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ORIGINAL ARTICLE

Loss of fibrinogen in zebrafish results in an asymptomatic embryonic hemostatic defect and synthetic lethality with thrombocytopenia

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Essentials

- Loss of fibrinogen in zebrafish has been previously shown to result in adult onset hemorrhage
- Hemostatic defects were discovered in early *fga*$^{-/-}$ embryos but well tolerated until adulthood
- Afibrinogenemia and thrombocytopenia results in synthetic lethality in zebrafish.
- Testing human *FGA* variants of uncertain significance in zebrafish identified causative mutations

Summary. *Background:* Mutations in the alpha chain of fibrinogen (*FGA*), such as deficiencies in other fibrinogen subunits, lead to rare inherited autosomal recessive hemostatic disorders. These range from asymptomatic to catastrophic life-threatening bleeds and the molecular basis of inherited fibrinogen deficiencies is only partially understood. Zinc finger nucleases have been used to produce mutations in zebrafish *fga*, resulting in overt adult-onset hemorrhage and reduced survival. *Objectives:* To determine the age of onset of hemostatic defects in afibrinogenemic zebrafish and model human fibrinogen deficiencies. *Methods:* TALEN genome editing (transcription activator-like effector nucleases) was used to generate a zebrafish *fga* mutant. Hemostatic defects were assessed through survival, gross anatomical and histological observation and laser-induced endothelial injury. Human *FGA* variants with unknown pathologies were engineered into the orthologous positions in zebrafish *fga*. *Results:* Loss of Fga decreased survival and resulted in synthetic lethality when combined with thrombocytopenia. Zebrafish *fga* mutants exhibit a severe hemostatic defect by 3 days of life, but without visible hemorrhage. Induced thrombus formation through venous endothelial injury was completely absent in mutant embryos and larvae. This hemostatic defect was restored by microinjection of wild-type *fga* cDNA plasmid or purified human fibrinogen. This system was used to determine whether unknown human variants were pathological by engineering them into *fga*. *Conclusions:* These studies confirm that loss of fibrinogen in zebrafish results in the absence of hemostasis from the embryonic period through adulthood. When combined with thrombocytopenia, zebrafish exhibit synthetic lethality, demonstrating that thrombocytes are necessary for survival in response to hemorrhage.

Keywords: fibrinogen; genome editing; hemostasis; thrombocytopenia; zebrafish.

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Introduction

Hemostasis is a critical requirement for maintaining blood circulation and upon injury a cascade of enzymatic reactions is initiated. Platelets adhere to the site of injury and are activated, forming the primary plug, after which coagulation factors trigger a burst of thrombin production, driving the conversion of soluble fibrinogen to an insoluble fibrin clot. The regulation of coagulation factor activation and inhibition provides checks and balances that assure proper clot formation and eventual dissolution. Aberrations to these pathways result in both

bleeding or clotting disorders, which contribute to hemorrhage, ischemia, surgical complications and even death [1].

Fibrinogen is a liver-derived hexameric glycoprotein encoded by paralogous genes *FGA*, *FGB* and *FGG* (coding for Aα, Bβ and γ chains, respectively) on human chromosome 4 (4q31.3–4q32.1) [2–4]. In the presence of thrombin, fibrinopeptides (A and B) are removed, leaving fibrin monomers, which are stabilized by FXIII cross-linking [3,5–8], driving insoluble clot formation. Mutations in *FGA*, *FGB* or *FGG* can all affect the synthesis, assembly, intracellular processing, stability or secretion of fibrinogen. Congenital deficiency of fibrinogen, either quantitatively (hypofibrinogenemia and afibrinogenemia) or qualitatively (dysfibrinogenemia), is an inherited autosomal bleeding disorder with heterogeneous penetrance, although the latter has been shown to result in thrombosis in some cases [9]. Hemorrhaging can occur in various tissues such as skin, soft tissues, muscles, joints, the gastrointestinal tract or genitourinary tract, with intracranial bleeding being a major cause of death [4,10–12]. Afibrinogenemia is estimated to occur in 1 per 1 000 000 individuals [11,13] and cumulatively congenital fibrinogen disorders are thought to make up 8% of all rare coagulation disorders [14]. A majority of diagnosed dysfibrinogenemia and afibrinogenemia cases have been attributed to mutations and modifications within the FGA sub-chain [15].

Current therapeutic strategies for congenital fibrinogen deficiency include replacement with fresh-frozen plasma, cryoprecipitate or plasma-derived fibrinogen concentrate. Unfortunately, these treatments have been linked to negative outcomes, including transfusion-associated complications, off-target effects on related plasma proteins and failed virus inactivation [10,16–18]. Consequently, increased risk and diminished treatment efficiency result in reduced clinical administration [19].

To improve treatments for fibrinogen-related disorders, further interrogation of the disease mechanisms in physiologically relevant model systems is required. In recent years, zebrafish (*Danio rerio*) has become an increasingly popular model for studying blood pathologies because of high genetic and physiologic homology to humans. Zebrafish are economical, easy to maintain and manipulate, and undergo rapid, external development as transparent embryos or larvae, making them amenable to hemostatic assays. We and others have demonstrated a high degree of conservation of the coagulation cascade between zebrafish and humans, identifying homologs for nearly all major factors [20–25], with strong data implicating mononuclear thrombocytes as functional equivalents to platelets [26,27]. However, thrombocytes have not been proven to be required for survival in response to hemorrhage. Recent advancements in genome editing technologies (e.g. transcription activator-like effector nucleases [TALENs] and clustered regularly interspaced short palindromic repeats [CRISPR]) have enabled the assessment of both individual and multigene knockouts to interrogate the genetic underpinnings of bleeding and clotting phenotypes [28–30].

We have previously shown that antisense morpholino-mediated knockdown of zebrafish fibrinogen chains results in frequent intracranial and intramuscular hemorrhage at 3 days post-fertilization (dpf) [31]. However, this was not observed in a zinc finger nuclease (ZFN)-mediated knockout of *fga* [28]. Instead, mutant fish survived into adulthood but were found to be underrepresented at 4–5 months of age, with the data suggesting ~40% survival. In this study we used TALEN-mediated genome editing to generate a transmissible mutation in *fga* similar to and recapitulating the phenotypes of the ZFN mutant. These include survival into adulthood with no gross larval bleeding, but eventual adult lethality due to hemorrhage in various tissues. Despite a lack of overt bleeding, hemostasis was defective as early as 3 dpf. Interestingly, we were able to reverse the hemostatic defect after treatment with an antifibrinolytic compound. We also showed that $fga^{-/-}$ fish could be used to discern human *FGA* variants that abrogate fibrinogen function. Finally, loss of the transcription factor Nfe2 results in severe thrombocytopenia in adult zebrafish [32], similar to mice [33–35]. Combined deficiency of Fga and Nfe2 results in synthetic adult lethality, which we believe is the first evidence indicating that thrombocytes are required *in vivo* for prevention of hemorrhage and hemostasis-related survival.

Methods

Zebrafish lines and maintenance

Experiments were performed using AB×TL F1 hybrid background zebrafish. Embryos derived from natural spawning were raised at 28.5 °C and developmental stages (embryo, larva, juvenile and adult [30]) were determined using age (hours post-fertilization [hpf] or dpf) and morphological characteristics [36]. Animal care and usage were in accordance with animal care guidelines approved by the University of Michigan Institutional Animal Care and Use Committee.

TALEN-induced mutagenesis of coagulation factors in zebrafish

TALEN-mediated genome editing was used to generate mutations in exon 2 of zebrafish *fga* at the target sequence CTTCAAGGCACAAGAC. Engineered TALENs were injected into one-cell-stage zebrafish embryos [30,37], raised to adulthood, and crossed with AB×TL wild-type fish to confirm germline transmission. The resulting *fga* mutants were used for further studies. *nfe2* mutants were previously generated using TALENs [32].

Genotyping

Zebrafish were anesthetized in tricaine (0.16 mg mL^{-1}, Western Chemical, Ferndale, WA, USA) and fin biopsies were obtained [38,39] or fish were humanely killed in high-dose tricaine (1.6 mg mL^{-1}) followed by DNA preparation. Tissue was lysed in buffer (10 mM Tris-Cl, pH 8.0; 2 mM EDTA, 2% Triton X-100 and 100 μg mL^{-1} proteinase K) at 55 °C overnight [29]. Samples were heated to 95 °C for 5 min to inactivate proteinase K, followed by PCR (primers in Table S1). PCR products were resolved using agarose gel electrophoresis or a Qiaxcel capillary electrophoresis system (Qiagen, Hilden, Germany).

Laser-induced endothelial injury

Laser-induced thrombi were generated after injury of the endothelium of the posterior cardinal vein (PCV) of larvae as previously described [40–42]. In brief, zebrafish larvae were anesthetized at 3 dpf, embedded in agarose (0.8%), and the endothelium was ablated with a laser at the fifth somite distal to the anal pore (Olympus IX71 and MicroPoint Pulsed Laser System, Andor Technology, Belfast, UK). Time to occlusion was recorded up to 2 min, followed by removal of larvae from agarose and subsequent genotyping.

ε-aminocaproic acid treatment

Mutant offspring were derived from $fga^{+/-}$ and $fga^{-/-}$ intercrosses. At 24 hpf, embryos were treated with 100 mM ε-aminocaproic acid [29,30]. At 3 dpf, laser injury and genotyping were performed.

Construction and microinjection of expression vectors

Expression constructs and primers used to generate them are listed in Supplemental Tables S1 and S2. Constructs were made on the p*ubi:p2A-EGFP* backbone [30] with wild-type and mutant human and zebrafish *fga* cDNAs under control of the zebrafish *ubiquitin* (*ubi*) promoter [43], with bicistronic expression of *egfp* using the *2A* self-cleaving peptide. Mutations were generated using site-directed mutagenesis [44], verified by restriction digestion and sequencing (primers in Table S1), and injected into one-cell-stage embryos. Mutations are numbered according to the human amino acids.

Retro-orbital fibrinogen infusions

Larvae were anesthetized with tricaine at 3 dpf and placed upon an agarose mold for infusions as previously described [29]. In brief, human fibrinogen (Sigma, St. Louis, MO, USA) or bovine serum albumin as a control (BSA, Sigma) were dissolved in 0.9% NaCl to a final concentration of 25 mg mL^{-1} and 2 nL were infused into the retro-orbital space. Larvae were allowed to recover in system water for 1 h before undergoing laser injury and genotyping.

Quantitative (qPCR) and qualitative PCR analysis

Zebrafish embryos and larvae were homogenized with a 21-gauge needle in lysis buffer. Total RNA was isolated with the PureLink® RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) or RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and used for first-strand cDNA synthesis with oligo $(dT)_{12\text{-}18}$ primers using Superscript-II (Invitrogen). The cDNA was used for RT-PCR (Eppendorf MasterCycler) and qPCR (Bio-Rad, iCycler, Hercules, CA, USA) reactions (primers in Table S1). qPCR data were analyzed as previously described [45]. Qualitative PCR analysis was also performed on cDNA derived from 5 dpf larva.

Blood collection and western blot analysis

Adult zebrafish blood was collected and western analysis performed as described [29,46,47]. Briefly, blood was collected into EDTA-coated microcapillaries (ThermoFisher). One microliter of pooled plasma (at least two fish per genotype) in buffer with β-mercaptoethanol was resolved on a 4–20% SDS-PAGE gel (Bio-Rad) and transferred to nitrocellulose (Bio-Rad). Membranes were probed with zebrafish anti-fibrinogen antibody, followed by HRP-conjugated secondary antibody (Santa Cruz, Dallas, TX, USA), then developed with chemiluminescent substrate (Super Signal West Femto; ThermoFisher, Waltham, MA, USA) and analyzed on a FluorCheM system (Protein Simple, San Jose, CA, USA).

Histological examination and o-dianisidine staining

Zebrafish from $fga^{+/-}$ in-crosses were fixed with 4% PFA/PBST at 4 °C overnight, embedded in paraffin, sectioned at 4–5 μm, and stained with hematoxylin and eosin (H&E). Embryos from $fga^{+/-}$ in-crosses were stained at 7 dpf for hemoglobin with o-dianisidine as previously described [48,49]. Images were collected on a Leica MZFL III stereoscope or Olympus BX51 and recorded using an Olympus DP70 digital camera.

Assessment of human FGA *variants*

Variants in human *FGA* were identified from patients manifesting with hemorrhage and hypofibrinogenemia as documented in the Human Fibrinogen Database [50] and other sources [51]. These were engineered into the orthologous positions of zebrafish wild-type *fga* cDNA constructs under the control of the *ubi* promoter (Table S2) and injected into one-cell-stage mutant offspring

from $fga^{+/-}$ to $fga^{-/-}$ intercrosses, followed by laser injury at 3 dpf.

Statistical analysis

Statistical analysis was performed using Mann–Whitney *U* or two-tailed Student *t*-tests. Survival was evaluated by log-rank (Mantel-Cox) testing for significance. Charts and survival curves were generated using Prism (GraphPad Software, San Diego, CA, USA).

Results

Targeted mutagenesis of fga using TALENs results in a null allele

Mutations in human *FGA* result in inherited hypofibrinogenemia with variable manifestations in humans [1,12,15]. We inactivated the zebrafish *fga* gene by TALEN-mediated genome editing in the second exon of *fga* (Fig. 1A). A mutant line was identified with a net 26-nucleotide insertion, resulting in a frameshift and premature stop codon (Fig. 1B). Frameshifts often result in loss of expression due to nonsense-mediated decay [29,30] and we tested this possibility using qPCR. We found that transcription of *fga* mRNA in 3-day-old $fga^{-/-}$ larvae was nearly undetectable compared to $fga^{+/+}$, and $fga^{+/-}$ siblings were reduced by ~40% ($P = 0.0001$ and $P < 0.05$, respectively, Fig. 1C). A qualitative assessment of cDNA isolated from $fga^{-/-}$ embryos showed no detectable $fga^{+/+}$ expression remaining in mutants (Fig. 1D), and no residual Fga was observed in plasma isolated from $fga^{-/-}$ adult fish (Fig. 1E). Furthermore, qPCR data indicate that relative transcription levels of other coagulation factors, prothrombin (*f2*), factor X (*f10*) and antithrombin

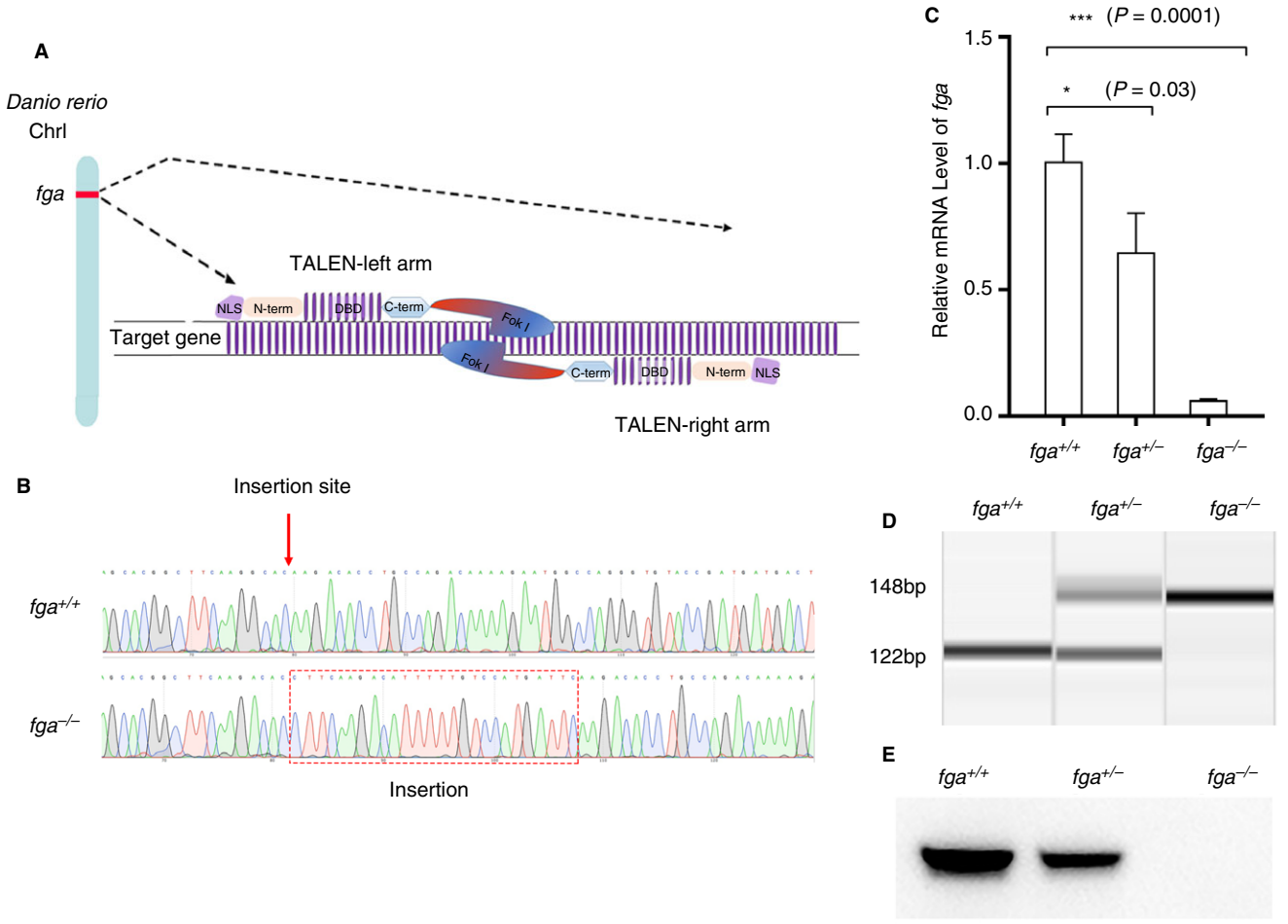

Fig. 1. Generation of a null allele in *fga* using genome editing with transcription activator-like effector nucleases (TALENs). (A) Schematic of TALENs targeting exon 2 of *fga*, located on zebrafish chromosome 1. (B) cDNA sequencing reveals a 26 base pair insertion in *fga* (indicated by red box), which generates a frameshift mutation and subsequent nonsense mutation, resulting in truncation. (C) Transcription of *fga* is remarkably reduced in $fga^{-/-}$ mutants when compared to $fga^{+/+}$ and/or $fga^{+/-}$ siblings, as evaluated by qPCR ($n = 25$ larvae per genotype at 3 days post fertilization (dpf), $P = 0.0001$ and $P < 0.05$, respectively, unpaired two-tailed Student's *t*-test). (D) Qualitative PCR of cDNA isolated from $fga^{-/-}$ embryos reveals that there is no detectable $fga^{+/+}$ transcript. (E) Western blot analysis shows no residual Fga present in plasma isolated and pooled from adult $fga^{-/-}$ mutants, in contrast to $fga^{+/+}$ and $fga^{+/-}$ control siblings. Chr1, chromosome 1; C-term, C-terminal domain; DBD, DNA-binding domain; Fok I, Fok I nuclease; NLS, nuclear localization signal; N-term, N-terminal domain.

III (*at3*), are also significantly reduced in $fga^{-/-}$ larvae ($P < 0.05$, Fig. S1).

fga *mutant survival drops off as fish reach breeding age and is exacerbated by loss of Nfe2*

Targeted mutation of *FGA* in mice results in a risk of fatal abdominal hemorrhage, but overall survival is variable and dependent on genetic background [52]. ZFN-generated fish mutants produced on a pure AB background demonstrate ~40% survival at 4–5 months of age [28]. We tracked the survival of our $fga^{-/-}$ mutants and their $fga^{+/-}$ siblings daily. $fga^{-/-}$ mutant survival was progressively reduced compared to the $fga^{+/-}$ siblings over the first year of life (Fig. 2A; $P < 0.0001$). Loss of *fga* in zebrafish did not manifest in lethality until $fga^{-/-}$ mutants approached breeding age. All $fga^{-/-}$ fish were alive at 45 dpf (Fig. 2A), but survival declined to 90% at 60 dpf and to 40% shortly after 1 year of age.

In mammals Nfe2 is required for megakaryocyte maturation and platelet formation [33–35], and zebrafish lacking Nfe2 have severe thrombocytopenia with normal survival [32]. Knockout mice deficient in fibrinogen or Nfe2 have severe hemostatic defects, with variable survival into adulthood. Combined deficiency results in death within 48–72 h after birth [53]. We intercrossed *fga* and *nfe2* mutant fish and found that loss of both Fga and Nfe2 resulted in synthetic lethality by 1 year of age (Fig. 2B; $P < 0.0001$).

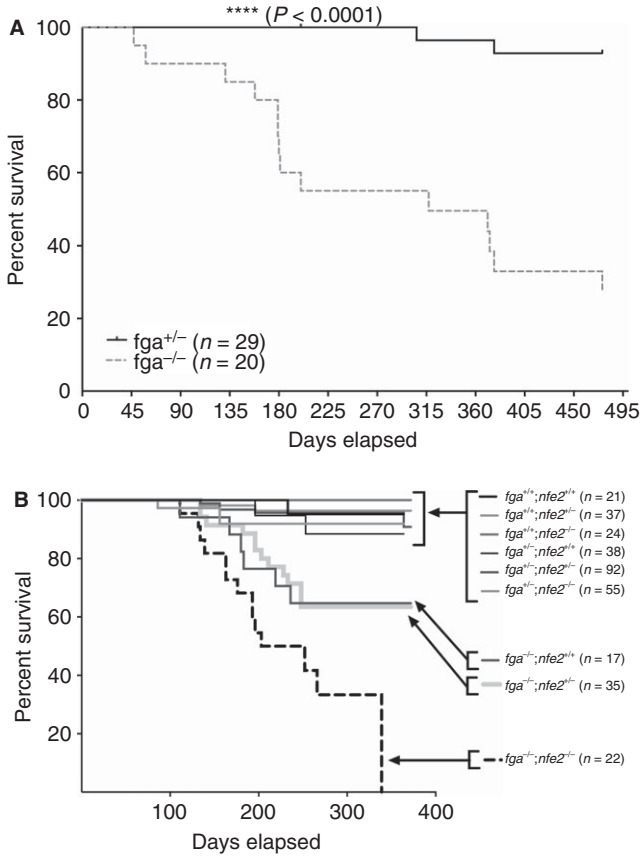

Fig. 2. $fga^{-/-}$ mutants demonstrate synthetic lethality with loss of *nfe2*. (A) Offspring derived from $fga^{+/-}$ and $fga^{-/-}$ intercrosses were genotyped at 3 days post-fertilization (dpf) and examined daily. Compared to heterozygous siblings ($n = 29$), homozygous mutants ($n = 20$) exhibit a significant decrease in survival over time ($P < 0.0001$ by log-rank testing). (B) Offspring derived from $fga^{+/-}$; $nfe2^{+/-}$ intercrosses were genotyped at 86 dpf and tracked for survival. Combined loss of *fga* and *nfe2* results in complete synthetic lethality by 1 year of age ($P < 0.0001$ by log-rank testing).

Mutation in zebrafish fga *leads to hemorrhage in multiple tissues*

In contrast to other coagulation protein deficiencies [29,30], loss of *fga* results in a relatively mild hemorrhagic phenotype in zebrafish. As seen previously [28], we also did not detect grossly visible bleeding by o-dianisidine staining in $fga^{-/-}$ larval mutants (data not shown). At 41 dpf juvenile $fga^{-/-}$ and $fga^{+/+}$ siblings were randomly selected for histological analyses. In homozygous mutants, 64% manifested with mild or moderate bleeds in various tissues, including brain, jaw, muscle, fin and abdomen (Fig. 3A). In addition to previously observed bleeding in the eyes, brain and abdominal cavity [28], massive hemorrhage was observed in muscle and mild bleeding in the jaw, mouth and fins (Fig. 3A). Hemorrhaging in the brain progressively worsened by 47 dpf (Fig. 3B) and was occasionally accompanied by ocular bleeds. Death was typically preceded by severe intracranial hemorrhage.

The hemostatic defect of fibrinogen deficiency manifests in the embryonic period

Previously, *fga* was targeted using ZFNs, resulting in overt hemorrhage and reduced survival [28]. To address whether this affects intravascular physiologic hemostasis, we measured the time to occlusion after laser-induced endothelial injury of the PCV. Normally, venous thrombus formation under these conditions occludes the vessel within 2 minutes, but this did not occur in $fga^{-/-}$ larvae ($P < 0.0001$, Fig. 4A). To rescue the hemostatic defect and confirm that it was due to loss of Fga, we injected a Tol2-mediated zebrafish *fga* cDNA expression plasmid into one-cell-stage $fga^{+/-}$ in-cross offspring. At 3 dpf, over 50% of the injected $fga^{-/-}$ larvae displayed PCV occlusion times similar to their injected and uninjected $fga^{+/+}$ and $fga^{+/-}$ siblings, which was significantly different to the uninjected homozygous mutants ($P < 0.05$ by Mann–Whitney *U*-test, Fig. 4B).

We previously demonstrated that loss of At3 in larvae results in a consumptive coagulopathy that is rescued by infusion of human fibrinogen [29]. To determine whether

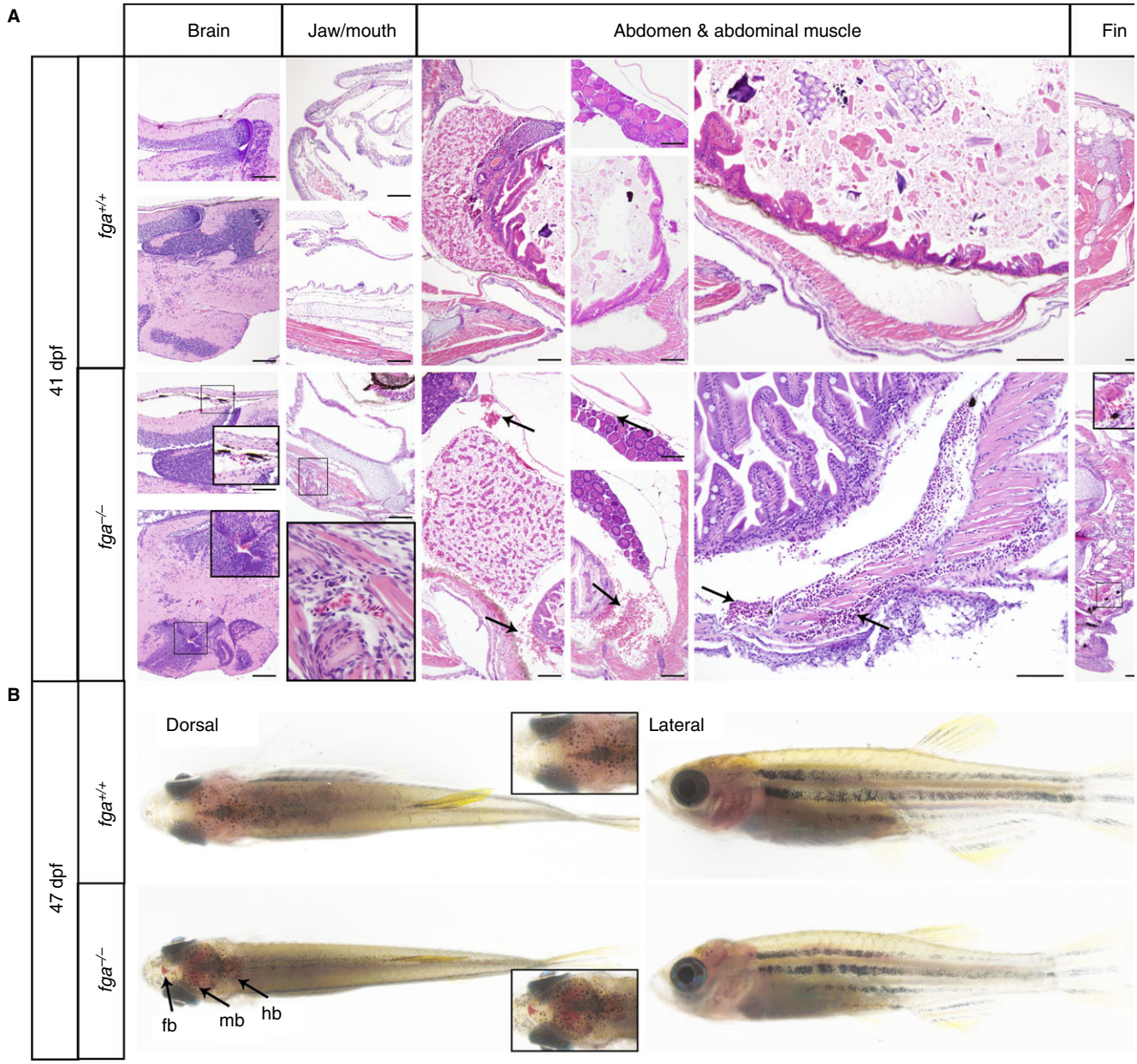

Fig. 3. *fga*$^{-/-}$ mutants develop spontaneous hemorrhage in multiple tissues. (A) Fish were fixed, sectioned, and stained with hematoxylin and eosin, demonstrating that *fga*$^{-/-}$ mutants ($n = 11$) develop mild bleeding in the brain and jaw/mouth, but severe bleeding within the abdomen and abdominal musculature by 41 days post-fertilization (dpf) (compared to wild-type siblings, $n = 3$). Insets show magnified regions of interest with sites of hemorrhage, as do arrows. (B) Severe bleeds identified in the forebrain (fb), midbrain (mb) and hindbrain (hb) in viable *fga*$^{-/-}$ mutant fish at 47 dpf, in contrast with wild-type siblings. Arrows indicate sites of bleeding. Anterior is towards the left and dorsal is at the top. Scale bar: 100 μm.

human fibrinogen could replace zebrafish fibrinogen, we infused human fibrinogen concentrate into *fga*$^{-/-}$ larvae. Approximately half of the infused *fga*$^{-/-}$ mutants showed significantly shorter occlusion times in comparison with non-infused *fga*$^{-/-}$ siblings ($P < 0.05$, Fig. 4C). Together, our findings confirm that the *fga* mutation leads to an intravascular hemostatic defect, similar to that seen in mouse and human pathologies, which can be reversed by transient expression of wild-type *fga* cDNA or infusion of human fibrinogen protein.

Inhibition of fibrinolysis alleviates the fga$^{-/-}$ *hemostatic defect*

Fibrinolysis inhibitors are believed to function through stabilization of the fibrin clot. However, we previously showed that ε-aminocaproic acid can reverse the hemostatic defect in our model of consumptive coagulopathy, which is characterized by hypofibrinogenemia [29]. At 24 hpf, *fga*$^{-/-}$ mutants were incubated in ε-aminocaproic acid and then subjected to laser injury at 3 dpf. No

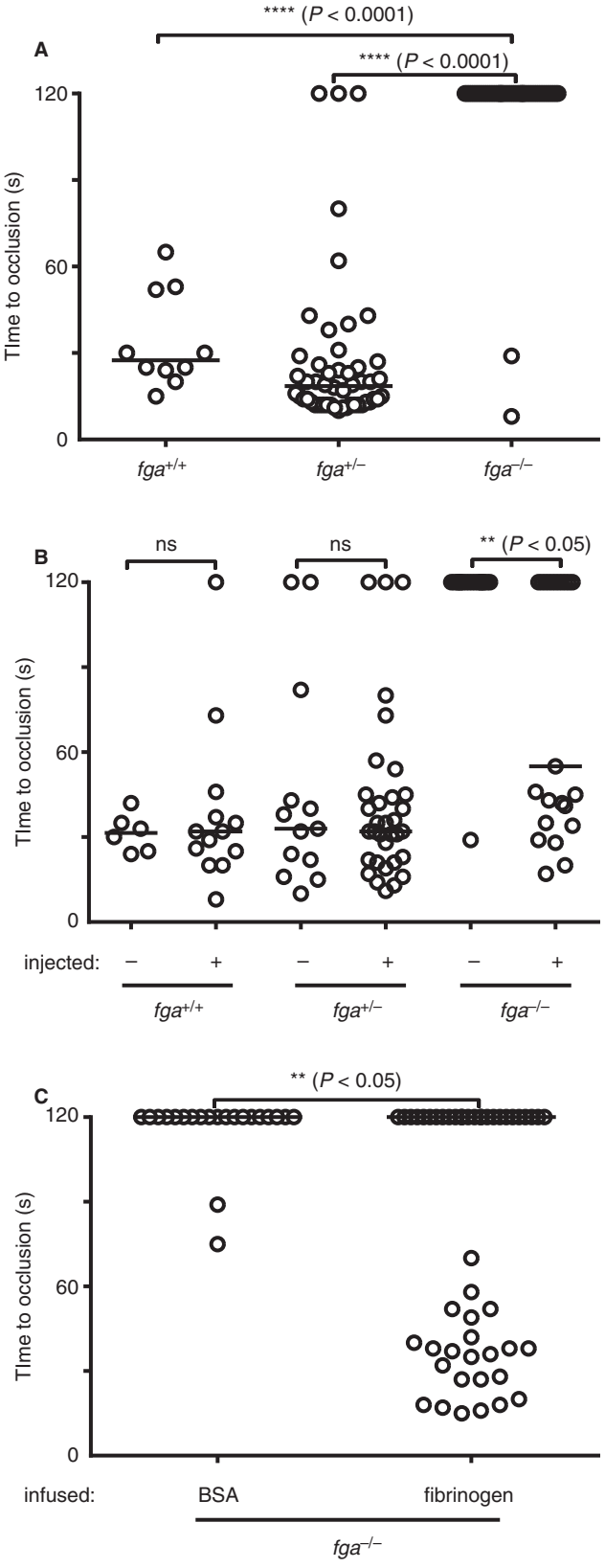

Fig. 4. Laser-mediated injury of the vascular endothelium reveals a hemostatic defect in *fga* mutant zebrafish. (A–C) Hemostasis was evaluated by measuring time to occlusion up to 120 s after laser-induced endothelial injury of the posterior cardinal vein (PCV) in 3 days post-fertilization (dpf) larvae. Time to occlusion was significantly prolonged in $fga^{-/-}$ ($n = 50$) larvae in comparison with $fga^{+/+}$ ($n = 10$) and $fga^{+/-}$ ($n = 50$) siblings ($P < 0.0001$) (A). Injection of wild-type *fga* cDNA into the offspring of $fga^{+/-}$ in-crosses rescued the hemostatic defect in $fga^{-/-}$ compared to uninjected $fga^{-/-}$ counterparts ($n = 19$–23 per genotype, $P < 0.05$). Hemostasis in injected $fga^{+/+}$ and $fga^{+/-}$ was unaffected compared to uninjected $fga^{+/+}$ and $fga^{+/-}$ ($n = 6$–33 per genotype) (B). (C) Retro-orbital infusion of human fibrinogen into 3-dpf mutant larvae ($n = 49$) rescued time to occlusion compared to bovine serum albumin (BSA)-infused $fga^{-/-}$ siblings ($n = 21$, $P < 0.05$). *P*-values determined by the Mann–Whitney *U*-test. Horizontal bars represent the median time to occlusion. ns, not significant.

control $fga^{-/-}$ larvae were able to occlude within 2 minutes of injury. In contrast, 33% of ε-aminocaproic acid-treated $fga^{-/-}$ larvae occluded within 60 s of laser-induced injury ($P = 0.0003$, Fig. 5).

Evaluation of human FGA *variants in* fga*-deficient zebrafish helps link genetic causation to pathology* in vivo

We previously showed that we can use zebrafish as an *in vivo* system for the identification of the causative mutations in coagulation disorders [29,30]. Because zebrafish Fga deficiency closely parallels human pathology, human *FGA* variants, including C55G, C64Y, Y809C (Human Fibrinogen Database) [50,54,55] and M1V [51], were assessed in $fga^{-/-}$ zebrafish. These variants have been associated with hemorrhage in human populations (Table 1); however, they have not been functionally validated *in vivo*. A multispecies alignment of humans, mice, chickens and zebrafish demonstrates conservation of these amino acids across all species. To evaluate each variant's ability to rescue the hemostatic defect in *fga* mutants, a series of zebrafish *fga* cDNA constructs were generated to express the variants at positions orthologous to the human *FGA* mutations (Table S2). Plasmids were injected into one-cell-stage mutant offspring and subjected to laser-induced endothelial injury followed by genotyping. M1V and Y809C were not able to reverse the occlusion defects of $fga^{-/-}$ mutant. In contrast, C55G and C64Y were able to rescue the mutant hemostatic defect (Fig. 6). By assessing known human gene variants in *fga* mutant zebrafish, gene regions that convey phenotypic deficiencies can be identified, shedding light on the mechanistic cause of pathologies.

Discussion

We have produced a mutation in exon 2 of the *fga* gene in zebrafish using TALENs in a nearly identical location to that of the three mutant *fga* lines generated using ZFNs. The TALENs and ZFN mutations overlap with differences of eight base pairs or less [28]. In the previous report, the authors identified spontaneous hemorrhage in adult fish, but examination of 3 and 5 dpf larvae found no evidence of bleeding [28]. This suggested the possibility that hemostasis in early development does not require the presence of fibrinogen. However, we have previously

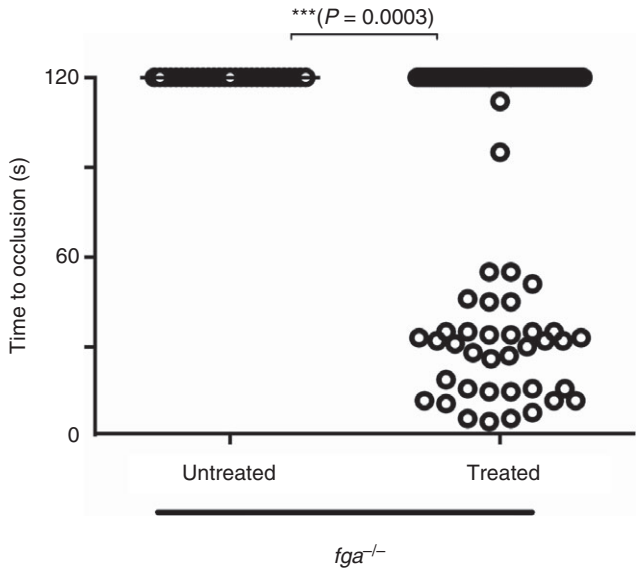

Fig. 5. Treatment of *fga* mutants with ε-aminocaproic acid rescues the hemostatic defect. Mutant offspring were treated with 100 mM ε-aminocaproic acid at 24 hours post-fertilization (hpf). Time to occlusion after endothelial injury was measured at 3 days post-fertilization (dpf). Treated $fga^{-/-}$ larvae (n = 110) exhibited a statistically significant rescue compared to untreated $fga^{-/-}$ (n = 29). P = 0.0003 by the Mann–Whitney U-test.

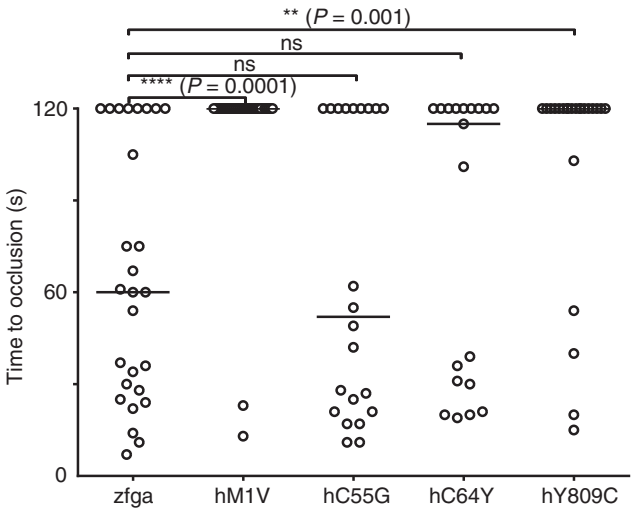

Fig. 6. Functional analysis of human FGA variants in a fibrinogen deficient zebrafish model. Human FGA substitutions M1V, C55G, C64Y and Y809C were genetically engineered into the orthologous positions of zebrafish *fga* cDNA in pubi-zfga-p2A-EGFP. Wild-type (*zfga*) or variant cDNAs were injected into one-cell-stage embryos from $fga^{+/-}$ and $fga^{-/-}$ intercrosses and time to occlusion was recorded after laser-induced endothelial injury, up to 120 s. Data shown are results from $fga^{-/-}$ larvae. Statistical significance was determined by comparing variant (hM1V, n = 33; hC55G, n = 29; hC64Y, n = 19; hY809C, n = 23) and *zfga* (n = 27) injected $fga^{-/-}$ larvae using the Mann–Whitney U-test. Horizontal bars represent the median time to occlusion. ns, not significant.

Table 1 Human *FGA* variants

Human amino acid variant	Human mutation	Orthologous amino acid position (zebrafish)	Hemorrhage	Reference
hM1V	c.1A>G	1	No	[43]
hC55G	c.221T>G	51	Yes	[45]
hC64Y	c.249G>A	60	Yes	[45]
hY809C	c.2425A>G	640	Yes	[46]

demonstrated that induced larval venous thrombi are fibrin rich [31], suggesting an important role in embryonic hemostasis. In this study our endothelial injury experiments confirm that fibrinogen is indeed required for induced thrombus formation, consistent with our previous studies of F10 deficiency, as well as observed hypofibrinogenemia in At3-deficient fish [29,30]. In all three mutants (*fga*, *f10* and *at3*), embryonic/larval zebrafish tolerate severe coagulopathies into early adulthood before succumbing, although death occurs at a much later time-point in the *fga* mutants. Fish *et al.* [28] found ~40% survival of homozygous mutants at 4–5 months of age, whereas at a similar age we recovered 80%, with 40% remaining around 1 year of age. These differences are likely to be caused by variations in housing or aquaculture systems and/or genetic background, because the prior mutant was generated on a pure AB background whereas we used an AB×TL hybrid. Similar survival variations have been observed with targeted mutation of *Fga* in mice, which exhibit variable timing of fatal abdominal hemorrhage depending on the genetic background. At 2 months of age, 91% of C57BL/6J mice, but only 57% of the mixed 129/CF-1 background, were found to be alive [52]. The fish *fga* mutant data are in sharp contrast to F10 deficiency, which resulted in death beginning as early as 1 month of age, with complete loss by 4 months [30]. However, this difference is consistent with observations in human patients [1] and mouse knockouts [56–59] demonstrating that common pathway mutations are more severe than defects in fibrinogen.

One of the most intriguing findings of this study was the synthetic lethality observed from combined loss of fibrinogen and Nfe2. Thrombocytes have clearly been shown to express similar genes and function like platelets [26], but definitive proof that they are active in preventing hemorrhage has not been shown. The lethality in $fga^{-/-}$; $nfe2^{-/-}$ mutants bolsters the belief that thrombocytes are the functional equivalent of mammalian platelets and, to our knowledge, these are the first *in vivo* data showing that they are required for hemostasis-related survival. In mice, $fga^{-/-}$; $nfe2^{-/-}$ results in lethality in the immediate neonatal period, but zebrafish initially survive with a steady decline until ~300 dpf. This trend is consistent with coagulation factor knockouts that are lethal in embryonic mice, but exhibit extended survival in zebrafish [29,30]. We hypothesize this may be a result of differential

species-specific factors regulating hemostatic balance, which could be leveraged to improve our understanding of coagulopathies. Presumably *fga*$^{-/-}$; *nfe2*$^{-/-}$ fish die from hemorrhage, although given the broad timespan of lethality and low genotypic frequency of double mutants, definitive confirmation will require significantly larger pedigrees.

Visible hemorrhage was noted after 1 month of age in *fga* mutants, most commonly in the brain, muscle and fins. The timing, location and extent of hemorrhage are similar to the previous ZFN-induced *fga* knockout [28], as well as our results for F10 deficiency [30]. In both mutants, this appears to be the cause of lethality, but the timing is significantly different, as all *f10* homozygous mutants die by 4 months of age, as opposed to 40–80% survival at that time-point for *fga* mutants. Unlike mice, there is great variability within standard zebrafish strains [60]. Even so, genetic background is less likely to be a factor in the difference between these two strains, as the mutant lines were produced from the same AB×TL colony. It seems unlikely that differences in the genetic background of the *fga* and *f10* mutants would cause such a large difference in timing of lethality, suggesting that there may be additional factors beyond the observed hemorrhage.

The reversal of the larval hemostatic defect by ε-aminocaproic acid was surprising. Clinically, ε-aminocaproic acid is thought to stabilize clots by binding to plasmin, preventing fibrinolysis. We have now observed rescue of hypofibrinogenemic [29] and afibrinogenemic zebrafish using ε-aminocaproic acid, which suggests there may be alternate mechanisms for its modulation of hemostasis.

Our previous studies of fibrinogen depletion in zebrafish employed antisense morpholino technology, targeting all three fibrinogen chains, *fga*, *fgb* and *fgg* [31]. Individual knockdown resulted in 1–5% rates of intramuscular and intracranial hemorrhage in 3 dpf larvae. When all three morpholinos were co-injected, the rate increased to nearly 20%. However, in both our TALEN-induced and the ZFN-induced knockouts, these phenotypes were not present. One possible explanation for this discrepancy is that morpholino and germline targeting yield differential phenotypic effects. There have been some reports that suggest there is genetic compensation for germline mutations, but not morpholino knockdowns [61].

Cross-regulation of coagulation factors at the mRNA level was identified in the *f10* knockout [30]. Loss of F10 resulted in statistically significant increases of *fga* and *at3* mRNA by 75 and 100%, respectively, but no significant effect on *f2*. We saw the opposite results through loss of Fga, with decreases in *f10, f2* and *at3* mRNAs by ~50%. These data suggest the possibility of a gene expression feedback loop for the common pathway. In both mutants, fibrin production is reduced, thus the presence or absence of fibrinogen itself may be an important determinant of common pathway mRNA expression. In the *fga* mutant larvae, we might have expected to see a severe bleeding phenotype, given the decrease in *f10* and *f2*, but this could be balanced by the decrease in *at3* expression.

We have previously used zebrafish larvae for qualitative *in vivo* analysis of human *AT3* and *F10* coagulation factor mutations. In this work we first showed the ability to rescue the *fga*$^{-/-}$ defect using zebrafish *fga* mRNA, followed by human fibrinogen protein. Given the similarities between human and zebrafish fibrinogen deficiencies, we were able to use our model to discriminate the functional consequences of human mutations, finding that M1V and Y809C were unable to rescue the hemostatic defect. M1V is a novel mutation that has been recently identified in patients [51] and here we confirm it *in vivo* as a pathological mutation. Surprisingly, two cysteine substitutions, C55G (Fibrinogen La Seyne and Fibrinogen Quimper) and C64Y (Fibrinogen Marseilles II), were able to rescue *fga*$^{-/-}$ mutants despite the fact that both positions are highly conserved and patients with these mutations are hypofibrinogenemic (activity levels down to 20–50% of the lower limit of the normal range [54]). The cysteine at position 55 has been clearly shown to be important for disulfide formation with the Bβ subunit of fibrinogen. Its substitution results in impaired hexamer assembly, yet continued secretion of half molecules of fibrinogen in cell culture [62], although not in patients [54]. Hanss *et al.* postulate that this is a result of overexpression in culture. This is consistent with our data as we used the *ubi* promoter, which drives robust expression in zebrafish embryos and larvae [43]. The Y809C substitution was originally an incidental finding in a pedigree with Fibrinogen Montreal (D515N), and segregated separately from the affected family member [55]. Y809C is only present in fibrinogen$_{420}$, a variant comprising only ~1% of circulating fibrinogen [63], although it is 3-fold higher in fetal plasma [64]. No functional studies had been performed on Y809C until this report in which we demonstrate that this variant abrogates fibrinogen function. One limitation of these experiments is that causative mutations that reduce human FGA expression without affecting function would be expected to rescue the defect in thrombus formation in this overexpression system.

These data expand our view of coagulation pathophysiology in aquatic species. Earlier studies demonstrated significant conservation of coagulation physiology in zebrafish [24–26,65,66]. With the advent of genome editing, we have now expanded on this foundation and developed genetic models of coagulation disorders and used these to identify novel functional human mutations [29,30,66]. The phenotypes align with those seen in mammals, but demonstrate a significant shift in survival. Further understanding of the underlying biology that explains why fish can tolerate such severe coagulation defects, as well as *in vivo* analysis of clotting factor variants, may lead to novel therapeutic approaches for patients with the associated disorders.

Addendum

Z. Hu designed and performed research, analyzed data and wrote the manuscript; K. I. Lavik performed research, analyzed data and wrote the manuscript; Y. Liu, C. E. Richter and A. H. Vo performed research and analyzed data; J. Di Paola provided unpublished reagents and edited the manuscript; and J. A. Shavit designed and supervised research, analyzed data and wrote the manuscript.

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Disclosure of Conflict of Interests

J. A. Shavit has been a consultant for Bayer, Shire, CSL Behring, Grifols and Octapharma in the last 3 years.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1. Comprehensive list of primers.
Table S2. Expression vectors.
Figure S1. Transcription of coagulation cascade factors was significantly reduced in *fga* mutants, as determined by qPCR.

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