, Osaka, Japan) and the set of primers to amplify the target region covering the mutated sequences: for FXIIIAdel7 forward primer; 5-1F 5'-GTACAACTTATACGTCGCTGTGGTC-3', reverse primer; 5-1R 5'-TTCCCTTAGAACACTATTGAGGTCC-3', for FXIIIAdel8 forward primer 2-1F 5'-CATGTCTGATACCAGTGCCAC-3'; reverse primer 2-1R 5'-CAGGAAAGTTGGCAAACCTCTG-3'. The reaction condition was following: 98 °C 10 sec, 60 °C 10 sec, 68°C 20 sec; 35 cycles. The products of amplicon were separated on 12% PAGE so that their migration pattern was compared.

Clot analysis of the medaka blood

Medaka body was cut by razor and the blood was aspirated by hematocrit tube to the tube containing EDTA used as anticoagulant, within a few minutes. After brief centrifugation, the supernatant was harvested as plasma. Twenty to 30 µL of blood harvested from 5-6 fish was used for the

analysis. Clot was formed from plasma by the addition of CaCl₂ at the final concentration of 10 mM and thrombin, and then prepared by centrifugation as pellet. After washing with a buffer containing 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl, the clot was homogenized in a buffer containing 6 M Urea, 2% SDS, and 50 mM Tris-HCl (pH 8.0). After mixing vigorously, the supernatant was obtained by centrifugation and treated with SDS buffer. The samples were analyzed by 10% SDS-PAGE and immunoblotting using anti-human γ fibrinogen antibody (GeneTex, Irvine, CA USA).

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Author contributions

RH, RO, YW, TH performed a series of experiments for biochemical characterization and development of mutant. HH and MK provided technical suggestions on genome-editing system. KH planned experiments

and wrote a paper.

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Figure legends

Fig. 1. Sequence alignment of medaka FXIIIa with human FXIIIa and phylogenetic tree including medaka TGases.

(A) Amino acid sequences from human Factor XIIIa (NP_000120) and medaka FXIIIa (LC068825) were aligned. Human and medaka sequences for FXIIIa were indicated HsFXIIIa and OIFXIIIa, respectively. The identical and similar sequences are boxed and marked with dot, respectively. The arrow indicates the possible cleavage site by thrombin for activation via limited proteolysis. The closed triangles represent the catalytic triad (C330, H389, and D412 in medaka FXIIIa sequence). The open circles indicate the amino acid residues responsible for calcium binding in human FXIIIa. Four larger closed triangles indicate the boundary of four domains: activation peptide, β -sandwich, catalytic core, β -barrel 1 and β -barrel 2.

(B) The phylogenetic tree was depicted (1000 bootstrap trials, Neighbor-Joining Method plot) based on the deduced primary sequences of human FXIIIa, rat FXIIIa (NP_067730.2), mouse FXIIIa (NP_001159863.1), OITGT (LC068826), OITGK1 (LC068829), OITGK2 (LC068830), OITGK3 (LC068831), OITGF (LC068827), OITGO

(LC068828) and medaka FXIIIa.

Fig. 2. Purification of recombinant medaka FXIIIa and its enzymatic activation by limited proteolysis.

(A) The cellular extract from Sf9 (A; applied sample) was applied to TALON gel. The flow-through, washed and eluted fractions are indicated as T, W and E, respectively. The eluted fraction was subjected to size fractionation and the peak fraction is indicated as S. Each sample was subjected to 7.5% SDS-PAGE. (B) The primary sequences around the predicted cleavage site in human FXIIIa (HsFXIIIa) and medaka FXIIIa (OIFXIIIa) were shown. The preserved amino acid residues are indicated by vertical lines. The arrow indicates the possible cleavage site. (C) The FXIIIa purified protein was digested by thrombin. The proteolyzed (+) and zymogen (-) forms were subjected to 7.5% SDS-PAGE analysis. (D) The enzymatic activity of recombinant FXIIIa was compared between with (closed column) or without (open column) limited proteolysis by thrombin. After digestion followed by addition of protease inhibitor, three kinds of amounts of the protein were measured for their enzymatic activity by incorporation of BPA into β -casein coated on microtiter well. The OD450 shows the TMB color development value as a result of streptavidin-peroxidase binding after reaction of BPA incorporation. The data show the mean \pm SD based on triplicate samples.

Fig. 3. Enzymatic activity of activated form of recombinant medaka

FXIII A.

The proteolyzed recombinant protein for FXIII A was analyzed for its enzymatic activity, by measuring the incorporated amounts of BPA as described in the legend to Fig. 2D. In the well, FXIII A was incubated in the presence of BPA (A: 400 μ M; B: 0 - 400 μ M). Incorporated amounts of BPA was evaluated at indicated times (A) and various concentrations of BPA for 30 min (B). In (A), the reaction was carried out in the absence (closed circle) or presence (open circle) of 15 mM EDTA at final concentration with 1 ng/ μ l FXIII A. In (B), three amounts of proteolyzed FXIII A were used: The closed rectangle, triangle, and circle indicate 0.625, 1.25, and 2.5 ng/ μ l, respectively.

In both analyses, the data show the mean \pm SD based on triplicate samples.

Fig. 4. Analysis of tissue distribution by immunoblotting.

The extracts from several tissues of wild-type fish were subjected to 7.5% SDS-PAGE following immunoblotting using affinity-purified polyclonal antibody against medaka FXIII A. The similar amounts of the extracted proteins were loaded with recombinant FXIII A (rFXIII A) used as a positive control. Lanes: gill, kidney, spleen, bone, clot, eye, brain, intestine, liver, and heart.

Fig. 5. Immunohistochemical analysis on whole medaka section.

The serial sections were reacted with affinity-purified polyclonal antibody against medaka FXIII A (middle) or with the same amount of normal rabbit

IgG (lower) as a negative control (NC) following the secondary antibody reaction and DAB staining. H&E staining section was done in parallel (upper). In (A), the staining pattern of the whole section is shown. The box indicates the specific immunostained areas {(i), (ii), and (iii)} which are shown as enlarged images in (B): (i) pharyngeal cavity, (ii) vertebra, and (iii) distal pterygiophore. The scale bars indicate 1 mm (A) and 50 μ m (B), respectively.

Fig. 6. Generation of deletion mutants for FXIII_A by CRISPR/Cas9 system.

The target sites are depicted for the gene-editing by two gRNAs. Both successfully established homozygotes show a deletion of 7 bp in exon 3 and 8 bp in exon 1 designated as FXIII_Adel7 and FXIII_Adel8, respectively. The deleted sequence is indicated by bold letters with the number of the genome sequence. Each deduced encoded polypeptide, shown with wild-type, is a truncated form with a frame-shifted sequence (underlined in the primary sequence), which do not contain the catalytic triad (circle) as indicated (A). Immunoblotting of the tissue extracts from the gene-deficient mutants (FXIII_Adel7 and FXIII_Adel8) were carried out on 7.5% SDS-PAGE. The tissues in which FXIII_A was expressed in wild type were examined: lane 1; kidney, lane 2, bone; lane 3, clot; lane 4, heart. WT, del7 and del8 indicate the tissue extracts from the wild-type and the mutants (FXIII_Adel7 and FXIII_Adel8), respectively (B). The recombinant FXIII_A was used as a positive control (rFXIII_A). The blotted membrane stained with CBB is shown in (C).

Fig. 7. Immunostaining of medaka in blood cells for the wild-type and the mutant medaka fish.

The fixed blood cells were reacted with polyclonal antibody against medaka FXIIIA (center) or with the same amount of normal rabbit IgG (right). May-Giemsa staining was carried out to show blood cells (left). The arrows indicate medaka possible thrombocytes. Among the blood cells (mainly erythrocytes) for immunoreaction, the possible thrombocytes from wild type fish (upper) showed immunological staining in contrast to those from the mutants (FXIIIAdel7 and FXIIIAdel8). The scale bar indicates 10 μm .

Fig. 8. Clot analysis on the blood from the wild-type and the mutant medaka fish.

Each blood sample harvested from wild-type (WT) and the mutants (del7; FXIIIAdel7, del8; FXIIIAdel8) was promoted for coagulation. The proteins extracted from the clot were subjected to 10% SDS-PAGE and to immunoblotting using antibody against human γ -fibrinogen. As controls, non-treated human fibrinogen (3 ng), human FXIIIA-treated fibrin (3 ng) and plasma from each fish were also subjected. The closed and open arrowheads indicate monomer and dimerized γ -fibrin, respectively. The asterisks are non-specific binding proteins.

Fig. 9. Analysis on bone from the wild-type and the mutant medaka fish.

Fish bone was obtained by dissection, fixation, and trypsin digestion. Alizarin-red staining was carried out by a standard method for whole body section of each fish (left): wild-type, FXIIIAdel7 and FXIIIAdel8. The enlarged images are shown with arrows indicating osteophyte-like excrescence (right). The scale bar indicates 2 mm.

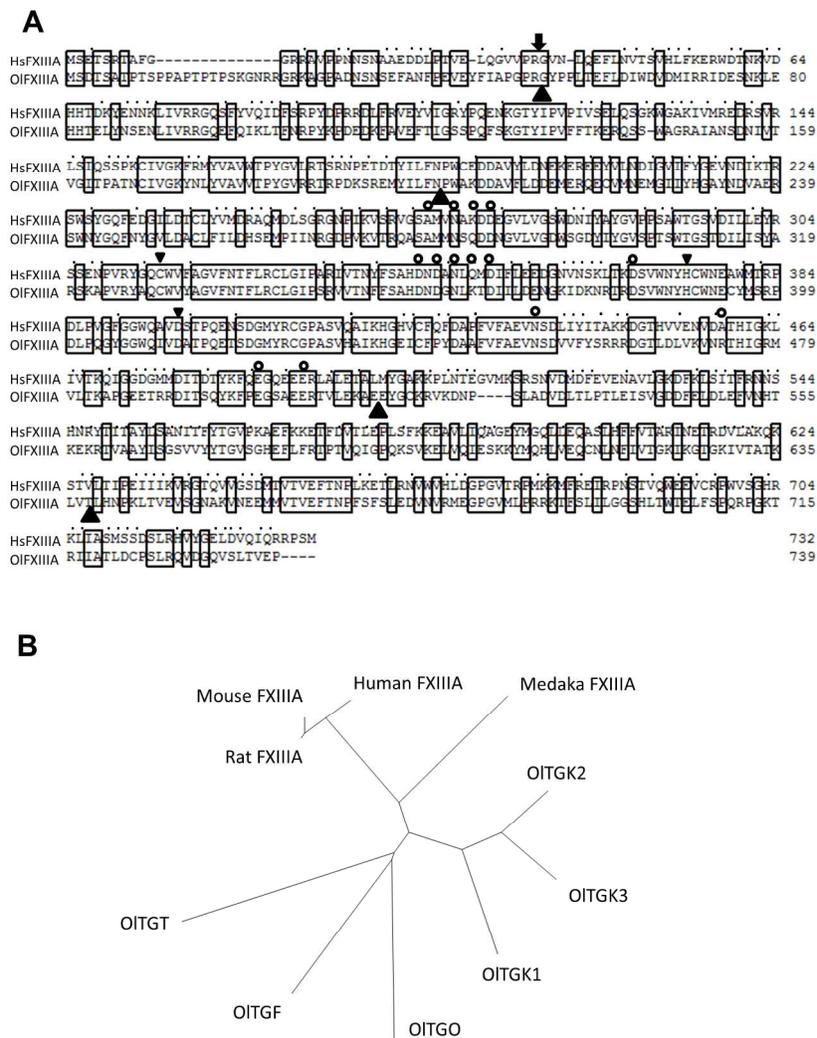


Fig. 1

Fig.1

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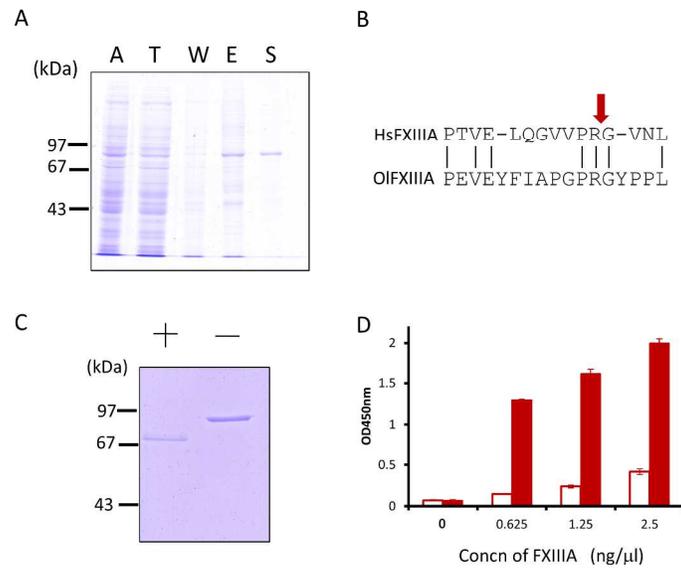


Fig. 2

Fig.2

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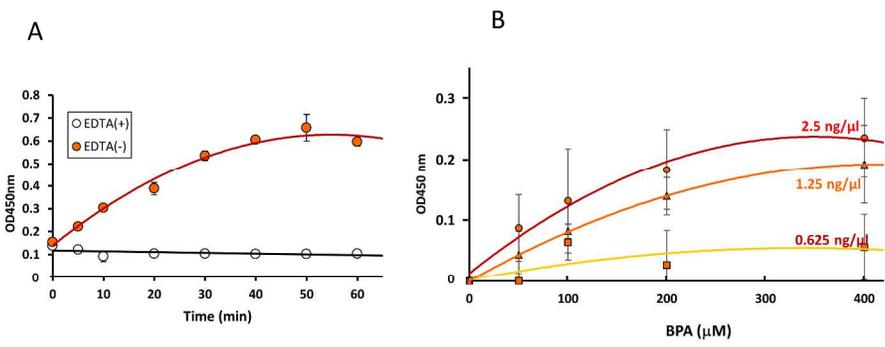


Fig. 3

Fig. 3

254x190mm (300 x 300 DPI)

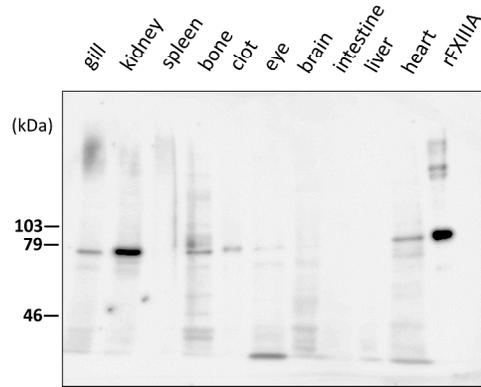


Fig. 4

Fig.4

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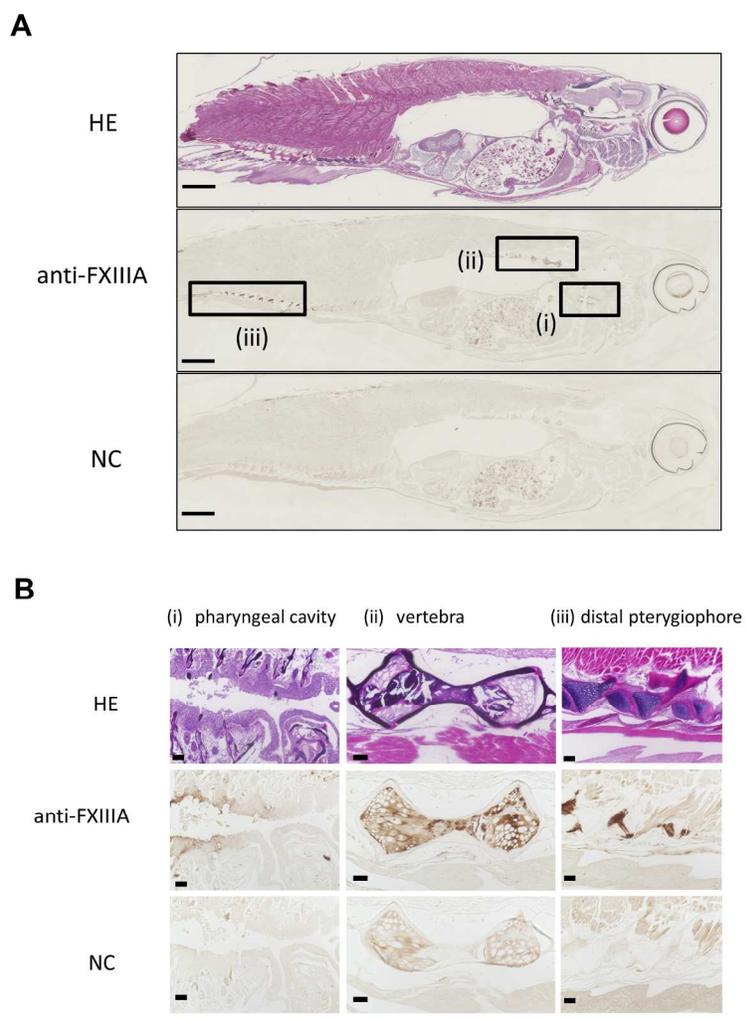


Fig. 5

Fig.5

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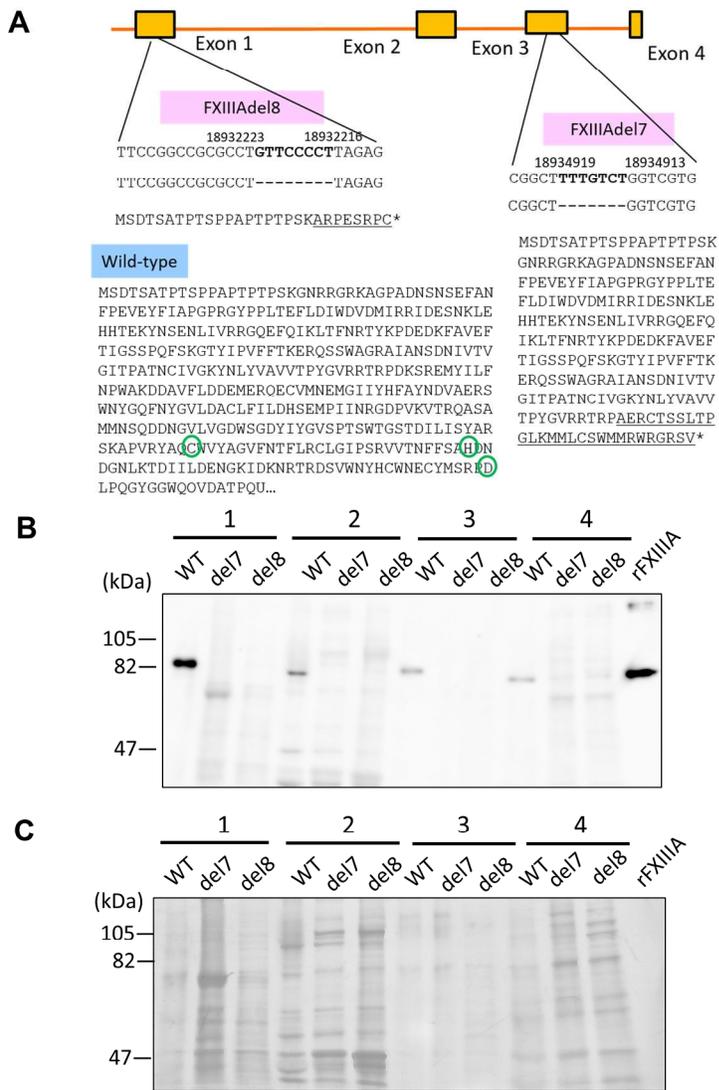


Fig. 6

Fig.6

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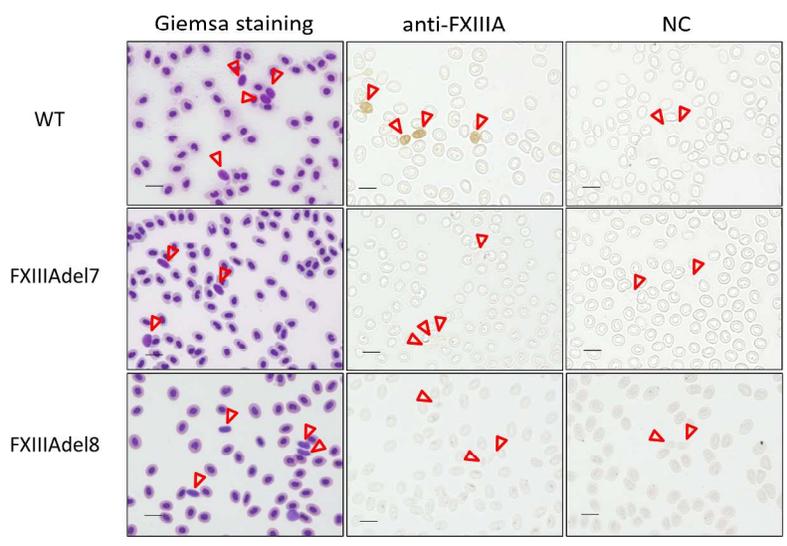


Fig. 7

Fig7

254x190mm (300 x 300 DPI)

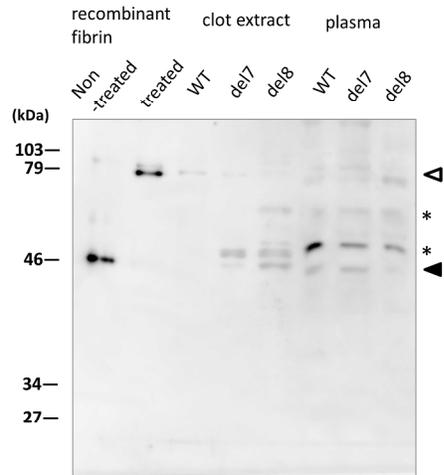


Fig. 8

Fig.8

254x190mm (300 x 300 DPI)

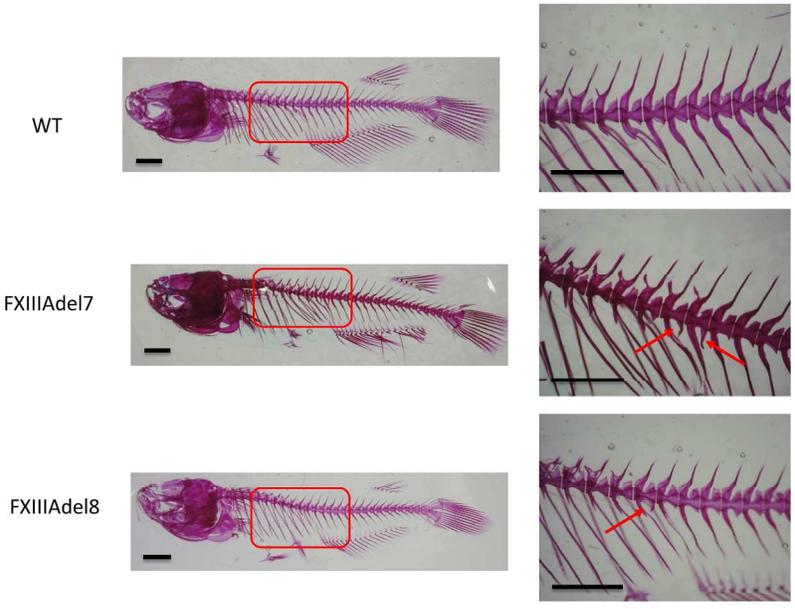


Fig. 9

Fig 9 (newly produced from S7)
254x190mm (300 x 300 DPI)