

## THROMBOSIS AND HEMOSTASIS

# Hypofibrinogenemia with preserved hemostasis and protection from thrombosis in mice with an *Fga* truncation mutation

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## KEY POINTS

- Genetic truncation of the fibrinogen  $\alpha$ C-region in mice results in hypofibrinogenemia from nonsense-mediated decay of mutant *Fga* mRNA.
- Induced hypofibrinogenemia preserves hemostasis and antimicrobial function, but protects against venous thrombosis.

Genetic variants within the fibrinogen A $\alpha$  chain encoding the  $\alpha$ C-region commonly result in hypodysfibrinogenemia in patients. However, the (patho)physiological consequences and underlying mechanisms of such mutations remain undefined. Here, we generated *Fga*<sup>270</sup> mice carrying a premature termination codon within the *Fga* gene at residue 271. The *Fga*<sup>270</sup> mutation was compatible with Mendelian inheritance for offspring of heterozygous crosses. Adult *Fga*<sup>270/270</sup> mice were hypofibrinogenemic with ~10% plasma fibrinogen levels relative to *Fga*<sup>WT/WT</sup> mice, linked to 90% reduction in hepatic *Fga* messenger RNA (mRNA) because of nonsense-mediated decay of the mutant mRNA. *Fga*<sup>270/270</sup> mice had preserved hemostatic potential in vitro and in vivo in models of tail bleeding and laser-induced saphenous vein injury, whereas *Fga*<sup>-/-</sup> mice had continuous bleeding. Platelets from *Fga*<sup>WT/WT</sup> and *Fga*<sup>270/270</sup> mice displayed comparable initial aggregation following adenosine 5'-diphosphate stimulation, but *Fga*<sup>270/270</sup> platelets quickly disaggregated. Despite ~10% plasma fibrinogen, the fibrinogen level in *Fga*<sup>270/270</sup> platelets was ~30% of *Fga*<sup>WT/WT</sup> platelets with a compensatory increase in fibronectin. Notably, *Fga*<sup>270/270</sup> mice showed complete protection from thrombosis in the inferior vena cava

stasis model. In a model of *Staphylococcus aureus* peritonitis, *Fga*<sup>270/270</sup> mice supported local, fibrinogen-mediated bacterial clearance and host survival comparable to *Fga*<sup>WT/WT</sup>, unlike *Fga*<sup>-/-</sup> mice. Decreasing the normal fibrinogen levels to ~10% with small interfering RNA in mice also provided significant protection from venous thrombosis without compromising hemostatic potential and antimicrobial function. These findings both reveal novel molecular mechanisms underpinning fibrinogen  $\alpha$ C-region truncation mutations and highlight the concept that selective fibrinogen reduction may be efficacious for limiting thrombosis while preserving hemostatic and immune protective functions.

## Introduction

Congenital hypodysfibrinogenemia is a rare autosomal disorder characterized by both altered fibrinogen structure and significantly reduced circulating fibrinogen levels. The disorder is often driven by mutations in the C-terminal region of the A $\alpha$  chain (ie, residues 220-610,  $\alpha$ C-region), including single nucleotide polymorphisms, truncations, and insertions. The clinical manifestations of  $\alpha$ C region truncation mutations in 24 reported cases vary, but are generally associated with mild bleeding from reduced circulating fibrinogen levels.<sup>1</sup> For example, patients with Fibrinogen Otago (lacking A $\alpha$  271-610) or Fibrinogen Marburg (lacking A $\alpha$  464-610) have significantly prolonged plasma clotting times, with circulating fibrinogen levels of 0.06 mg/mL

and 0.6 mg/mL, respectively, compared with 2 to 4 mg/mL in healthy individuals.<sup>2,3</sup> Notably, the molecular basis of the reduced fibrinogen levels is currently unclear.<sup>1</sup>

A significant complication to understanding the biology of hypodysfibrinogenemia is that 2 variables are simultaneously imposed (ie, low levels and mutant form of fibrinogen). Consequently, whether low circulating levels of fibrinogen, the mutated fibrinogen protein, or both, are tied to changes in the hemostatic and thrombotic properties of patients is an unresolved question. Analyses of patient plasma and recombinant fibrinogen have implicated the  $\alpha$ C region in lateral aggregation of fibrin fibrils and interaction with fibrinolytic

proteins such as plasminogen, tissue plasminogen activator (tPA), and antiplasmin.<sup>2-10</sup> However, these studies are unable to recapitulate all of the complex interactions mediated by fibrinogen and other blood components (eg, platelets) that modulate hemostatic and thrombotic outcomes in the proper microenvironment (eg, within vessels under arterial or venous flow conditions).

To analyze the (patho)physiological implications and the underlying mechanisms of a genetic truncation mutation in the  $\alpha$ C region of fibrinogen, we generated *Fga*<sup>270</sup> mice that carry a stop codon at residue 271 of the  $\alpha$ A chain, analogous to Fibrinogen Otago.<sup>2</sup> Similar to patients with  $\alpha$ C region truncation mutations, *Fga*<sup>270/270</sup> mice were hypodysfibrinogenemic. Studies of *Fga*<sup>270/270</sup> mice were coupled with a new model of small interfering RNA (siRNA)-mediated reduction of normal fibrinogen in wild-type (WT) mice to determine whether alterations in hemostasis, thrombosis, and antimicrobial function were mechanistically coupled to low fibrinogen levels and/or the loss of the  $\alpha$ C region.

## Materials and methods

Detailed materials and methods can be found in the supplemental Methods and Materials on the *Blood* Web site.

### Generation of *Fga*<sup>270</sup> gene-targeted mice

Single-cell C57Bl/6J embryos were injected with Cas9 protein, donor single-stranded DNA replacement vector and single guide RNA targeting the mouse *Fga* gene within exon 5. Injected embryos were subsequently implanted into pseudo-pregnant female mice to generate mice carrying the *Fga*<sup>270</sup> mutation. Homologous recombinants were identified by polymerase chain reaction (PCR) analysis followed by a diagnostic *AflIII* restriction enzyme digest. In all animal studies, sex- and age-matched (8-12 weeks) mice were used. Animal experiments and protocols were approved by the Animal Care and Use Committees of University of North Carolina at Chapel Hill and Michigan State University.

### Fibrinogen knockdown with siRNA

Mice were administered 2 mg/kg of lipid nanoparticles containing siRNA against fibrinogen (siFga) or luciferase (siLuc). Lipid nanoparticles were prepared as previously described.<sup>11</sup> For each experiment, the decrease in fibrinogen was confirmed by enzyme-linked immunosorbent assay (ELISA) and/or western blot. For clotting and infection studies, siRNA was administered at 10 days and 3 days before each experimental challenge to ensure stable knockdown of fibrinogen.

### In vivo hemostasis and thrombosis analyses

Tail bleeding times were measured as described.<sup>12</sup> Briefly, a 3-mm tail tip was excised from anesthetized mice and submerged in Tris-buffered saline (pH 7.5) containing 2 mM CaCl<sub>2</sub> at 37°C until cessation of bleeding was sustained for more than 30 seconds. Laser-induced saphenous vein injury model was performed as previously described.<sup>13</sup> Briefly, mice were injected with Alexa Fluor 488-conjugated antibodies to GPIIb/IIIa (2.5  $\mu$ g, clone XIA.B4, Emfret Analytics) to label circulating platelets and with Alexa Fluor 647-conjugated antibodies against fibrin

(2  $\mu$ g/mouse; gift from Rodney Camire). Platelet and fibrin accumulation at laser injury sites were assessed by intravital microscopy. The inferior vena cava (IVC) stasis model was performed as described.<sup>14</sup> Side branches were ligated and the lumbar branches cauterized. After 24 hours, mice were anesthetized and the thrombi were separated from the isolated IVC and weighed.

## Statistical analyses

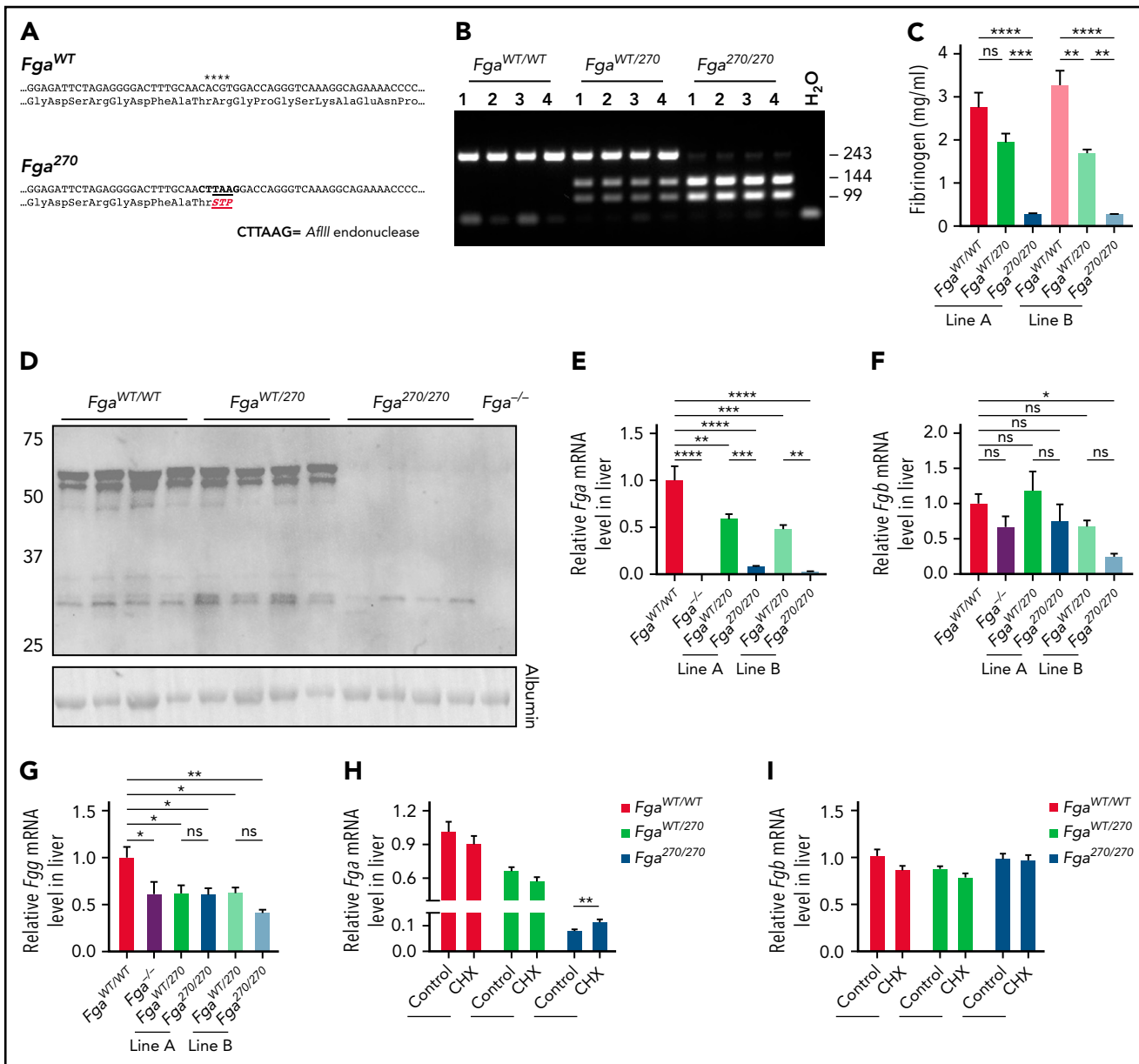
All analyses were performed using Prism 9. Comparisons of multiple groups were performed using analysis of variance (ANOVA) and Sidak multiple comparison test. Analyses of samples containing censored values were performed using the Kruskal-Wallis or Mantel-Cox tests. Analyses of survival were performed using the Kaplan-Meier method. Results were considered significant when  $P < .05$ .

## Results

### *Fga*<sup>270</sup> mice that carry a premature termination codon in the *Fga* gene are hypodysfibrinogenemic

Mice carrying a truncation mutation in the  $\alpha$ C region of fibrinogen were generated by introducing a premature termination codon in exon 5 of the *Fga* gene (Figure 1A). Incorporation of the mutation was confirmed by PCR analysis of genomic DNA (representative data in Figure 1B) and direct sequence analysis in 2 independent genetic lines (termed lines A and B). Plasma fibrinogen levels of *Fga*<sup>270/270</sup> mice were  $\sim$ 10% of *Fga*<sup>WT/WT</sup> mice (Figure 1C; supplemental Figure 1A-C). The truncated fibrinogen  $\alpha$ A chain had the expected molecular weight of  $\sim$ 30 kDa by western blot analysis of plasma collected from mice of line B under reducing conditions using a fibrinogen  $\alpha$ A chain-specific antibody (Figure 1D). When nonreduced samples were analyzed by western blot against fibrinogen  $\beta$  $\beta$  chain, the mutant fibrinogen (ie, FibA $\alpha$ <sup>270</sup>) had the predicted molecular weight, indicating that FibA $\alpha$ <sup>270</sup> was able to assemble into a mature fibrinogen molecule (supplemental Figure 1D). Similar results were obtained with plasma from mice of line A (data not shown).

Hepatic *Fga*, *Fgb*, and *Fgg* messenger RNA (mRNA) levels were analyzed to determine the cause of hypofibrinogenemia in *Fga*<sup>270</sup> mice. Compared with *Fga*<sup>WT/WT</sup> mice, *Fga* mRNA was decreased to 59% and 48% in *Fga*<sup>WT/270</sup> mice, and 8% and 3% in *Fga*<sup>270/270</sup> mice in lines A and B, respectively (Figure 1E). Hepatic *Fgb* mRNA levels were decreased in *Fga*<sup>270/270</sup> mice only in line B, whereas *Fgg* mRNA levels were decreased in both *Fga*<sup>WT/270</sup> and *Fga*<sup>270/270</sup> mice (Figure 1F-G). To analyze for possible nonsense-mediated decay of the mutant mRNA, a protein translation-dependent process, primary hepatocytes were isolated and treated with cycloheximide.<sup>15</sup> *Fga* mRNA levels were significantly increased by 40% in hepatocytes of *Fga*<sup>270/270</sup> mice, whereas no changes were detected in those of *Fga*<sup>WT/WT</sup> and *Fga*<sup>WT/270</sup> mice (Figure 1H). *Fgb* expression levels in hepatocytes of all genotypes did not change with cycloheximide treatment (Figure 1I). As expected, no histological differences were observed in livers of *Fga*<sup>270</sup> mice (supplemental Figure 2A).



**Figure 1. Generation of *Fga*<sup>270</sup> mice that express low levels of fibrinogen with a truncated form of  $\alpha$  chain after residue 270.** (A) Summary of the nucleic acid substitutions introduced by Crispr-Cas9 gene editing and resulting amino acid changes for the mutated fibrinogen  $\alpha$ -chain gene of the *Fga*<sup>270</sup> mice. Asterisks highlight positions of the nucleotide substitutions. (B) Representative PCR analysis to establish animal genotypes of *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, and *Fga*<sup>270/270</sup> mice. (C) ELISA measurement of plasma fibrinogen levels from *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, and *Fga*<sup>270/270</sup> mice from 2 independent lines (n = 3-4 per genotype). One-way ANOVA was used to determine statistical significance. (D) Western blot analysis of plasma (reducing conditions) from *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice using antibodies directed against the  $\alpha$  chain of fibrinogen. Analysis of hepatic mRNA levels by quantitative reverse transcriptase PCR for (E) *Fga*, (F) *Fgb*, and (G) *Fgg* in *Fga*<sup>-/-</sup> mice and 2 independent lines of *Fga*<sup>270</sup> mice (n = 4 per genotype). One-way ANOVA test was used to determine statistical significance. Analysis of fibrinogen (H) *Fga* and (I) *Fgb* gene expression from primary mouse hepatocytes isolated from *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, and *Fga*<sup>270/270</sup> mice treated with or without 100  $\mu$ M cycloheximide (CHX) for 3 hours (n = 3-4 per treatment group). Two-tailed Student t test was used to determine statistical significance. \**P* < .05, \*\**P* < .01, \*\*\**P* < .001, \*\*\*\**P* < .0001. ns, no statistical significance.

## *Fga*<sup>270</sup> mice display postnatal success, but a fraction of *Fga*<sup>270/270</sup> dams succumb to the challenge of pregnancy

Crosses of heterozygous *Fga*<sup>WT/270</sup> mice produced offspring in a Mendelian distribution. For line A, 20.8% were *Fga*<sup>WT/WT</sup>, 54.8% were *Fga*<sup>WT/270</sup>, and 24.4% were *Fga*<sup>270/270</sup> for the first 169 pups. Similar results were found with the first 165 pups of line B: 22.4% *Fga*<sup>WT/WT</sup>, 55.2% *Fga*<sup>WT/270</sup>, and 22.4% *Fga*<sup>270/270</sup> (Table

1). The survival profile of offspring from crosses of *Fga*<sup>270/270</sup> males and *Fga*<sup>WT/270</sup> females produced *Fga*<sup>270/270</sup> mice at 66% (*P* < .05) and 75% (*P* = .11) of expected for lines A and B, respectively. In lines A and B, 8 of 127 (6.3%) and 7 of 127 (5.1%) of *Fga*<sup>270/270</sup> offspring, respectively, suffered fatal spontaneous perinatal abdominal hemorrhagic events and soft-tissue bleeds within 3 days of birth (supplemental Figure 2B). Mating of *Fga*<sup>270/270</sup> dams produced mixed results. Contrary to *Fga*<sup>-/-</sup>

**Table 1. Analysis of postnatal survival of offspring of  $Fga^{WT/270}$  dams in 2 independent lines**

$\text{♀}Fga^{WT/270} \times \text{♂}Fga^{WT/270}$	Line A			Line B		
Offspring genotype	$Fga^{WT/WT}$	$Fga^{WT/270}$	$Fga^{270/270}$	$Fga^{WT/WT}$	$Fga^{WT/270}$	$Fga^{270/270}$
Expected Mendelian ratio	1	2	1	1	2	1
Number observed (at weaning)	35	92	41	37	91	37
% of expected	100%	131%	117%	100%	123%	100%
$\text{♀}Fga^{WT/270} \times \text{♂}Fga^{270/270}$	Line A		Line B			
Offspring genotype	$Fga^{WT/270}$	$Fga^{270/270}$	$Fga^{WT/270}$	$Fga^{270/270}$		
Expected Mendelian ratio	1	1	1	1		
Number observed (at weaning)	164	109	126	94		
% of expected	100%	66%*	100%	75%		

\* $P < 0.05$ ,  $\chi^2$  analysis.

dams that uniformly die of hemorrhagic events during pregnancy, 9 of 21  $Fga^{270/270}$  dams survived to sustain pregnancies and deliver pups. However,  $Fga^{270/270}$  dams that gave birth either died of postpartum hemorrhage or were unable to nurse the newborn pups, resulting in loss of the offspring (Table 2). The pups resulting from  $Fga^{270/270}$  dams that died after delivery were able to survive to weaning when fostered to healthy nursing females. Adult  $Fga^{270/270}$  mice did not display spontaneous bleeding events nor altered survival rate compared with  $Fga^{WT/WT}$  mice.

### $Fga^{270/270}$ mice exhibit preserved hemostasis

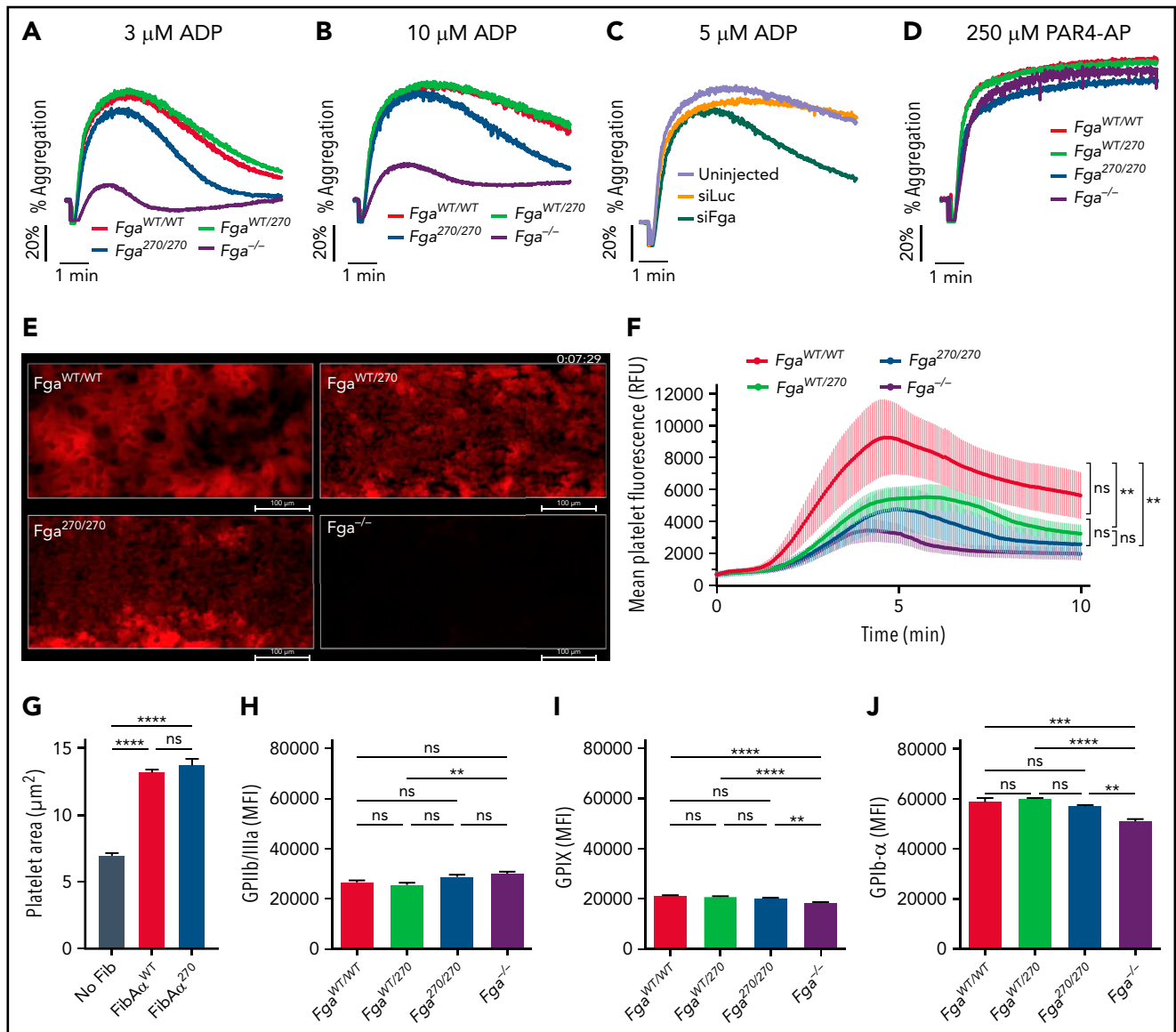
Two distinct bleeding models were performed on  $Fga^{270}$  and  $Fga^{-/-}$  mice to quantify the impact of  $Fga^{270}$  mutation on

hemostasis. In a tail bleeding assay,  $Fga^{WT/WT}$  and  $Fga^{WT/270}$  mice rapidly achieved hemostasis and stopped bleeding in  $100.7 \pm 14.2$  and  $91.5 \pm 10.1$  seconds, respectively. Brief rebleeding was observed in each of  $Fga^{WT/WT}$  and  $Fga^{WT/270}$  mice. Notably, 5/6  $Fga^{270/270}$  mice achieved hemostasis with one mouse bleeding during the entire 5-minute observation period (Figure 2A). In contrast, none of the  $Fga^{-/-}$  mice achieved hemostasis, consistent with previous reports.<sup>16</sup> To determine whether 10% of circulating normal fibrinogen was sufficient to maintain hemostasis, tail bleeding assays were performed on mice treated with siFga or siLuc. With this model, circulating fibrinogen was successfully reduced to ~10% in siFga-treated mice (supplemental Figure 3). Notably, both siLuc- and siFga-treated mice readily achieved hemostasis. Using a

**Table 2. Analysis of postnatal survival of offspring of  $Fga^{270/270}$  dams in 2 independent lines**

$\text{♀}Fga^{270/270} \times \text{♂}Fga^{WT/270}$	Line A	Line B
Total impregnated dams	2	2
Gave birth and supported pups to weaning	—	—
Gave birth, but dam died before weaning	1	—
Gave birth, but pups died before weaning	—	—
Died during pregnancy	1	2
$\text{♀}Fga^{270/270} \times \text{♂}Fga^{270/270}$	Line A	Line B
Total impregnated dams	9	8
Gave birth and supported pups to weaning	—	—
Gave birth, but dam died before weaning	3	3
Gave birth, but pups died before weaning	2	—
Died during pregnancy	4	5





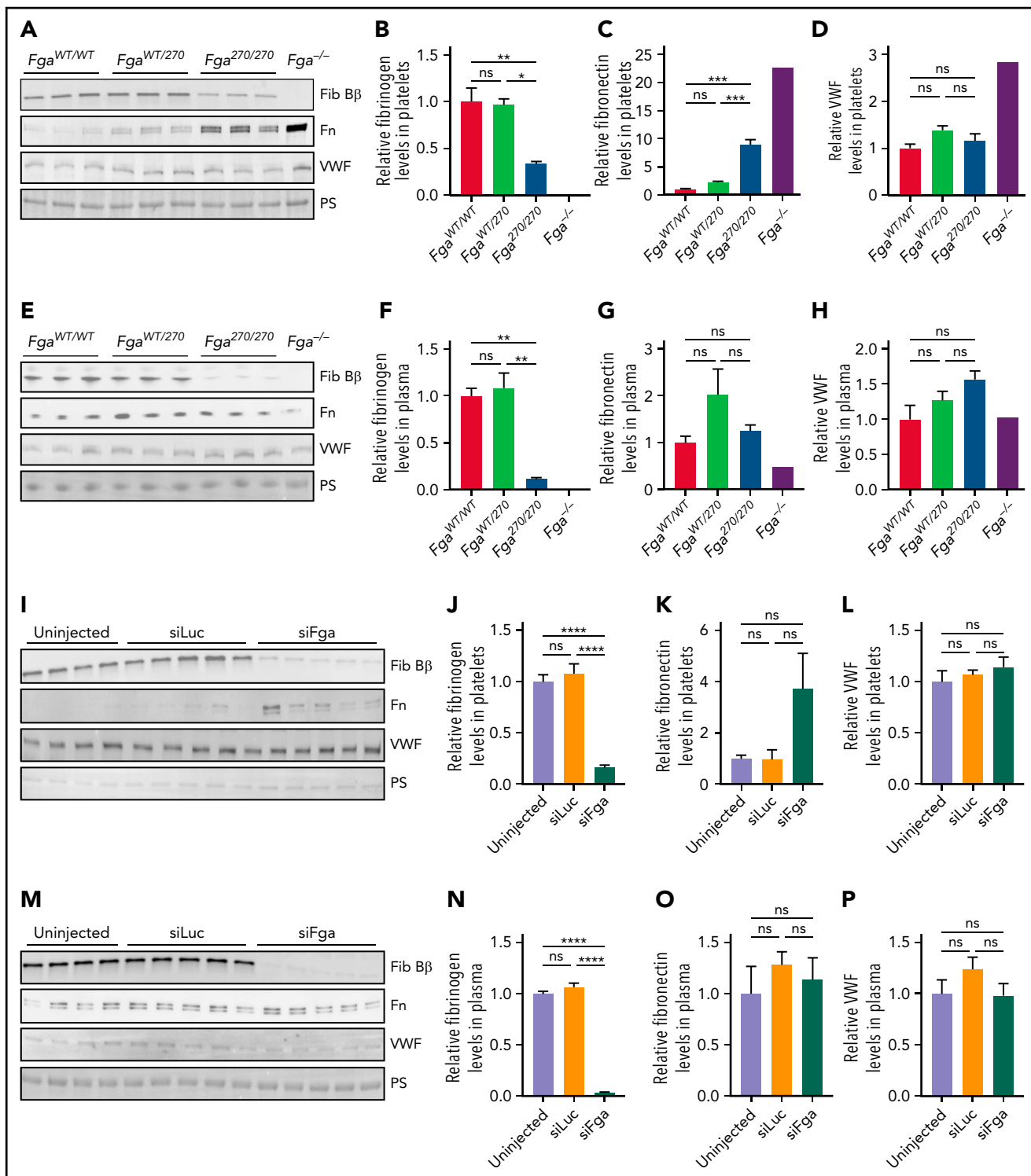
**Figure 3. *Fga*<sup>270/270</sup> platelets form weaker aggregates in response to ADP stimulation.** Representative aggregation traces of platelet-rich plasma collected from *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice following stimulation with (A) 3 μM ADP or (B) 10 μM ADP (n = 4 per genotype). (C) Representative aggregation traces of platelet-rich plasma collected from untreated or siLuc- or siFga- treated mice following stimulation with 5 μM ADP (n = 4-5 per group). (D) Representative aggregation traces of platelet-rich plasma collected from *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice following stimulation with 250 μM of protease-activated receptor 4 activating peptide (PAR4-AP) (n = 4 per genotype). (E) Representative images and (F) quantification of platelet adhesion (indicated in red) to collagen coated surface at venous (400 seconds<sup>-1</sup>) shear rate using heparinized whole blood from *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice (n = 4 per genotype). (G) Adhesion and spreading of *Fga*<sup>-/-</sup> platelets on uncoated or FibAα<sup>WT</sup>- or FibAα<sup>270</sup>- coated coverslips following stimulation with 50 μM of ADP for 30 minutes (n = 3 per fibrinogen). One-way ANOVA was used to determine statistical significance. Expression of (H) GPIIb/IIIa, (I) GPIX, and (J) GPIb-α on platelet membrane of *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice (n = 5 per genotype). One-way ANOVA was used to determine statistical significance. \*\*P < .01, \*\*\*\*P < .0001. ns, no statistical significance.

was initially low following the first injury, but progressively increased after the second and third injuries.

### FibAα<sup>270</sup> supports unstable platelet aggregation in vitro

To determine whether the *Fga*<sup>270</sup> mutation alters platelet biology, aggregation studies were performed in vitro using platelet-rich plasma. Although aggregation of *Fga*<sup>-/-</sup> platelets was markedly impaired when activated with adenosine 5'-diphosphate (ADP), *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, and *Fga*<sup>270/270</sup> platelets aggregated at similar rates. However, *Fga*<sup>270/270</sup> platelets disaggregated

faster compared with *Fga*<sup>WT/WT</sup> and *Fga*<sup>WT/270</sup> platelets (Figure 3A-B). Platelets from siFga-treated mice displayed a similar profile to that of *Fga*<sup>270/270</sup> platelets with an initial aggregation rate similar to platelets of siLuc-treated mice, but faster disaggregation following ADP stimulation (Figure 3C). Aggregation patterns were virtually identical for *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> platelets when stimulated with protease-activated receptor 4 activating peptide (Figure 3D). Platelet adhesion to collagen-coated surfaces under venous flow conditions was measured in a microfluidics chamber system by flowing heparinized whole blood obtained from *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>,



**Figure 4. *Fga*<sup>270/270</sup> platelets contain higher levels of fibrinogen relative to fibrinogen levels in plasma.** (A-D) Platelet lysates and (E-H) plasma harvested from *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice using antibodies against fibrinogen B $\beta$ -chain (Fib B $\beta$ ), fibronectin (Fn), and VWF. Ponceau S staining of albumin (PS) was used as a loading control (n = 3 per genotype). (I-L) Platelet lysates and (M-P) plasma harvested from untreated or siLuc- or siFga-treated mice using antibodies against fibrinogen B $\beta$ -chain, fibronectin, and VWF. Ponceau S staining of albumin was used as a loading control (n = 4-5 per treatment). One-way ANOVA test was used to determine statistical significance. \*P < .05, \*\*P < .01, \*\*\*\*P < .0001. ns, no statistical significance.

*Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice. *Fga*<sup>WT/WT</sup> platelets rapidly formed large and relatively stable thrombi. This phenotype was similar but less pronounced in *Fga*<sup>WT/270</sup> and *Fga*<sup>270/270</sup> blood, whereas

*Fga*<sup>-/-</sup> blood formed initial thrombi that were completely washed away (Figure 3E-F; supplemental Video 3). Changes in platelet adhesion and aggregation were not from differences

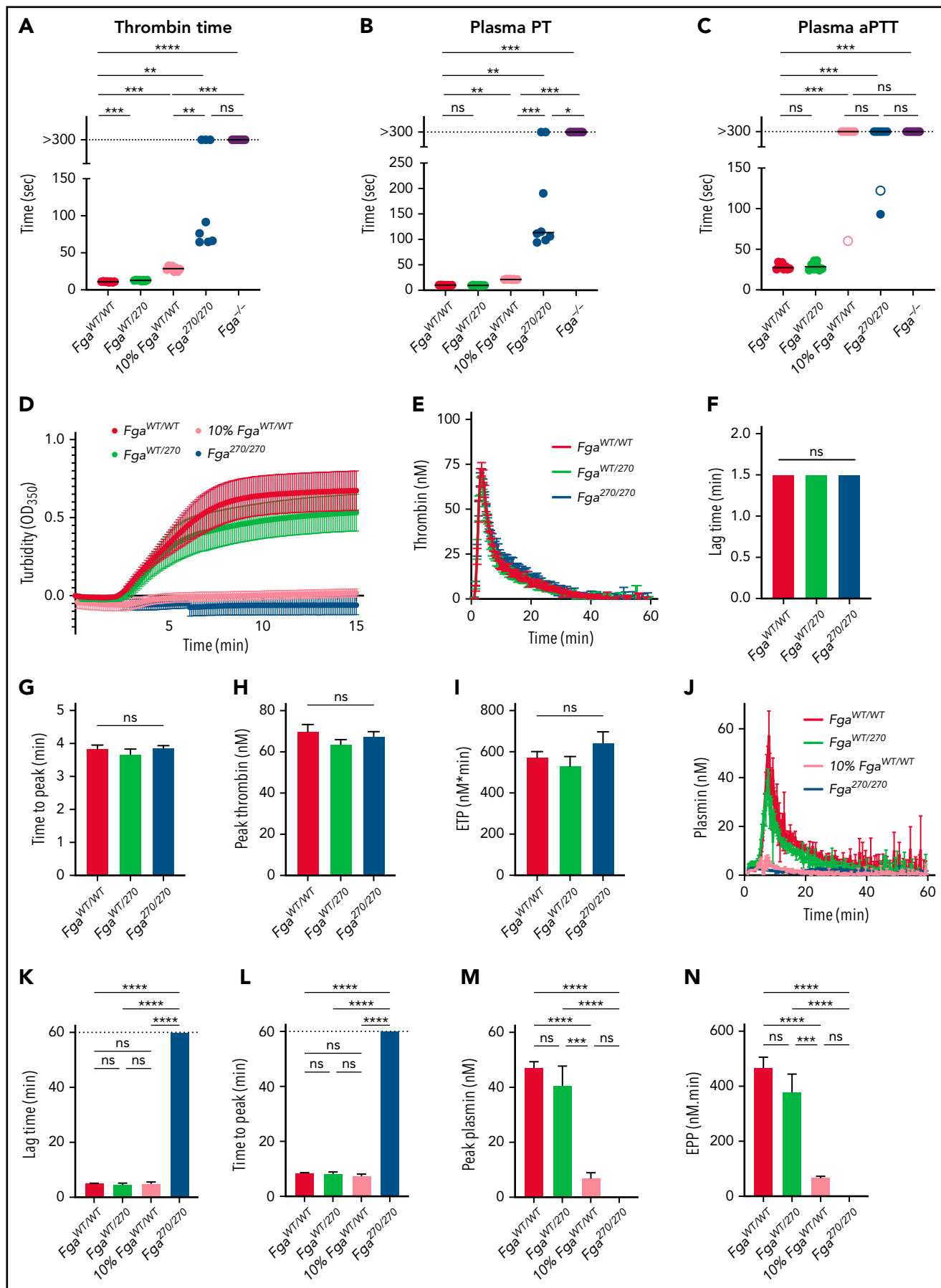
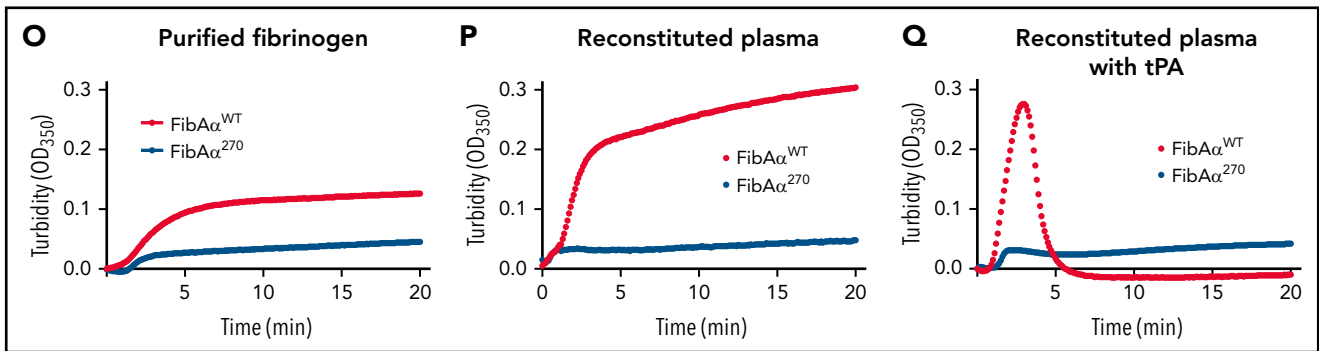


Figure 5.





**Figure 5. Altered clotting with *Fga*<sup>270/270</sup> plasma and FibA $\alpha$ <sup>270</sup> protein.** (A) TT, (B) PT, and (C) APTT of *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, 10% *Fga*<sup>WT/WT</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice (n = 4-6 per genotype). Kaplan-Meier analysis was used to determine statistical significance. (D) Turbidity analysis of *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, 10% *Fga*<sup>WT/WT</sup>, and *Fga*<sup>270/270</sup> plasma (n = 3-6 per genotype). (E) Representative thrombin generation curves and (F-I) associated parameters, and (J) representative plasmin generation curves and (K-N) associated parameters (n = 3-4 per genotype). One-way ANOVA was used to determine statistical significance. Turbidity analysis using (O) 2 mg/mL of purified FibA $\alpha$ <sup>WT</sup> and FibA $\alpha$ <sup>270</sup> fibrinogen in buffered system and 1 mg/mL of FibA $\alpha$ <sup>WT</sup> and FibA $\alpha$ <sup>270</sup> fibrinogen reconstituted in *Fga*<sup>-/-</sup> plasma in the (P) absence or (Q) presence of tPA (n = 1 per group). \**P* < .05, \*\**P* < .01, \*\*\**P* < .001, \*\*\*\**P* < .0001. ns, no statistical significance.

in platelet adhesion and spreading to purified FibA $\alpha$ <sup>WT</sup> and FibA $\alpha$ <sup>270</sup> (Figure 3G; supplemental Figure 4) nor membrane expression of GPIIb/IIIa, GPIX, and GPIb $\alpha$  (Figure 3H-J).

Comparative analyses of platelet lysates and plasma revealed that the fibrinogen content in *Fga*<sup>270/270</sup> platelets was ~30% of that in *Fga*<sup>WT/WT</sup> platelets (Figure 4A-B). Fibrinogen levels in *Fga*<sup>270/270</sup> plasma from the same set of mice were ~10% compared with *Fga*<sup>WT/WT</sup> plasma (Figure 4E-F), consistent with ELISA measurements (Figure 1C). The fibronectin content in *Fga*<sup>270/270</sup> platelets was increased approximately ninefold compared with *Fga*<sup>WT/WT</sup> platelets (Figure 4C), consistent with previous studies indicating elevated fibronectin levels in *Fga*<sup>-/-</sup> platelets.<sup>17</sup> There were no significant genotype-dependent differences in plasma fibronectin levels or platelet or plasma von Willebrand factor (VWF) levels (Figure 4D,G-H). The changes in protein content in *Fga*<sup>270/270</sup> platelets appear to be driven primarily by hypofibrinogenemia. In siFga-treated mice, reduction of plasma fibrinogen to ~4% resulted in decrease of intraplatelet fibrinogen content to ~16% relative to siLuc-treated mice (Figure 4I-J,M-N). There was a trend toward an increase in fibronectin levels in platelets of siFga-treated mice, but not in plasma fibronectin levels, and platelet or plasma VWF levels (Figure 4K-L,O-P).

### ***Fga*<sup>270/270</sup> mice display delayed clotting and impaired fibrinolysis in vitro**

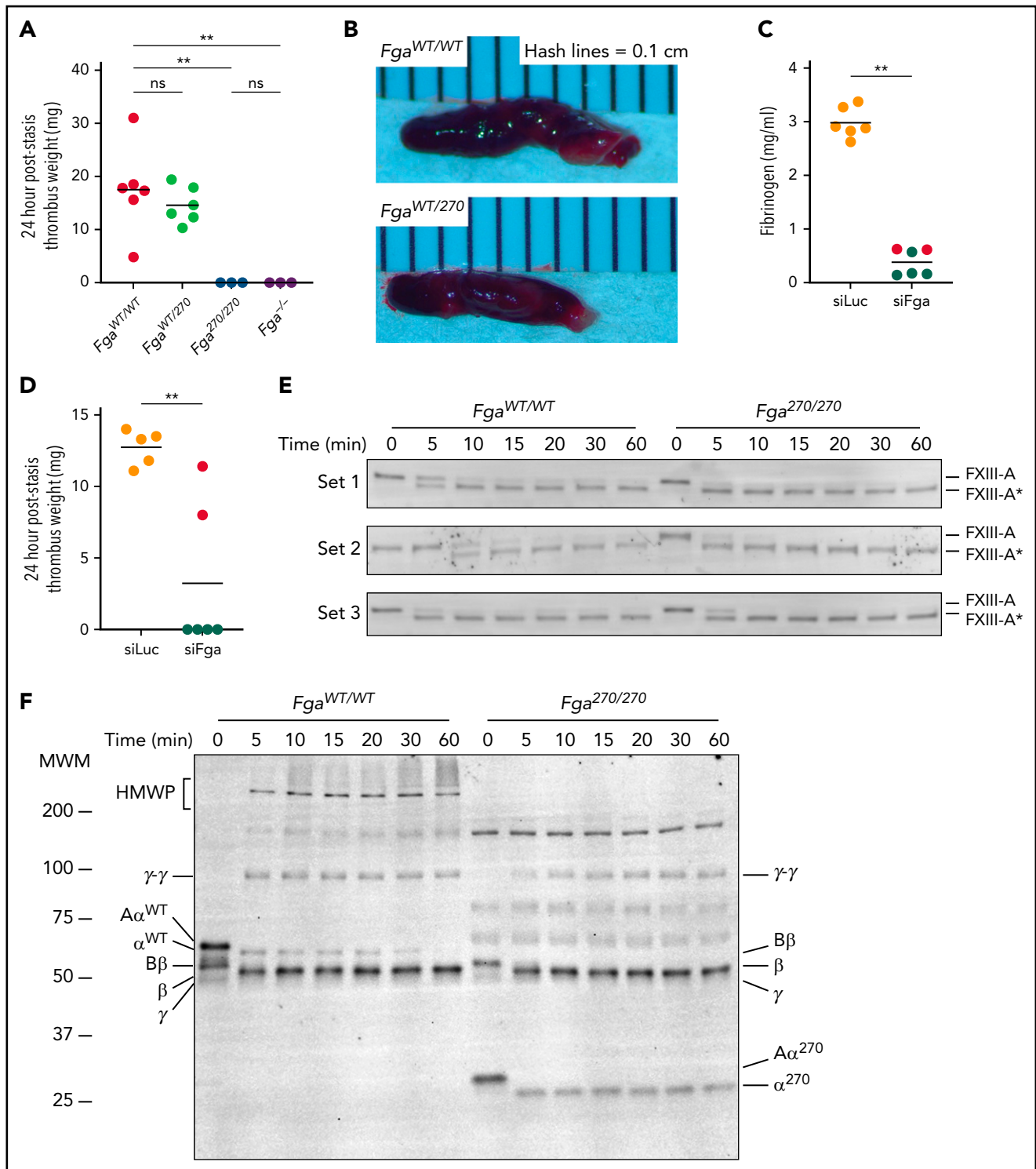
The impact of the *Fga*<sup>270</sup> mutation on clotting parameters was determined. First, thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured with platelet-poor plasma. *Fga*<sup>WT/WT</sup> and *Fga*<sup>WT/270</sup> plasma had comparable TT, PT, and APTT clotting times (Figure 5A-C). *Fga*<sup>270/270</sup> plasma had prolonged clotting times in each assay, and some individual samples failed to clot. When *Fga*<sup>WT/WT</sup> plasma was diluted to 10% with *Fga*<sup>-/-</sup> plasma (10% *Fga*<sup>WT/WT</sup>) to match the *Fga*<sup>270/270</sup> plasma fibrinogen concentration, there was a modest, but statistically significant delay in clotting time for TT and PT (Figure 5A-B). Significant differences between 10% *Fga*<sup>WT/WT</sup> and *Fga*<sup>270/270</sup> were observed for TT and PT, suggesting that both the low fibrinogen levels and the mutant protein were contributing to prolonged clotting times in *Fga*<sup>270/270</sup> samples. Both *Fga*<sup>270/270</sup> and 10% *Fga*<sup>WT/WT</sup> plasma

failed to clot in most APTT samples (Figure 5C). *Fga*<sup>-/-</sup> plasma failed to form clots over the entire observation periods for each assay. The turbidity profiles were significantly lower in *Fga*<sup>270/270</sup> and 10% *Fga*<sup>WT/WT</sup> plasma compared with *Fga*<sup>WT/WT</sup> and *Fga*<sup>WT/270</sup> plasma (Figure 5D). Thrombin generation of *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, and *Fga*<sup>270/270</sup> plasma produced identical curves with no differences in lag time, time to peak, peak thrombin concentration, or endogenous thrombin potential (Figure 5E-I). Plasmin generation<sup>18</sup> was undetectable in *Fga*<sup>270/270</sup> plasma. Whereas lag time and time to peak of 10% *Fga*<sup>WT/WT</sup> plasma were similar to *Fga*<sup>WT/WT</sup> plasma, peak plasmin concentration and endogenous plasmin potential were significantly diminished (Figure 5J-N), suggesting a contribution of both the low fibrinogen levels and the *Fga*<sup>270</sup> mutation.

In turbidity assays, FibA $\alpha$ <sup>270</sup> produced substantially lower turbidity profiles using purified protein in a buffer system (Figure 5O) or in reconstituted plasma (Figure 5P), suggesting an altered FibA $\alpha$ <sup>270</sup> clot structure relative to FibA $\alpha$ <sup>WT</sup> clots. FibA $\alpha$ <sup>WT</sup> reconstituted in *Fga*<sup>-/-</sup> plasma formed clots that were readily lysed within 5 minutes by tPA, whereas no lysis were observed with FibA $\alpha$ <sup>270</sup> clots (Figure 5Q). In a TT assay, FibA $\alpha$ <sup>WT</sup> formed stable fibrin clots, whereas FibA $\alpha$ <sup>270</sup> was unable to stop the movement of the magnetic beads within the 180-second observation period, even though gel-like fibrin was present upon examination after the assay (not shown). An altered clot structure was confirmed by scanning electron microscope analyses revealing FibA $\alpha$ <sup>270</sup> clots were generally less dense with substantial heterogeneity in fiber thickness (supplemental Figure 5).

### ***Fga*<sup>270/270</sup> mice are protected from venous thrombosis**

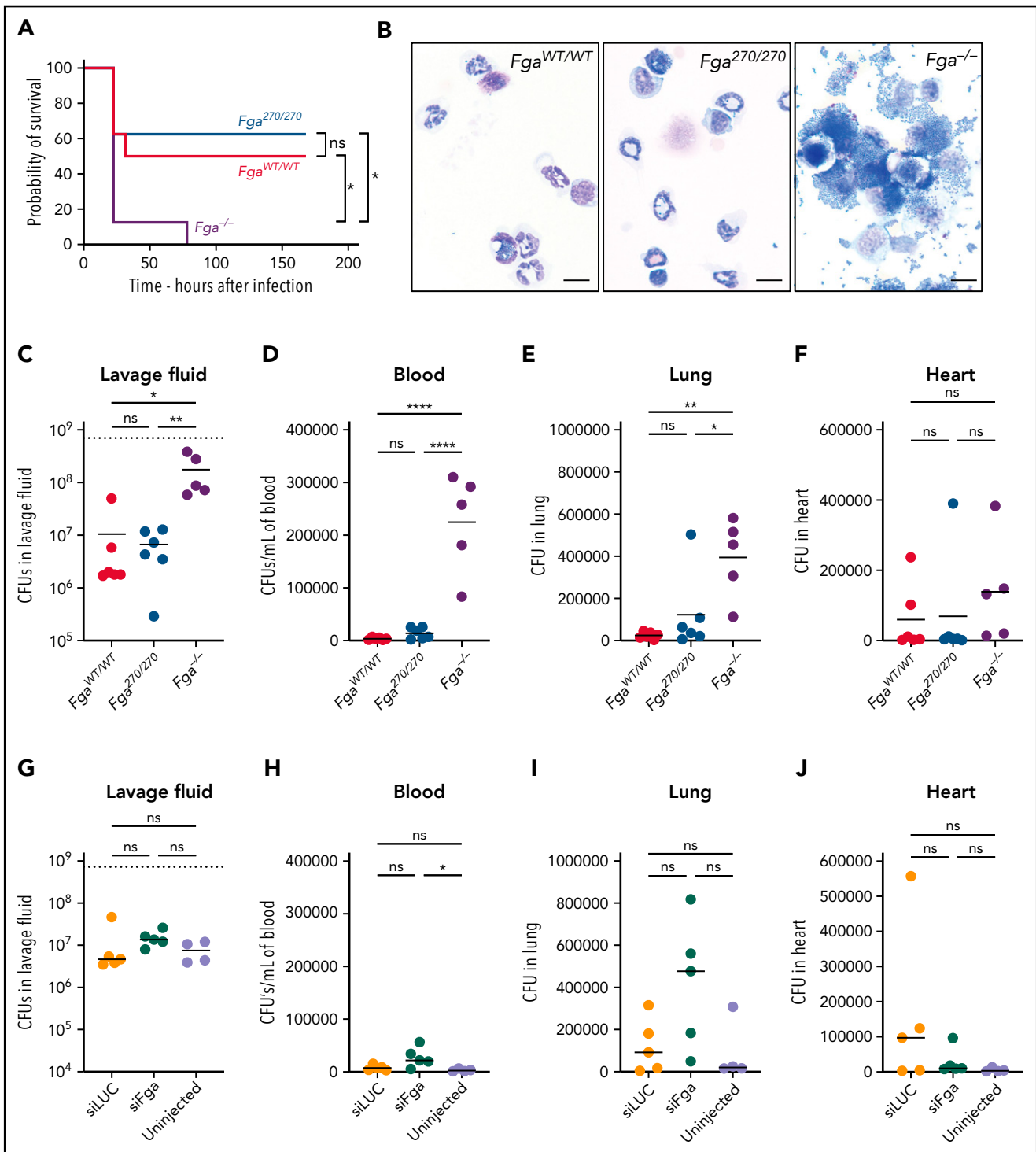
To determine the potential impact of the *Fga*<sup>270</sup> mutation on thrombus formation, mice were subjected to an IVC stasis model of venous thrombosis. All *Fga*<sup>WT/WT</sup> and *Fga*<sup>WT/270</sup> mice developed thrombi 24 hours after IVC ligation (Figure 6A-B). In contrast, neither *Fga*<sup>270/270</sup> nor *Fga*<sup>-/-</sup> mice developed thrombi. The platelet count of *Fga*<sup>270/270</sup> and *Fga*<sup>-/-</sup> mice at baseline were slightly lower than *Fga*<sup>WT/WT</sup> mice (supplemental Table 1). To determine if hypofibrinogenemia alone was protective against venous thrombosis, mice were treated with siLuc or



**Figure 6. *Fga*<sup>270/270</sup> and siFga-treated mice are protected from venous thrombosis.** (A) Thrombus weights from *Fga*<sup>WT/WT</sup>, *Fga*<sup>WT/270</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice 24 hours after IVC ligation (stasis model). Kruskal-Wallis test was used to determine statistical significance. (B) Representative images of thrombi isolated 24 hours after IVC ligation. (C) Circulating fibrinogen levels of siLuc- and siFga-treated mice immediately before the IVC ligation, measured by ELISA. Red dots indicate mice that later developed occlusive thrombi. (D) Thrombus weights from siLuc- and siFga-treated mice 24 hours after ligation. Mantel-Cox test was used to determine statistical significance. Time course of (E) FXIII activation and (F) fibrin crosslinking in *Fga*<sup>WT/WT</sup> and *Fga*<sup>270/270</sup> plasma. HMWP, high-molecular-weight polymer; MWM, molecular weight marker. \*\**P* < .01. ns, no statistical significance.

siFga to deplete circulating fibrinogen before IVC stasis (Figure 6C). Although all 5 siLuc-treated mice developed thrombi, only 2 of 6 siFga-mice developed thrombi that were smaller than those of siLuc-treated mice (Figure 6D). Factor XIIIa

(FXIIIa)-mediated crosslinking of fibrin A $\alpha$  chains contributes significantly to venous thrombosis.<sup>19,20</sup> The rate of FXIIIa activation was similar between *Fga*<sup>WT/WT</sup> and *Fga*<sup>270/270</sup> plasma (Figure 6E). Importantly, although  $\alpha$  chain in *Fga*<sup>WT/WT</sup> plasma was readily



**Figure 7. *Fga*<sup>270/270</sup> and siFga-treated mice have preserved fibrinogen-dependent antimicrobial function.** (A) Kaplan-Meier analysis of survival of *Fga*<sup>WT/WT</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice after intraperitoneal infection with  $0.7 \times 10^9$  CFUs of *S. aureus* (n = 8 per genotype). (B) Representative photomicrographs of cytopsin preparations of peritoneal lavage fluid 1 hour after intraperitoneal infection with  $0.7 \times 10^9$  CFUs of *S. aureus* from *Fga*<sup>WT/WT</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice (n = 6 per genotype). Note the cell-associated and free bacteria in samples from *Fga*<sup>-/-</sup> mice that are absent in samples from *Fga*<sup>WT/WT</sup> and *Fga*<sup>270/270</sup> mice. Scale bar: 10  $\mu$ m. CFUs within (C) lavage fluid, (D) blood, (E) lung homogenates, and (F) heart homogenates after intraperitoneal infection of *S. aureus* in *Fga*<sup>WT/WT</sup>, *Fga*<sup>270/270</sup>, and *Fga*<sup>-/-</sup> mice. CFUs within (G) lavage fluid, (H) blood, (I) lung homogenates, and (J) heart homogenates after intraperitoneal infection with *S. aureus* in untreated, siLuc- and siFga-treated mice. The dashed lines in panels C and G indicate the initial inoculum dose in CFUs. One-way ANOVA test was used to determine statistical significance. \*P < .05, \*\*P < .01, \*\*\*\*P < .0001. ns, no statistical significance.

crosslinked to high-molecular-weight polymers,  $\alpha$ -chain high-molecular-weight polymers were not detected in *Fga*<sup>270/270</sup> plasma (Figure 6F). Collectively, these findings are consistent

with the concept that the *Fga*<sup>270</sup> mutation is protective against venous thrombosis because of both hypofibrinogenemia and the loss of  $\alpha$ C-region.

## Fga<sup>270/270</sup> mice have preserved fibrinogen-dependent antimicrobial function

To determine whether the Fga<sup>270</sup> mutation influences fibrin(ogen)-dependent activities independent of hemostasis and thrombosis, we analyzed fibrin(ogen)-mediated antimicrobial function. Fga<sup>WT/WT</sup>, Fga<sup>270/270</sup>, and Fga<sup>-/-</sup> mice were challenged with an intraperitoneal infection of *Staphylococcus aureus* USA300, a methicillin-resistant *S aureus* strain. All Fga<sup>-/-</sup> mice succumbed to the infection within 78 hours, whereas 50% of Fga<sup>WT/WT</sup> mice and 62% of Fga<sup>270/270</sup> mice survived the 1-week observation period (Figure 7A). In a parallel study evaluating fibrinogen-dependent bacterial clearance, Fga<sup>WT/WT</sup> mice eliminated ~99% of the initial inoculum from the peritoneal cavity within 1 hour of infection, whereas Fga<sup>-/-</sup> failed to clear the microbe (Figure 7B-C).<sup>12,21</sup> Notably, Fga<sup>270/270</sup> mice cleared the microbes from the peritoneal cavity as efficiently as Fga<sup>WT/WT</sup> mice. The loss of fibrinogen also promoted bacterial dissemination with significantly higher colony-forming units (CFUs) in blood and lung, and a trend of higher CFU in the heart of infected Fga<sup>-/-</sup> mice relative to Fga<sup>WT/WT</sup> mice (Figure 7D-F). Importantly, the CFU burden in the blood, lung, and heart tissue of Fga<sup>270/270</sup> mice was comparable to Fga<sup>WT/WT</sup> mice. Similarly, reduction of circulating normal fibrinogen to ~10% (supplemental Figure 6) was sufficient to retain fibrin(ogen)-mediated antimicrobial function because siLuc- and siFga-treated mice challenged with *S aureus* had no differences in bacterial CFUs in the peritoneal lavage, blood, lungs, and heart (Figure 7G-J).

## Discussion

In this study, we generated Fga<sup>270/270</sup> mice with a truncated  $\alpha$ C region of fibrinogen and confirmed these mice have severe hypodysfibrinogenemia, consistent with reports of patients with homozygous truncation mutations within the  $\alpha$ C region of fibrinogen, particularly within A $\alpha$  220-447.<sup>1,22-24</sup> Low circulating fibrinogen levels in patients were previously attributed to decreased assembly, secretion and/or plasma half-life of the mutant fibrinogen protein, based on observations of low ratios of mutant:WT A $\alpha$  chains in plasma of heterozygous patients.<sup>1-3,7,25,26</sup> An important caveat of previous reports is that analyses of patient hepatic fibrinogen mRNA levels are not available because liver tissue biopsies are not feasible. Our findings indicate that reduced fibrinogen in circulation of Fga<sup>270/270</sup> mice was due to elimination of mutant mRNA. Indeed, Fga<sup>270/270</sup> mice, which encode a mutation comparable to Fibrinogen Otago, have Fga mRNA levels at ~10% of normal, matching the reduction in circulating protein. The observed rescue of Fga<sup>270/270</sup> mRNA with cycloheximide was consistent with studies of truncation mutations in other coagulation factors, including Fibrinogen Shizuoka III and Kanazawa II, FXIII, and tissue factor.<sup>27-30</sup> Notably, whereas previous analyses used transgene expression in heterologous cell lines, we analyzed nonsense-mediated decay of an endogenous coagulation factor gene using primary cells.

Despite profound hypofibrinogenemia, Fga<sup>270/270</sup> mice had preserved hemostatic potential that could be appreciated from birth. The expected Mendelian distribution of Fga<sup>270/270</sup> mice were observed at weaning from crosses of heterozygous parents. This is notable because a substantial number of Fga<sup>-/-</sup> mice do not survive to weaning but instead suffer fatal abdominal hemorrhages within 3 days of birth.<sup>16</sup> Intriguingly, we

observed a modest reduction in the number of Fga<sup>270/270</sup> mice at weaning in crosses of Fga<sup>270/270</sup> sires with Fga<sup>WT/270</sup> dams. The reason for this modest difference based on breeding strategy is unknown. The pregnancy profile in Fga<sup>270/270</sup> dams is also markedly distinct from that of Fga<sup>-/-</sup> animals. Although all Fga<sup>-/-</sup> dams die of hemorrhages during pregnancy, a subset of Fga<sup>270/270</sup> dams were able to sustain pregnancies and deliver pups, indicating sufficient fibrinogen concentration and function required for pregnancy.<sup>16</sup> The occasional miscarriages and postpartum hemorrhages of Fga<sup>270/270</sup> dams mimic observations of miscarriages in Fibrinogen Otago and Fibrinogen Mannheim V patients, and postpartum hemorrhages in Fibrinogen Perth and Fibrinogen Lincoln patients.<sup>2,6,7</sup>

Functional studies in Fga<sup>270/270</sup> mice and the siFga model supported a conclusion of a mildly perturbed, but largely preserved hemostatic potential. Fga<sup>270/270</sup> and siFga-treated mice, but not Fga<sup>-/-</sup> mice, achieved hemostasis in the tail bleeding model. A possible mechanism for the preserved hemostasis was linked to platelet-fibrinogen interactions. Both Fga<sup>270/270</sup> and siFga platelets readily aggregated initially, albeit both disaggregated faster following ADP stimulation. Fibrinogen-mediated platelet aggregation is dependent on the  $\alpha_{IIb}\beta_3$  integrin, which was present at similar levels on the platelet membrane in all Fga<sup>270</sup> mice. Additionally,  $\alpha_{IIb}\beta_3$ -dependent adhesion and spreading on immobilized FibA $\alpha^{WT}$  and FibA $\alpha^{270}$  were similar.<sup>31</sup> Unexpectedly, fibrinogen uptake in Fga<sup>270/270</sup> platelets exceeded the anticipated amounts based solely on plasma levels. Integrin  $\alpha_{IIb}\beta_3$  plays a major role in plasma protein uptake, but mechanisms that dictate the relative amounts of specific plasma factors within platelets remain largely undefined.<sup>32,33</sup> Elevated fibrinogen levels in platelets relative to plasma levels was also observed in siFga-treated mice, indicating this is a phenomenon of hypofibrinogenemia and not linked specifically to the  $\alpha$ C-region truncation. Intraplatelet fibronectin levels in Fga<sup>270/270</sup> mice were significantly increased when compared with Fga<sup>WT/WT</sup> mice, in an apparent compensation for the reduced fibrinogen. This observation is similar to what has been described in Fga<sup>-/-</sup> mice and patients with afibrinogenemia.<sup>17,34</sup> Moreover, platelets from mice expressing mutant fibrinogen lacking the binding site for integrin  $\alpha_{IIb}\beta_3$  (Fgg <sup>$\Delta$ 5 $\Delta$ 5</sup>) have significantly reduced fibrinogen levels with elevated fibronectin levels.<sup>33</sup> Given that the plasma fibrinogen levels of Fgg <sup>$\Delta$ 5 $\Delta$ 5</sup> mice are normal, these findings suggest that intraplatelet fibronectin levels are regulated by and inversely correlated to intraplatelet fibrinogen levels.<sup>33</sup>

Truncation of the  $\alpha$ C-region in FibA $\alpha^{270}$  resulted in profound changes in clot structure and stability. Turbidity analyses using purified fibrinogens indicated that the FibA $\alpha^{270}$  mutant protein produced clots with decreased optical density, consistent with studies using Fibrinogen Keokuk, Nieuwegein, Guarenas, Perth, Marburg, and Otago.<sup>2,3,7-10</sup> In prior scanning electron microscope studies, recombinant human FibA $\alpha^{251}$  produced denser clots with thinner fibers.<sup>35</sup> However, FibA $\alpha^{270}$  produced heterogenous clots that are generally less dense with thicker fibers, an observation similar to that for fibrinogen truncated at residue 220 of the  $\alpha$  chain.<sup>36</sup> The basis for the structural differences in clots with different fibrinogen  $\alpha$ C-region truncation mutants remains unknown. The  $\alpha$ C region of fibrinogen also binds to components of the plasminogen activation system, and fibrin lacking the  $\alpha$ C region has been suggested to have reduced

susceptibility to fibrinolysis.<sup>5</sup> Consistent with these findings, tPA/plasmin-mediated fibrinolysis of FibA $\alpha$ <sup>270</sup> clots and plasmin generation in *Fga*<sup>270/270</sup> plasma did not occur in our in vitro analyses. Although previous studies suggested that reducing or eliminating plasmin(ogen) has little impact on hemostasis,<sup>37,38</sup> it is possible that, in combination with the altered clot structure of FibA $\alpha$ <sup>270</sup>, the loss of fibrinolytic potential facilitates hemostasis. Consistently, fibrin accumulation progressively increased in the hemostatic plugs of *Fga*<sup>270/270</sup> mice following successive saphenous vein laser injury despite low plasma fibrinogen, which could have contributed to the preservation of hemostatic potential.

Previous research indicated that hyperfibrinogenemia exacerbates thrombosis.<sup>39</sup> A largely untested hypothesis is whether selectively lowering fibrinogen levels confers protection from thrombotic disease. We found no reports of thrombosis in  $\alpha$ C-region truncation patients in the absence of interventions. There are reports of hypofibrinogenemic Fibrinogen Keokuk and Fibrinogen Marburg patients developing thrombosis, but only after cryoprecipitate or fresh blood transfusion.<sup>3,10</sup> Animal models and in vitro systems each indicate that fibrin(ogen) supports the development of occlusive thrombi.<sup>12,16</sup> *Fga*<sup>-/-</sup> and mice expressing a mutant form of fibrinogen that cannot support fibrin formation (*Fga*<sup>AEK/AEK</sup>) fail to develop occlusive thrombi, but each exhibit profound bleeding.<sup>12</sup> Our studies document for the first time that genetically or pharmacologically lowering fibrinogen levels (while leaving other coagulation system components unaltered) is protective against venous thrombosis, while preserving hemostatic potential. All *Fga*<sup>270/270</sup> mice and 4/6 siFga-treated mice developed no quantifiable thrombi in the IVC stasis model. Notably, 2/6 siFga-treated mice, whose circulating fibrinogen levels were ~0.6 mg/mL, developed thrombi, but had smaller thrombi compared with siLuc-treated mice. Thus, pharmacologically lowering fibrinogen levels is beneficial even if venous thrombosis does occur. These findings also suggest that the loss of the fibrinogen  $\alpha$ C-region itself may independently contribute to the protection against venous thrombosis. FXIIIa-mediated crosslinking of fibrinogen  $\alpha$  chain contributes to thrombus size by promoting red blood cell retention in clots.<sup>19,20</sup> Although *Fga*<sup>270/270</sup> plasma supports FXIIIa activation equivalently to *Fga*<sup>WT/WT</sup> plasma, the primary FXIIIa crosslinking residues on the A $\alpha$ -chain that reside within the  $\alpha$ C region are deleted in FibA $\alpha$ <sup>270</sup>, resulting in inability to form fibrinogen  $\alpha$ -chain high-molecular-weight polymers.<sup>19,20,30,40</sup> Furthermore, the  $\alpha$ C region of fibrinogen binds the glycoprotein VI receptor on the platelet surface, which also contributes to the pathogenesis of venous thrombosis.<sup>41,42</sup> Thus, *Fga*<sup>270/270</sup> mice seem to be protected from experimental venous thrombosis through multiple mechanisms.

Fibrinogen plays an essential role in antimicrobial host defense. In an *S aureus* peritoneal infection model, *Fga*<sup>-/-</sup> and *Fga*<sup>AEK/AEK</sup> mice displayed compromised survival and *S aureus* clearance within the peritoneal cavity, highlighting an essential role of fibrin(ogen) in antimicrobial mechanisms.<sup>12,43</sup> Here, we show that both *Fga*<sup>270/270</sup> and *Fga*<sup>WT/WT</sup> mice had similar bacterial clearance and survival following intraperitoneal *S aureus* infection. Furthermore, bacterial killing and suppression of dissemination were maintained in siFga-treated mice. Together, these findings highlight that neither a 90% reduction of circulating fibrinogen nor loss of the fibrinogen  $\alpha$ C region compromise

fibrin(ogen)-dependent host defense mechanisms against *S aureus* infection.

In conclusion, our study reveals that *Fga*<sup>270/270</sup> mice are hypodysfibrinogenemic with preserved hemostasis and fibrin(ogen)-dependent antimicrobial function, while exhibiting near complete protection from venous thrombosis. The  $\alpha$ C-region variants may have clinical significance in nonhemostatic functions, given the observations implicating the  $\alpha$ C region in the pathogenesis of Alzheimer's disease and familial renal amyloidosis.<sup>44,45</sup> *Fga*<sup>270</sup> mice establish a promising model to examine the functional significance of an  $\alpha$ C-region truncation mutation and to investigate the impact of profound hypofibrinogenemia in vivo.

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## Authorship

Contribution: W.S.H., E.G.B., D.S.P., E.G.C., O.A.N., J.-M.M., L.G.P., H.M.C.-F., J.P.L., and M.J.F. designed the research, performed experiments, and analyzed the data; L.J.J., J.L., and C.J.K. provided valuable reagents; W.S.H. and M.J.F. wrote the manuscript; and T.P.U., A.S.W., W.B., and J.P.L. provided critical guidance on experimental procedures and helped write the manuscript. All authors read and approved the final manuscript.

Conflict-of-interest disclosure: C.J.K. is a director and shareholder of NanoVation Therapeutics, Inc., which is developing other RNA-based therapies. C.J.K., L.J.J., and J.L. are inventors on pending intellectual property related to RNA-based therapies. The remaining authors declare no competing financial interests.

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## Footnotes

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