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Limiting prothrombin activation to meizothrombin is compatible with survival but significantly alters hemostasis in mice

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Key Points

1. Mice expressing a form of prothrombin with limited activation potential to meizothrombin are viable and are reproductively successful.
2. Meizothrombin directly activates platelets but has diminished positive regulation of hemostatic factor activation.

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Abstract

Thrombin-mediated proteolysis is central to hemostatic function but also plays a prominent role in multiple disease processes. The proteolytic conversion of fII to α-thrombin (fIIa) by the prothrombinase complex occurs through two parallel pathways: i) the inactive intermediate, prethrombin, or ii) the proteolytically active intermediate, meizothrombin (fIIa^{MZ}). FIIa^{MZ} has distinct catalytic properties relative to fIIa, including diminished fibrinogen cleavage and increased protein C activation. Thus, fII activation may differentially influence hemostasis and disease depending on the pathway of activation. To determine the *in vivo* physiologic and pathologic consequences of restricting thrombin generation to fIIa^{MZ}, mutations were introduced into the endogenous fII gene resulting in expression of prothrombin carrying three amino acid substitutions (R157A, R268A, and K281A) to limit activation events to yield only fIIa^{MZ}. Homozygous fII^{MZ} mice are viable, express fII levels comparable to fII^{WT} mice, and have reproductive success. Although *in vitro* studies revealed delayed generation of fIIa^{MZ} enzyme activity, platelet aggregation by fII^{MZ} is similar to fII^{WT}. Consistent with prior analyses of human fIIa^{MZ}, significant prolongation of clotting times was observed for fII^{MZ} plasma. Adult fII^{MZ} animals displayed significantly compromised hemostasis in tail bleeding assays, but did not demonstrate overt bleeding. More notably, fII^{MZ} mice had two significant phenotypic advantages over fII^{WT} animals: protection from occlusive thrombosis after arterial injury and markedly diminished metastatic potential in a setting of experimental tumor metastasis to the lung. Thus, these novel animals will provide a valuable tool to assess the role of both fIIa and fIIa^{MZ} *in vivo*.

Abstract word count: 247

Introduction

The activation of prothrombin (fII) is the penultimate step of hemostasis. Thrombin (fIIa) cleaves fibrinogen and directly activates platelets, via protease-activated receptors (PARs).[1] However, fIIa also controls its own production, via activation of factors V (fV), VIII (fVIII), XI (fXI), and protein C.[2] Through these targets and others (e.g., fXIII, thrombin-activatable fibrinolysis inhibitor), fIIa not only plays a pivotal role in hemostasis, but in other physiologic and pathologic processes (e.g., development, inflammation, cancer biology).[3-7] Mouse studies have underlined the seminal importance of fII, as the genetic elimination of fII is not compatible with life.[3,4,8]

Prothrombin activation proceeds through two parallel activation pathways, depending on the site of first cleavage by the prothrombinase complex. One pathway features an intermediate that is not an active enzyme, prethrombin, while the second pathway is via an active enzyme precursor, meizothrombin (fIIa^{MZ}).[9-11] FIIa^{MZ}, as an active enzyme, is capable of participating in hemostasis and thrombosis, as well as other physiologic processes. FIIa^{MZ}, like fIIa, activates fV,[12] fVIII,[13] and fXI.[14] Thus, fII^{MZ} may contribute to positive feedback of physiologic hemostasis. Both human and murine rfIIa^{MZ} demonstrate reduced fibrinogen cleavage capacity.[15,16] Further, rfIIa^{MZ} from both species demonstrates an increased activation potential for protein C, in the presence of thrombomodulin. Thus, fIIa^{MZ} has potentially distinct effects on hemostasis regulation from that of the mature fIIa enzyme. At least two described human mutations result in a partial limitation of the activation of prothrombin to meizothrombin. Both prothrombin-Dharhan (R271H)[17] and prothrombin-Barcelona/Madrid (R271C)[18-21] limit fII activation to meizothrombin-like enzymes by removing a cleavage site. For each of the described pedigrees, individuals that were heterozygotes did not have a bleeding diathesis, while homozygotes had a mild to moderate bleeding tendency. Thus, while limitation of fII activation has a clear hemostatic effect, the broader biologic impact of meizothrombin *in vivo* has not been defined. The fact that the spontaneous human mutations were viable in a homozygous state supports the feasibility for examining meizothrombin in a homozygous state in mice.

To better understand the biology of fII overall, and fIIa^{MZ}, specifically, we generated a mouse with knock-in targeted mutations in the endogenous *F2* allele resulting in three amino acid substitutions (R157A, R258A, and K281A) that lock fII after activation by factor Xa (fXa) to meizothrombin. These mutations in murine fII have previously been identified to limit activation to meizothrombin.[16] The working hypothesis was that mice expressing a form of fII that is limited to activation to fIIa^{MZ}, would i) be viable without spontaneous hemorrhage, ii) allow examination of the role of fIIa^{MZ} in hemostasis *in vivo*, and iii) provide a novel tool for study of the biology of prothrombin in physiologic and pathologic conditions.

Methods

Generation of fII^{MZ} gene-targeted mice

For details of the generation of fII^{MZ} gene-targeted mice, please see the online supplemental methods. Mice that are heterozygous for the fII null allele (fII$^{+/-}$) and compound heterozygotes for the conditional fII allele and fII null (fII$^{lox/-}$) were previously described.[8] *In vivo* and *ex vivo* (i.e., PT, aPTT and quantitative PCR) experiments utilized outbred hybrid (129/Ola:C57/Bl6) littermate controls, except as noted using at least six generations inbred C57/Bl6 animals. All experiments were approved by the Cincinnati Children's Hospital Research Foundation Animal Care and Use Committee and complied with National Institutes of Health guidelines.

Hematological profile, determination of prothrombin levels, thrombin generation assay, and bleeding times

For details of the hematologic analysis, please see the online supplemental methods.

Prothrombin activation assay

For the prothrombin activation assay, citrated PPP was diluted 1:10 with sterile TBS. The peptide Gly-Pro-Arg-Pro (EMD Millipore) was added to a final concentration of 4 mM to prevent fibrin polymerization. Thromboplastin (Diapharma), reconstituted in sterile water, was added to the plasma. Aliquots were taken from this solution and directly added to SDS sample buffer to stop the reaction, at time points as indicated in the results section. Immunoblots of these samples were performed under reducing conditions, using an antibody specific for the N-terminus of prothrombin (antigen was a peptide within the first 50 amino acids of prothrombin, sc-23340, Santa Cruz Biotechnology). Detection would yield, in decreasing size, intact prothrombin, F1.2.A, F1.2, or F1.

Thromboplastin-induced platelet aggregation and clot retraction

Please see the online supplemental methods.

Real-time analysis of in vivo thrombus formation by intravital microscopy

Cohorts of fII^{WT} and fII^{MZ} mice were infused with genotype controlled fluorescently-labeled platelets. Anesthetized animals then had mesenteric arterioles isolated and 10% topical ferric chloride solution was applied for 5 minutes. An observer blinded to genotype monitored vessels for the time to the first thrombus formation and the time until occlusion or for thirty minutes.

Lipopolysaccharide challenge and activated protein C levels

Please see the online supplemental methods.

Histopathology

Tissues were fixed in 10% buffered Formalin (Sigma) and embedded in paraffin. Sections were cut and subsequently stained with haemotoxylin and eosin (Sigma), Masson's Trichrome, or Prussian Blue (Electron Microscopy Services). Photomicrographs were captured using an Axioplan 2 microscope (Zeiss) equipped with an AxiocamHR camera and software (Axiovision 5.6.3; Zeiss).

Experimental Tumor Metastasis

As described,[22] a single cell suspension of 4×10^5 GFP-expressing Lewis lung carcinoma cells (LLC^{GFP}) was injected intravenously in cohorts of C57/Bl6 fII^{WT} and fII^{MZ} mice in parallel. Fluorescent pulmonary metastatic foci were evaluated 14 days after injection. For B16 melanoma experimental metastases, a single cell suspension was injected (8×10^4 cells) intravenously into cohorts of C57/Bl6 fII^{WT}, $fII^{MZ,}$ and $fII^{+/-}$ mice in parallel, as previously described.[23] Nineteen days after injection, lungs were removed, stained overnight in picric acid solution (Sigma), and metastatic foci counted.

Statistical analysis

All statistical analyses were generated using the Mann-Whitney U test, except as follows: the χ^2 test was utilized to analyze breeding data and the Fisher's exact test was utilized to analyze the tail bleeding times and percent occlusion in intravital microscopy experiments. Statistical analyses were performed on GraphPad Prism version 5.04 (GraphPad Software).

Results

Mice carrying the fII^{MZ} allele

To determine the *in vivo* consequences of expressing a form of prothrombin incapable of activation beyond the intermediate, meizothrombin, we introduced three amino acid substitutions into the endogenous allele, to remove both a fXa cleavage site and potential fIIa auto-cleavage sites: R157A, R268A, and K281A (Figure 1A).[24] The R157A and R268A cleavage site mutations have been previously identified to limit murine prothrombin activation to meizothrombin.[16] In addition to these three amino acid changes, we introduced a novel BamHI endonuclease site in Exon 7 and a novel EcoRI site in Exon 8, without further modification of the amino acid coding sequence. Founder mice transmitted the mutant allele through the germline to yield mice heterozygous for the mutation ($fII^{MZ/WT}$). Analyses of intercrosses of $fII^{MZ/WT}$ mice resulting in over 800 progeny revealed an approximate 50% decrease in the numbers of homozygous mutant animals (hereafter referred to as fII^{MZ}) relative to what would be expected based on a 1:2:1 Mendelian ratio (Table 1). Further studies were conducted to confirm the timing of failure of the homozygous animals. Analyses of embryos harvested at embryonic day 18.5 (E18.5; i.e., the day prior to birth) from $fII^{MZ/WT}$ breeding pairs did not reveal a significant difference in relative numbers of fII^{WT} and fII^{MZ} embryos (Table 2). This suggests the loss of homozygous fII^{MZ} animals occurs in the early postnatal timeframe. Consistent with this conclusion, evidence of abdominal hemorrhage in fII^{MZ} pups was observed (Supplemental Figure 1). Homozygous fII^{MZ} mice identified at weaning survived well into adulthood without excess mortality. Of the over 200 adult fII^{MZ} mice generated to date, none were found to suffer overt hemorrhage. Furthermore, homozygous fII^{MZ} females were capable of carrying litters to term, with 23 C57Bl/6 homozygous fII^{MZ} females successfully carrying 52 litters to term.

Prothrombin expression levels were determined to confirm that homozygous fII^{MZ} neonates had normal leves of circulating fII protein. Total RNA, harvested from fII^{WT} and fII^{MZ} livers, revealed no significant difference in fII mRNA in either fII^{WT} or fII^{MZ} mice, while the expected decreases in mRNA levels for both fII$^{+/-}$ and fII$^{lox/-}$ control samples were observed (Figure 1B). Immunoblot analyses of plasma obtained from fII^{WT} and fII^{MZ} homozygous animals revealed no discernible differences in fII protein levels between genotypes (Figure 1C). Citrated plasma was also assayed for chromogenic fII activity, as it has been previously determined that both α-thrombin and meizothrombin have similar affinities for S-2238 chromogenic substrate.[16] After activation of the fII in the plasma samples by ecarin, a similar level of chromogenic activity was observed between fII^{WT} and fII^{MZ} plasma samples (Figure 1D).

To confirm that the prothrombin generated from the fII^{MZ} animals was indeed limited to activation potential to meizothrombin, diluted plasma (1:10 with TBS) from fII^{WT} and fII^{MZ} animals was incubated with thromboplastin. Fibrin polymerization was blocked with the peptide Gly-Pro-Arg-Pro. Immunoblots (under reducing conditions) were performed using an antibody against an epitope at the N-terminus of the prothrombin molecule. Figure 2A details the expected fragments from fII activation in both fII^{WT} and fII^{MZ} plasma. Samples from fII^{WT} animals revealed that a fragment consistent with activation to meizothrombin (fragment 1.2.A), that then migrated to a size consistent with fragment 1.2 over time (Figure 2B). However, in fII^{MZ} animals, no conversion beyond fIIa^{MZ} was detected (only fragment 1.2.A was present), even after incubation for thirty minutes at 37ºC (Figure 2C).

Hematologic analysis of fII^{MZ} animals

CBC analyses, on blood from fII^{WT} and fII^{MZ} mice, revealed no significant difference in the white blood cell count (WBC), hemoglobin, or platelet count (Table 3) between genotypes. Additionally, analysis of the WBC differential revealed no significant difference between fII^{WT} and fII^{MZ} animals (data not shown) in terms of leukocyte subsets. Standard coagulation function analyses revealed a significant prolongation of both the PT and aPTT for fII^{MZ} animals compared to fII^{WT} animals (Table 3). This prolongation was expected given the known diminution of fIIa^{MZ} activity for fibrinogen. Predictably, there was no difference in the thrombin times between fII^{WT} and fII^{MZ} mice (Table 3) consistent with fII^{MZ} having no impact on plasma fibrinogen levels.

Comparative thrombin generation assays (TGA) were performed on plasma from both fII^{MZ} and fII^{WT} mice (representative curves, Figure 3A-B). The fII^{MZ} animals had both a prolonged lag time compared to fII^{WT} [e.g., a prolonged time to *any* (meizo)thrombin generation, (Figure 3C)] and a prolonged time to peak (meizo)thrombin production (Figure 3D). Furthermore, peak (meizo)thrombin production and area under the curve were found to be reduced in fII^{MZ} compared to fII^{WT} mice (Figure 3E-F, respectively). The Velocity Index, the rate of (meizo)thrombin generation, was also decreased in fII^{MZ} mice in contrast to fII^{WT} animals (data not shown).

To explore the contribution of fII^{MZ} toward activated protein C (aPC) generation, we challenged cohorts of fII^{MZ} and fII^{WT} mice with LPS, an inflammatory challenge known to activate the hemostatic cascade and result in aPC generation. Two hours after administration of LPS, fII^{WT} mice displayed the expected, and statistically significant, increase in aPC activity (Supplemental Figure 2). The fII^{MZ} animals challenged with LPS did not display a significant difference in aPC activity compared to unchallenged fII^{MZ} animals. Interestingly, there was also not a statistically significant difference in aPC generation between the fII^{MZ} and fII^{WT} mice after LPS. In light of the data regarding reduced meizothrombin generation in fII^{MZ} animals, these data do not necessarily contradict the previously reported findings regarding activity of $fIIa^{MZ}$ for PC; this finding would also be expected with diminished overall $fIIa^{MZ}$ activity secondary to diminished generation of $fIIa^{MZ}$ from fII^{MZ}.

Meizothrombin induced platelet aggregation and clot retraction

FIIa is a potent activator of platelet aggregation through activation of PARs.[1] To determine the potential of $fIIa^{MZ}$ to activate platelets, we initiated fIIa or $fIIa^{MZ}$ generation in platelet-rich plasma (PRP) derived from fII^{WT} and fII^{MZ} animals. Qualitative platelet aggregation was comparable between genotypes, with similar total aggregation (Figure 4A-B; fII^{WT} n=7, fII^{MZ} n=7). However, there was a prolonged time to both shape change and positive deflection in fII^{MZ} animals (Figure 4C-D). We postulate that the prolonged time to initiation of platelet aggregation in fII^{MZ} plasma was a function of the extended time to (meizo)thrombin generation. More notably, these data suggest that murine fIIa^{MZ} is capable of activating PAR-4.

Clot retraction is an important physiologic step in both hemostasis and wound repair that is dependent on fibrin polymer formation, platelet activation, and factor XIII (fXIII) activity.[25-27] To directly determine if fII^{MZ} supports clot retraction, we initiated coagulation with thromboplastin in both whole blood and platelet-rich plasma (platelet counts adjusted to 2.5 x 10^5 platelets/μL) from fII^{WT} and fII^{MZ} animals. While time to initial clot retraction was modestly delayed in fII^{MZ} blood and plasma samples, both fII^{WT} and fII^{MZ} displayed evidence of similar maximal clot retraction in both whole blood and platelet rich plasma (Supplemental Figure 3A-B; E-F). There was no qualitative difference in the size of clots by the end of the 1.5 hour observation period. Further, red blood cell (RBC) inclusion in thrombi was not significantly different between samples from fII^{WT} and fII^{MZ} mice (Supplemental Figure 3C-D).

In vivo hemostasis and thrombosis assessment

To determine the effect of limiting fII activation to meizothrombin on hemostasis *in vivo*, we employed a standard tail tip amputation assay. Cohorts of fII^{MZ} and fII^{WT} animals were challenged by amputation of 3 mm of the distal tail. All fII^{WT} mice had cessation of bleeding, with a mean time to cessation of 72 ± 6 s. However, all fII^{MZ} animals had bleeding for the entire ten-minute observation period (Figure 5A). Qualitative narrowing of the caliber of the blood stream from the amputation site was sometimes observed in fII^{MZ} animals, but bleeding universally persisted despite throughout the observation period.

In order to define thrombotic potential in FII^{MZ} mice, we challenged FII^{MZ} and fII^{WT} mice in parallel with a ferric chloride mesenteric artery injury. Here, fluorescein-labeled platelets from donor mice of the same genotype as the challenged animals were infused prior to the procedure to allow real-time tracking of thrombus size and time to occlusion by intravital fluorescent microscopy. FII^{WT} mice demonstrated rapid formation of platelet aggregates that progressed steadily to full occlusion (Figure 5B). While fII^{MZ} animals had clear evidence of platelet aggregation (Figure 5C), fII^{MZ} mice demonstrated a significantly increased time to first thrombus formation and prolonged time to occlusion relative to fII^{WT} mice, with a significant fraction of fII^{MZ} mice failing to form a stable occlusive thrombus during the 30-minute observation period (Figures 5D & E). In evaluation of the formation of thrombosis in the fII^{MZ} animals, the thrombi formed after ferric chloride injury were unstable with frequent emboli. In mice with no occlusive thrombi, a combination of delayed thrombus formation and embolization contributed to this phenotype (Supplemental video 1 represents fII^{WT} animals and video 2 represents fII^{MZ} animals). Taken together with the platelet aggregation data, these data suggest the hypothesis that diminished fibrin polymer formation in fIIa^{MZ} mice results in a failure to stabilize the growing platelet aggregate and a corresponding protection from arterial occlusion.

FII^{MZ} animals develop cardiac fibrosis

Mice with genetic deficiencies of TF, fII, fVII, fX, and fXIII[8,28-31] develop cardiac hemorrhage with iron deposition and subsequent fibrosis that progresses with age. To investigate if fII^{MZ} predisposes adult animals to spontaneous hemorrhage, we examined organs from cohorts (10-12 weeks of age) of C57/Bl6 fII^{WT} and fII^{MZ} mice. No gross or microscopic evidence of hemorrhage was observed in the pulmonary, gastrointestinal, genitourinary, or central nervous systems of fII^{WT} or fII^{MZ} animals. As expected, fII^{WT} mice did not show any histologic evidence of cardiac pathologies (Figure 6A-C). In contrast, cardiac fibrosis was readily identified in both male and female fII^{MZ} mice (Figure 6D-E) and was most notable in the sub-epicardial zones.

Prussian Blue staining of the heart tissue revealed co-localization of iron deposits with areas of fibrosis (Figure 6F), suggesting chronic hemorrhage leading to the etiology of fibrosis in this setting.

Additionally, cohorts of inbred C57/Bl6 fII^{WT} and fII^{MZ} mice were aged for a year prior to having organs harvested. Notably, no loss of fII^{MZ} animals occurred during this time. Grossly, evidence of one ovarian hemorrhagic cyst and one intestinal hemorrhage were noted in the fII^{MZ} (n=8) animals. There was no evidence of gross or microscopic hemorrhage in the fII^{WT} (n=7) mice (Supplemental Figure 4A-C). Abundant cardiac fibrosis was noted in both male and female fII^{MZ} animals (Supplemental Figure 4D-E), whereas minimal, if any, cardiac fibrosis was noted in the fII^{WT} mice. Again, sites of fibrosis co-localized with sites of iron deposition (Supplemental Figure 4F), likely due to local hemorrhage. There were no hemorrhagic findings in other organ systems.

FII^{MZ} mice have diminished experimental metastases

To investigate the biologic consequences of fII^{MZ} activity in a disease context distinct from a classical hemostasis or thrombosis challenge, we examined the contribution of fII^{MZ} to experimental tumor metastasis. FIIa can support metastasis through tumor cell-instrinsic mechanisms involving activation of PARs,[32] as well as through fibrinogen cleavage and platelet activation.[23,33,34] Our hypothesis was that fII^{MZ} animals would demonstrate reduced numbers of metastases. Cohorts of inbred C57Bl/6-derived fII^{MZ} and fII^{WT} mice were intravenously injected in parallel with LLC^{GFP} cells and the lungs harvested 14 days later. While abundant metastases were uniformly present among the fII^{WT} animals, few, if any, fII^{MZ} animals had any evidence of metastatic foci (Figure 7A-C). This was similar to previous findings of LLC experimental lung metastases in animals with lowered levels of prothrombin.[35] To expand on these findings and further compare fII^{MZ} to diminished levels of prothrombin, we compared experimental metastases of B16 melanoma in fII^{WT}, fII^{MZ}, and $fII^{+/-}$ animals. Again, fII^{MZ} animals demonstrated a significant protection from the development of lung metastases (Figure 7D; F-G). Evaluation of tumor metastases in mice with 50% prothrombin (fII$^{+/-}$) revealed an essentially identical pattern to fII^{MZ} animals (7E). This suggests that even modest diminutions in thrombin generation potential, either through diminution of circulating prothrombin levels or alteration of activation, have profound effects on metastatic potential of tumor cells.

Discussion

Activation of thrombin follows one of two pathways, which produce functionally different intermediate species. The intermediate of the alternative prothrombin activation pathway prethrombin, lacks detectable protease activity. In contrast, meizothrombin, a short-lived activation intermediate of prothrombin,[9,11] has readily detectable protease activity with notably distinct substrate specificities from α-thrombin. The pathway of thrombin activation is dictated in part by the microenvironment or cellular surface on which the prothrombinase complex is assembled. Recent data have suggested that thrombin generation on platelets primarily proceeds via prethrombin[36] whereas thrombin generation on the surface of RBCs tends to occur through meizothrombin[37]. While it has been hypothesized that the pathway of fII activation is biologically relevant, given the short half-life of $fIIa^{MZ}$, the specific contribution of $fIIa^{MZ}$-mediated proteolysis to hemostasis and other physiologic or pathologic processes has been difficult to assess *in vivo.* Here, we demonstrate for the first time the consequences of limiting the activation of endogenous fII to an activation intermediate *in vivo*. Mice that express fII^{MZ} are viable, survive well into adulthood, and have reproductive success. FII^{MZ} mice have partial perinatal lethality, unlike $fII^{lox/-}$ animals (mice with 10% of wildtype prothrombin levels). Similar to $fII^{lox/-}$ animals, fII^{MZ} animals that survive to weaning have an essentially normal lifespan. FII^{MZ} animals have significantly delayed and decreased (meizo)thrombin generation, compared to fII^{WT} mice. FII^{MZ} animals also have delayed time to occlusion following a ferric chloride arterial injury and prolonged bleeding times.

While we did not directly measure factor XIII (fXIII) activation by fII^{MZ}, indirect evidence points to at least adequate activation. Clot retraction in fII^{MZ} plasma was normal, both in platelet-rich plasma and in whole blood. FXIII activity is required in the process of clot retraction and RBC retention within the thrombus.[25-27] As additional evidence that fII^{MZ} activates fXIII, RBC retention in whole blood thrombi after clot retraction was not significantly different between fII^{WT} and fII^{MZ} animals. Similarly, PAR-1 activation by fII^{MZ} has not been directly measured. However, the homozygous fII^{MZ} animals do not have the mid-gestation loss seen in PAR-1^{-}[38] and fII^{-} animals,[3,4] which would suggest that activation of PAR-1 by fIIa^{MZ} is adequate to support successful embryonic development.

Both human and murine rfIIa^{MZ} have decreased polymerization potential of fibrin.[15,16] Our findings with *in vivo* fII^{MZ} are compatible with these results. Prolongation of the PT and aPTT are both suggestive of a decreased polymerization of fibrin (the endpoint of both assays). While delayed, fIIa^{MZ} also induces platelet aggregation qualitatively similar to α-thrombin. However, fII^{MZ} animals were unable to achieve hemostasis after a standard tail amputation bleeding time. This is in contrast to animals expressing a form of fibrinogen that cannot be cleaved by thrombin (Fib^{AEK}). Fib^{AEK} mice demonstrated partial cessation of bleeding in a standard tail amputation bleeding time.[39] This suggests that while inadequate fibrin polymerization contributes to abnormal hemostasis in fII^{MZ} mice, positive feedback for self-activation also likely participates in the failure to achieve hemostasis.

Intravital microscopy, following mesenteric arteriole ferric chloride injury, also reveals insight into the failure to form thrombi. While fII^{MZ} animals formed platelet aggregates at the site of vessel injury, these aggregates were not stable. The aggregates formed after ferric chloride injury in the fII^{WT} controls quickly evolved into occlusive thrombi. In stark contrast, fully occlusive thrombi never formed in fII^{MZ} mice, rather smaller aggregates embolized from the initial thrombus in fII^{MZ} animals precluding occlusion. Taken together, this suggests in the setting of animals expressing only fII^{MZ}, platelet aggregation is adequate, but insufficient positive feedback to the hemostatic cascade and diminished fibrinogen cleavage lead to unstable platelet aggregates. Thus, a lack of both physiologic hemostasis and diminished thrombosis is observed in fII^{MZ} mice.

Previous studies have reported the effects of recombinant human and murine meizothrombin (rhfIIa^{MZ} and rmfIIa^{MZ}, respectively), both *in vitro* and *in vivo*.[16,40-42] Similar to our current work, rmfIIa^{MZ} demonstrated significantly diminished fibrinogen cleavage capabilities *in* *vitro*. However, in the same *in vitro* studies of rmfIIa^{MZ}, increased protein C activation in the presence of thrombomodulin was observed.[16] Our data suggest that the activation of protein C is not significantly different in the fII^{MZ} and fII^{WT} animals after an LPS challenge. Direct comparison between the *in vitro* findings and the *in vivo* analysis of aPC generation reported here is complicated by the overall reduced activation potential of meizothrombin in the homozygous mutant mice. Thus, we cannot further address the activity of fIIa^{MZ} for protein C. The other significant difference in the published account of rmfIIa^{MZ} and our current findings using knock-in animals is that rmfIIa^{MZ} was evaluated not as a zymogen, but as a fully active enzyme. Here, we were able to explore the consequences of limiting all activation of fII to meizothrombin. While *in vitro* data suggested that rhfIIa^{MZ} would be capable of positive feedback of the hemostatic cascade, our data in murine meizothrombin suggest otherwise.[12,13] Further studies will be needed to assess the *in vivo* activation of factors V, VIII, and XI by meizothrombin. As (meizo)thrombin generation is significantly altered in the fII^{MZ} compared to fII^{WT} animals, this suggests meizothrombin is less effective at supporting activation of at least one of these factors. Our data challenges the hypothesis that fIIa^{MZ} contributes to physiologic hemostasis.

Another modification of fII leading to a specificity difference, fII^{WE}, has diminished fibrinogen cleavage while preserving activation of protein C.[43,44] Exploiting this difference in specificities, fIIa^{WE} has been administered pharmaceutically as an anticoagulant[43,45] and anti-inflammatory[46] agent. Additionally, animals have been generated that express only the fII^{WE} zymogen.[5] However, unlike the fII^{MZ} animals, mice expressing only fII^{WE} are not viable. The difference in viability between fII^{WE} and fII^{MZ} animals illustrates that fII^{MZ} does not impact physiologic hemostasis to the same degree. Further, fII^{MZ} animals may have an advantage in studying fII specificity alterations in physiologic or disease states.

Animals expressing fII^{MZ} developed cardiac fibrosis, worsening with age, similar to mice with low levels of tissue factor, fII, fVII, fX, and fXIII.[8,28-31] Areas of fibrosis co-localized with areas of iron deposition, likely related to recurrent small hemorrhages. Unlike mice with low levels of fII (both $fII^{lox/-}$ mice and transgenic mice expressing low levels of human prothrombin) that only develop modest cardiac fibrosis at over one year of age,[8] fII^{MZ} mice developed substantial cardiac fibrosis by 8-10 weeks of age. Also unlike previous accounts of cardiac fibrosis hemostatic factor deficiencies, we did not observe a gender predisposition to the development of cardiac fibrosis. Thus, while (meizo)thrombin-induced platelet aggregation is preserved, this was not sufficient to prevent the cardiac parenchymal bleeding that leads to cardiac fibrosis. That this bleeding is limited to the heart, as opposed to bleeding patterns seen in the low TF and low fVII animals, suggest tissue differences in hemostasis requirements to prevent hemorrhagic complications.[47]

As proof of concept that fII^{MZ} animals will provide a novel tool to study the biology of fII in disease, we examined the effect of limitation of the activation of fII in the setting of tumor metastasis. We found that mice with fII^{MZ} have dramatically reduced experimental metastasis compared to fII^{WT} animals. This was not necessarily a predictable result, since fibrin(ogen) and platelets both contribute to early protection of metastatic foci via protection from natural killer cells.[23,33] FII^{MZ} retains, albeit reduced, fibrinogen cleavage potential, and essentially normal platelet activation capacity. That mice with fII^{MZ} had very few metastases is most likely related to the low positive feedback in generation of (meizo)thrombin combined with diminished fibrinogen cleavage and polymerization and a delay in platelet activation. However, other possible mechanisms cannot be excluded, such as diminished PAR activation in either tumor cells or non-malignant stromal cells. Interestingly, $fII^{+/-}$ animals displayed a similar phenotype in tumor metastasis. $FII^{+/-}$ animals have approximately 75% of peak thrombin generation seen in $fII^{+/+}$ mice.[5] Protection from metastases in fII^{MZ} and $fII^{+/-}$ underscores that tumor metastasis is quite sensitive to derangements in thrombin generation.

The current studies demonstrate that mice expressing only fII^{MZ} have adequate hemostasis for successful survival and reproduction and that these animals provide a unique tool to further assess the role of both prothrombin and meizothrombin in physiologic and pathologic processes. As coagulation factors are more closely tied to non-hemostatic processes, the ability to study altered enzyme specificity *in vivo* will allow further elucidation of the mechanistic contribution of fII to disease. Tools, such as fII^{MZ} animals, will be crucial to determine how the activation and activity of fII modify disease severity.

Authorship: EM, MS, MF, JP, NE, CE, DW, and JD designed research. MS, KK, KM, DS, MC, AB and EM performed research. EM, MS, NE, CE, DW, and JD analyzed data. EM, MS, MF, and JP wrote the manuscript.

Conflict of interest: ESM has served on an advisory board for US WorldMeds and received honoraria from Baxalta for matters unrelated to this research. JSP has served on an advisory board for US WorldMeds on matters unrelated to this research. No other authors report conflicts of interest.

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Figure Legends

Figure 1. Alteration of the endogenous fII allele to limit activation to meizothrombin. (A) This map demonstrates the targeting vector used to alter the endogenous fII allele as well as a map of the endogenous allele. Incorporation of the targeting vector by homologous recombination targeting vector was confirmed in germline-competent mice by PCR. After transmission of the targeted allele by the chimeric animals, mice were crossed with CMV-Cre animals to remove the HPRT cassette, with the final targeted allele as illustrated. Loss of the HPRT cassette was also confirmed by PCR. (B) Liver fII mRNA levels in fII^{WT} and fII^{MZ} animals were assessed using qPCR. No significant difference in fII RNA levels were detected between the two genotypes. In contrast, significant decreases, as expected, were noted in $fII^{+/-}$ and $fII^{lox/-}$ animals (n = 6 in all cohorts). (C) Plasma from both fII^{MZ} and fII^{WT} mice was assayed for fII antigen levels via Western blot. No difference in fII levels between fII^{WT} and fII^{MZ} animals was appreciable on the immunoblot. (D) Chromogenic fIIa activity from both fII^{WT} and fII^{MZ} plasma was assayed following activation with ecarin. There was no significant difference in plasma fIIa activity derived from either fII^{MZ} or fII^{WT} animals (n = 7 in both cohorts). The expected decrease in fIIa activity in both $fII^{+/-}$ and $fII^{lox/-}$ (n = 4 in both cohorts) was readily detectable.

Figure 2. Evaluation of activation and activity of fII^{MZ}. (A) Schematic of prothrombin cleavage sites. An antibody was utilized that targets the N-terminus of prothrombin and the immunoblot was performed under reducing conditions. Therefore, an activation event resulting in meizothrombin would result in a product including fragment 1.2 (F1.2) and the A chain (F1.2.A). Upon further cleavage to α-thrombin, F1.2 is released from the A chain. (B-C) Activation of prothrombin was determined in both fII^{WT} and fII^{MZ} animals. Hemostatic factor activation was initiated with thromboplastin in plasma from fII^{WT} (B) and fII^{MZ} (C) animals. Aliquots were taken

at specified time points and assayed via immunoblot for fII and products of fII activation. While no remaining detectable fII or fragment 1.2.A is present in the fII^{WT} samples beyond 30 seconds, prothrombin from the fII^{MZ} animals remains at F1.2.A (representing meizothrombin) throughout the time period assayed. No further conversion/degradation was detected.

Figure 3. Determination of the activation of fII in fII^{WT} and fII^{MZ} plasmas. To evaluate the activation potential of fII in either fII^{WT} or fII^{MZ} plasma, we utilized thrombin generation assays to assess $fIIa/fIIa^{MZ}$ generation over time. Representative thrombin generation curves from fII^{WT} (A) and fII^{MZ} (B) plasma. Time to the start of thrombin generation was significantly delayed in fII^{MZ} plasma (C), as well as time to peak thrombin generation (D). Peak $fIIa/fIIa^{MZ}$ generation was also significantly decreased in the fII^{MZ} animals (E). Of note plasma from four fII^{MZ} animals did not have any appreciable (meizo)thrombin generation. Therefore, they were excluded from the time to peak (meizothrombin) analysis. Total $fIIa/fIIa^{MZ}$ generation, as represented by area under the curve, was also significantly reduced in fII^{MZ} plasma compared to fII^{WT} plasma (F).

Figure 4. Platelet aggregation in fII^{MZ} animals. Platelet aggregation in PRP derived from either fII^{WT} or fII^{MZ} animals in response to thromboplastin was determined. Fibrin polymerization was blocked with Gly-Pro-Arg-Pro. There was no qualitative difference between fII^{WT} and fII^{MZ} platelet aggregation (A and B, respectively). However, a modest, but statistically significant difference was noted in time to shape change (as determined by negative deflection, C) and time to positive deflection (D).

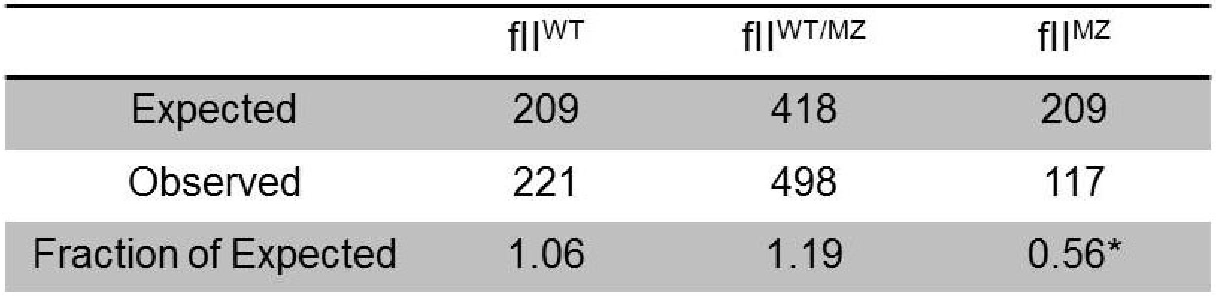
Figure 5. Impaired hemostasis and thrombus formation in fII^{MZ} animals. A. In a standard tail bleeding time analysis, fII^{WT} animals all achieved hemostasis, while no fII^{MZ} mice had cessation of bleeding during the 10 minute window of evaluation. In ferric chloride mesenteric arteriole injury, fII^{WT} animals developed occlusive thrombi (B). However, while fII^{MZ} mice formed platelet

aggregates, these were unstable and embolized, frequently without progression to occlusion of the vessel (C). FII^{MZ} animals also exhibited a prolonged time to first thrombus formation (D) and prolonged time to occlusion (E).

Figure 6. Cardiac fibrosis in fII^{MZ} mice. FII^{WT} mice did not have any evidence of cardiac fibrosis or hemorrhage/iron deposition (A-C). However fII^{MZ} animals developed evidence of cardiac fibrosis by the age of 10 weeks on both H & E and trichrome stains (D-E). At 10 weeks of age, the most pronounced area of fibrosis was in the subepicardial region. Areas of fibrosis co-localize with areas of iron deposition as shown here on Prussian blue stain (F). All scale bars represent 100 microns.

Figure 7. Diminished experimental tumor metastasis in fII^{MZ} animals. Cohorts of fII^{WT} and fII^{MZ} animals were challenged with an IV bolus of LLC^{GFP}. Two weeks later, metastatic foci in the lung were determined. A substantial, statistically significant diminution in the number of lung metastases was noted in the fII^{MZ} animals compared to fII^{WT} mice (A). FII^{WT} animals had prominent metastatic foci (B), while many fII^{MZ} animals demonstrated few, if any, metastases (C). Cohorts of fII^{WT} and fII^{MZ} animals were challenged with IV bolus of B16 melanoma. Eighteen days later, metastatic foci in the lung were determined. As was seen with LLC, fII^{MZ} animals had significantly fewer metastatic foci when compared to fII^{WT} mice (D). The same pattern was found when comparing fII^{WT} mice to mice with 50% prothrombin ($fII^{+/-}$) (E). Numerous metastatic foci were seen in fII^{WT} animals (F), while metastases were rare in fII^{MZ} mice (G).

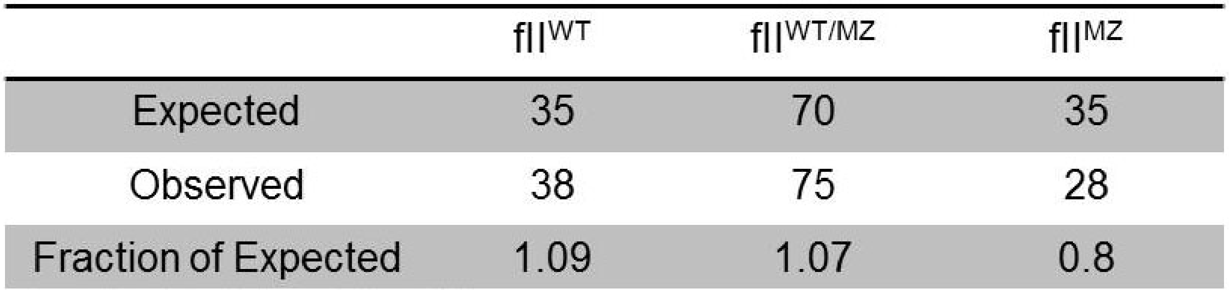
Table 1. fII^{MZ} and fII^{WT} animals at weaning



	fII^{WT}	$fII^{WT/MZ}$	fII^{MZ}
Expected	209	418	209
Observed	221	498	117
Fraction of Expected	1.06	1.19	0.56*

n = 836; *$P < 0.0001$, χ^2 analysis

Table 2. fII^{MZ} and fII^{WT} embryos at E18.5



	fII^{WT}	$fII^{WT/MZ}$	fII^{MZ}
Expected	35	70	35
Observed	38	75	28
Fraction of Expected	1.09	1.07	0.8

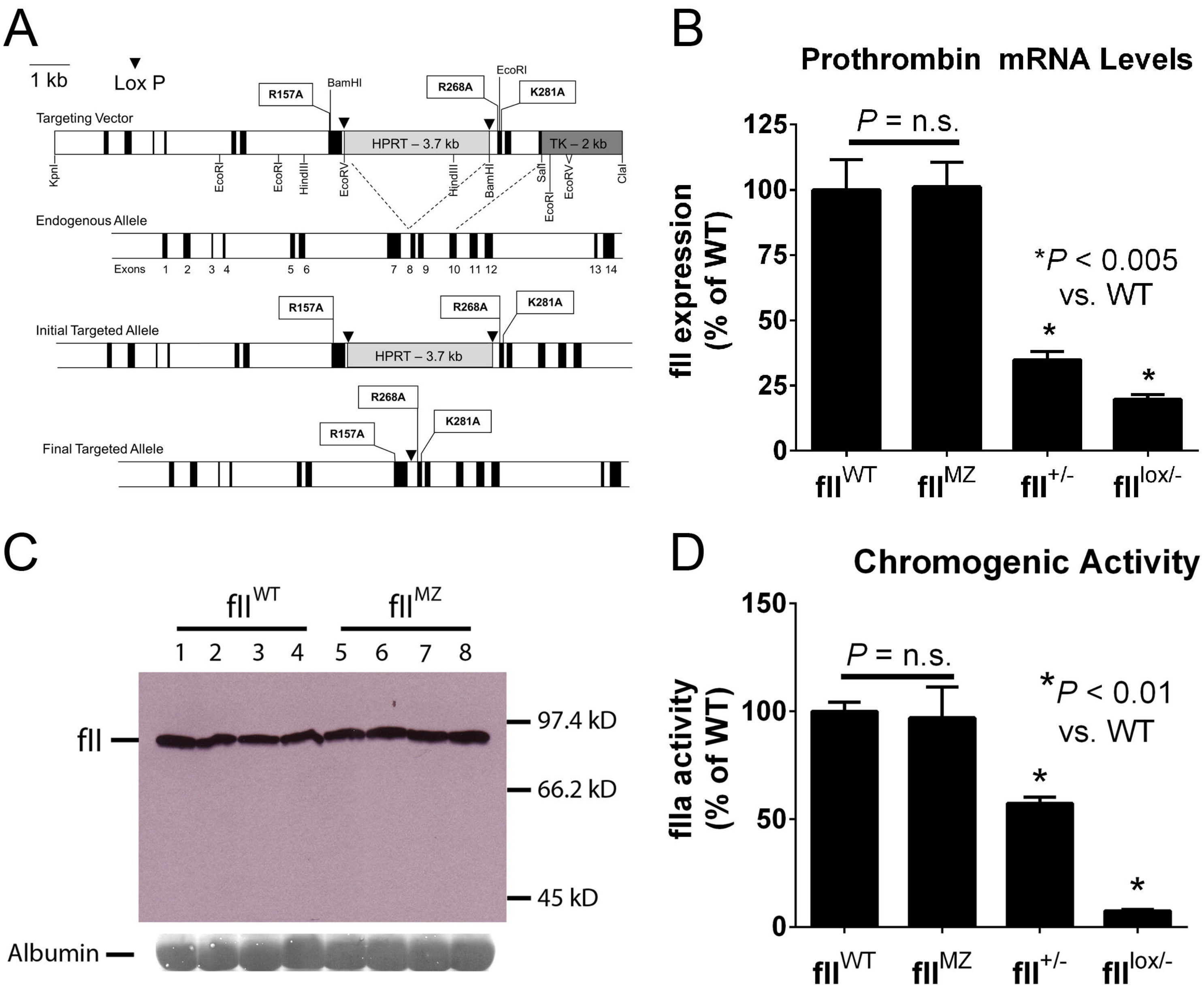
n = 141; P = n.s., χ^2 analysis

Table 3. Hematologic parameters of fII^{Mz} animals

	fII^{WT}	fII^{MZ}
White Blood Cell Count (x10^3/μL)	5.6 ± 1.5	5.2 ± 2.0
Hgb (g/dL)	12.0 ± 0.2	11.7 ± 0.5
Platelet Count (x10^3/μL)	681 ± 62	698 ± 42
Thrombin Time (s)	23.7 ± 0.6	24.0 ± 0.4
PT (s)	11.8 ± 0.2	23.4 ± 1.2*
PTT (s)	25.4 ± 2.4	64.5 ± 2.2*

*$P < 0.05$ compared to wildtype

Figure 1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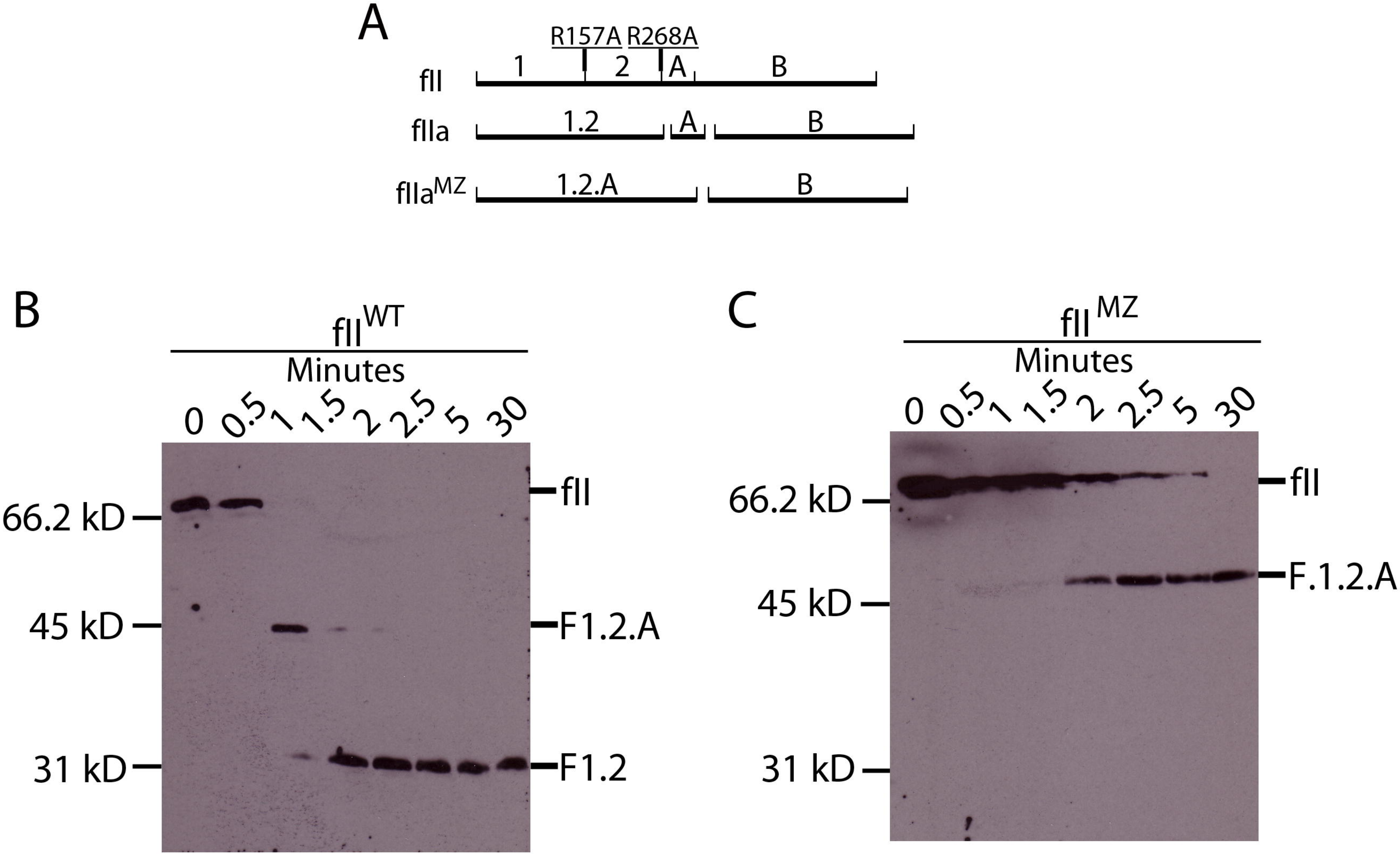
Lox P
Targeting Vector
R157A
BamHI
R268A
EcoRI
K281A
HPRT – 3.7 kb
TK – 2 kb
KpnI
EcoRI
EcoRI
HindIII
EcoRV
HindIII
BamHI
SalI
EcoRI
EcoRV
ClaI
Endogenous Allele
Exons 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Initial Targeted Allele
R157A
R268A
K281A
HPRT – 3.7 kb
Final Targeted Allele
R268A
R157A
K281A
B
Prothrombin mRNA Levels
fII expression (% of WT)
P = n.s.
*P < 0.005 vs. WT
125
100
75
50
25
0
fII^WT
fII^MZ
fII^+/-
fII^lox/-
C
fII^WT
fII^MZ
1 2 3 4 5 6 7 8
fII
97.4 kD
66.2 kD
45 kD
Albumin
D
Chromogenic Activity
fIIa activity (% of WT)
P = n.s.
*P < 0.01 vs. WT
150
100
50
0
fII^WT
fII^MZ
fII^+/-
fII^lox/-


A
R157A
R268A
1
2
A
B
fII
fIIa
1.2
A
B
fIIa^MZ
1.2.A
B
B
fII^WT
Minutes
0
0.5
1
1.5
2
2.5
5
30
66.2 kD
45 kD
31 kD
fII
F1.2.A
F1.2
C
fII^MZ
Minutes
0
0.5
1
1.5
2
2.5
5
30
66.2 kD
45 kD
31 kD
fII
F.1.2.A

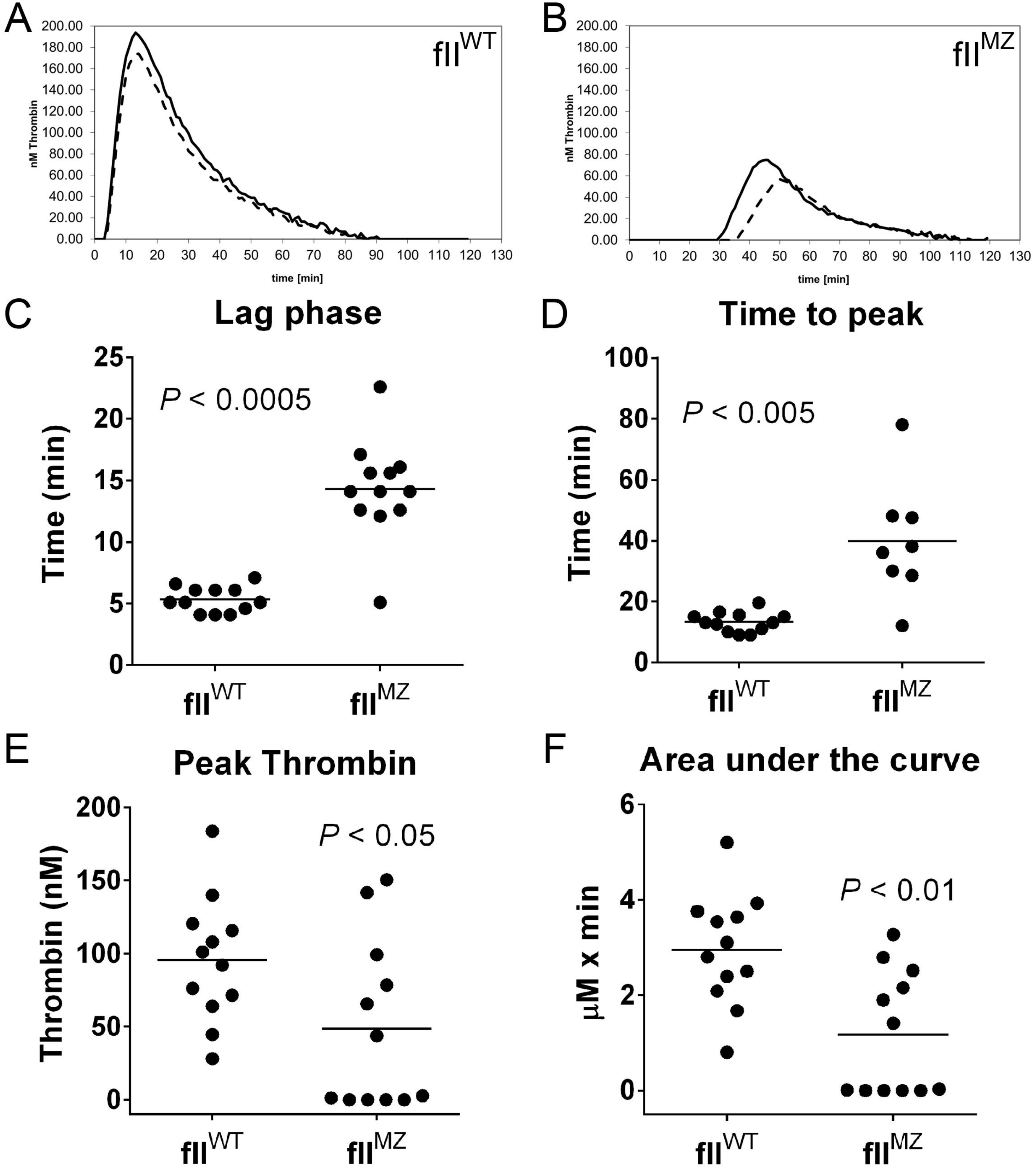
A
fII^WT
nM Thrombin
0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
160.00
180.00
200.00
time [min]
0 10 20 30 40 50 60 70 80 90 100 110 120 130
B
fII^MZ
nM Thrombin
time [min]
C
Lag phase
Time (min)
0 5 10 15 20 25
P < 0.0005
fII^WT
fII^MZ
D
Time to peak
Time (min)
0 20 40 60 80 100
P < 0.005
fII^WT
fII^MZ
E
Peak Thrombin
Thrombin (nM)
0 50 100 150 200
P < 0.05
fII^WT
fII^MZ
F
Area under the curve
μM x min
0 2 4 6
P < 0.01
fII^WT
fII^MZ

Figure 4

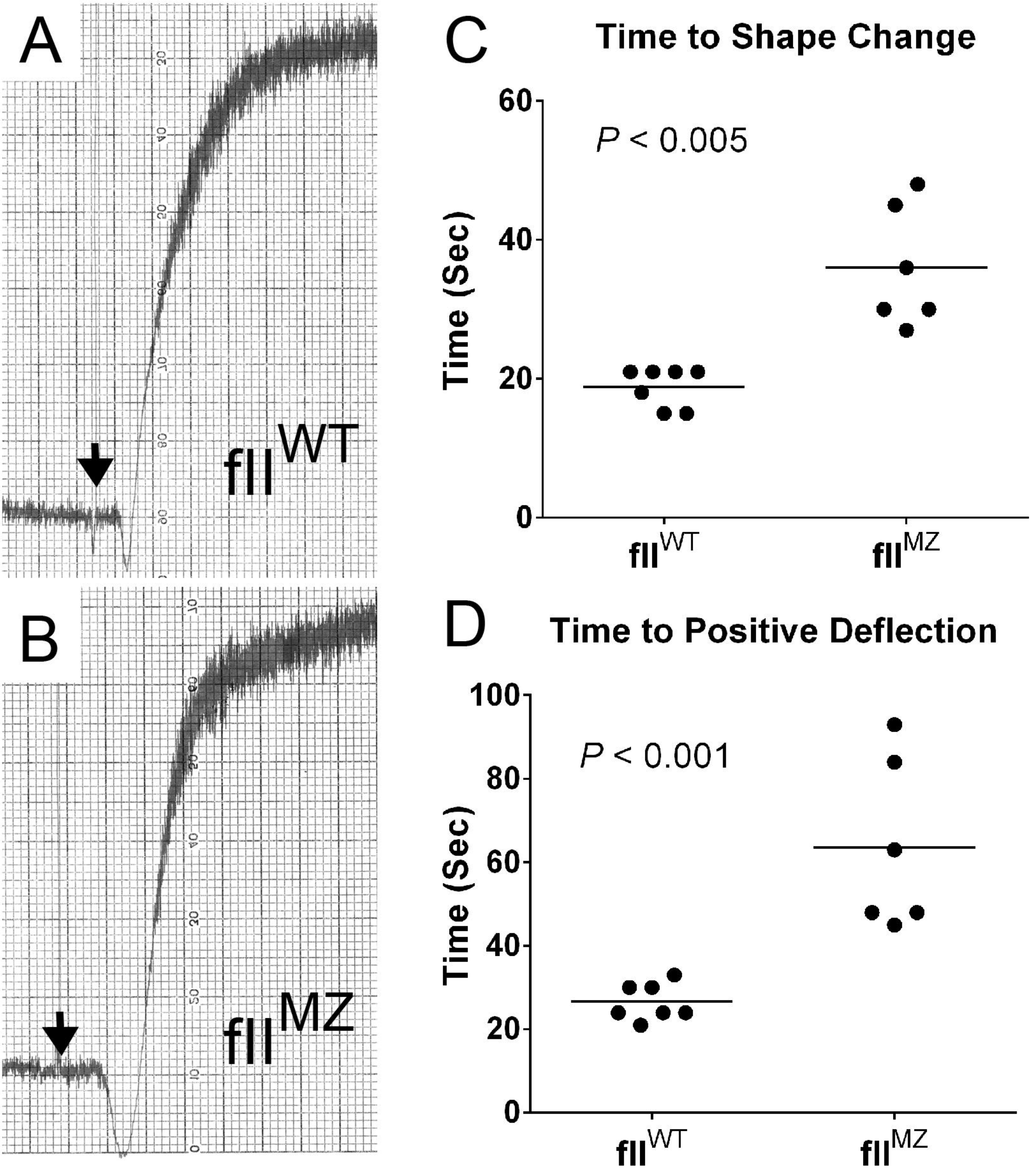

A
fII^WT
B
fII^MZ
C
Time to Shape Change
60
40
20
0
Time (Sec)
P < 0.005
fII^WT
fII^MZ
D
Time to Positive Deflection
100
80
60
40
20
0
Time (Sec)
P < 0.001
fII^WT
fII^MZ

Figure 5

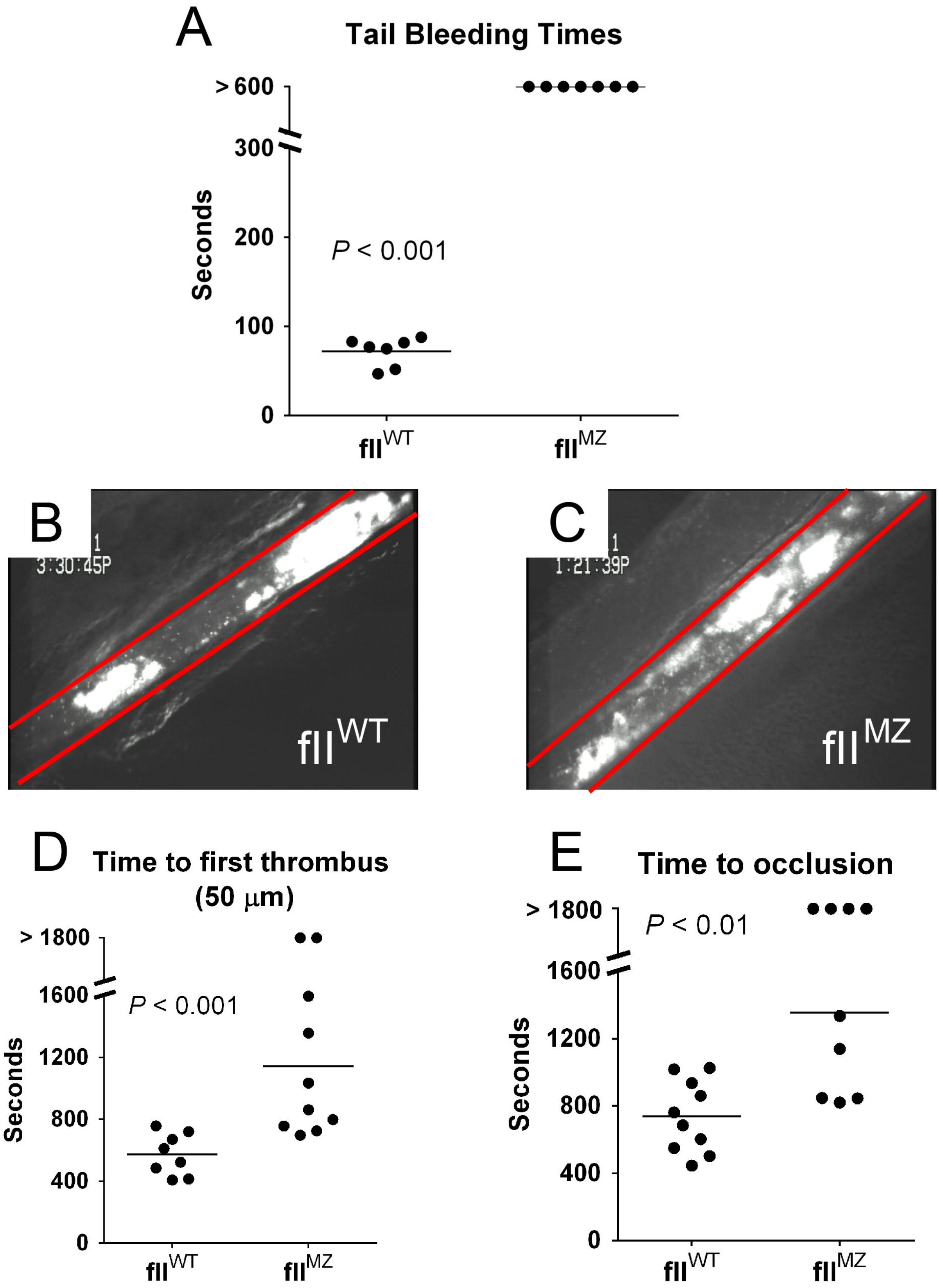
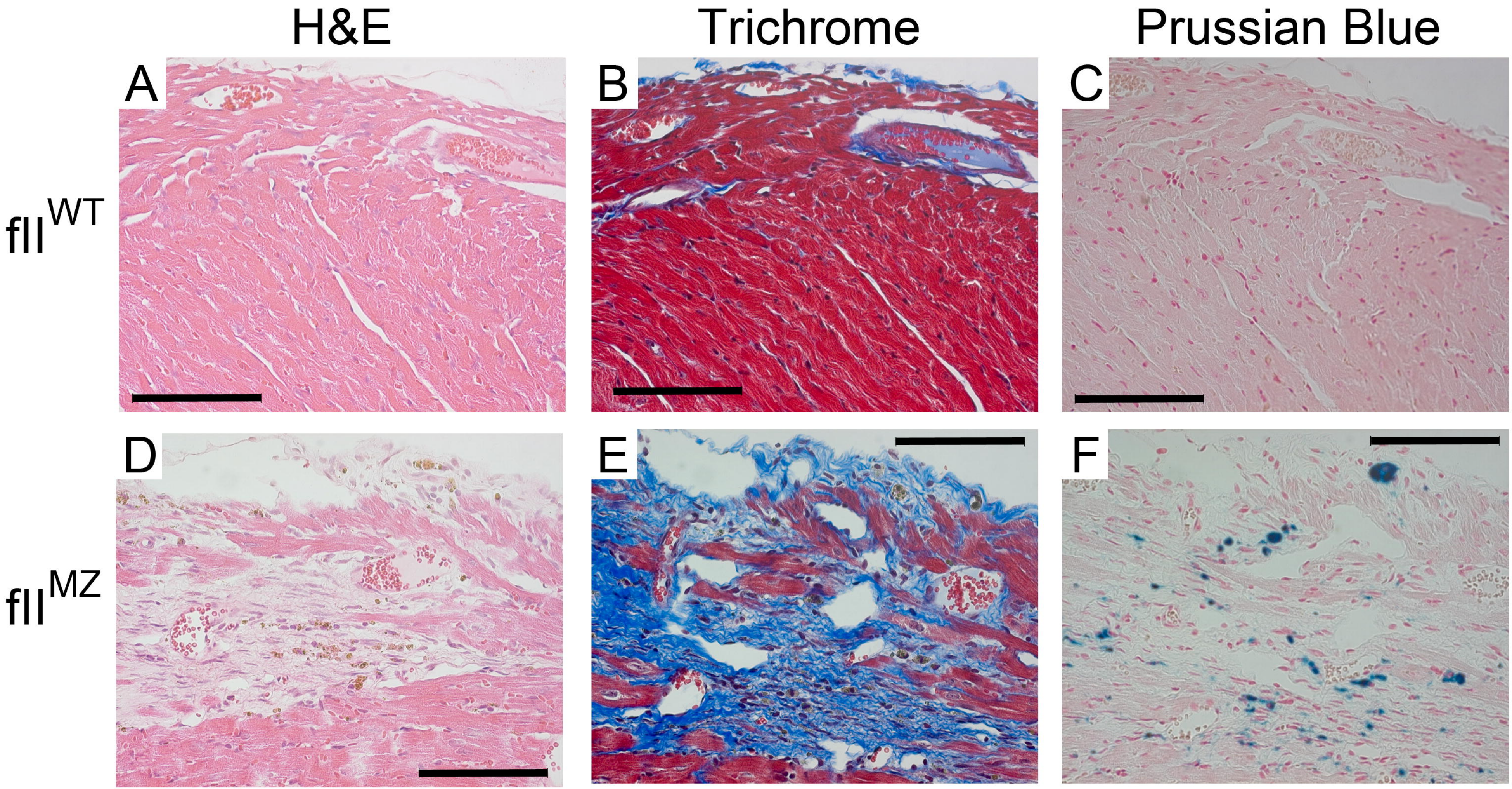
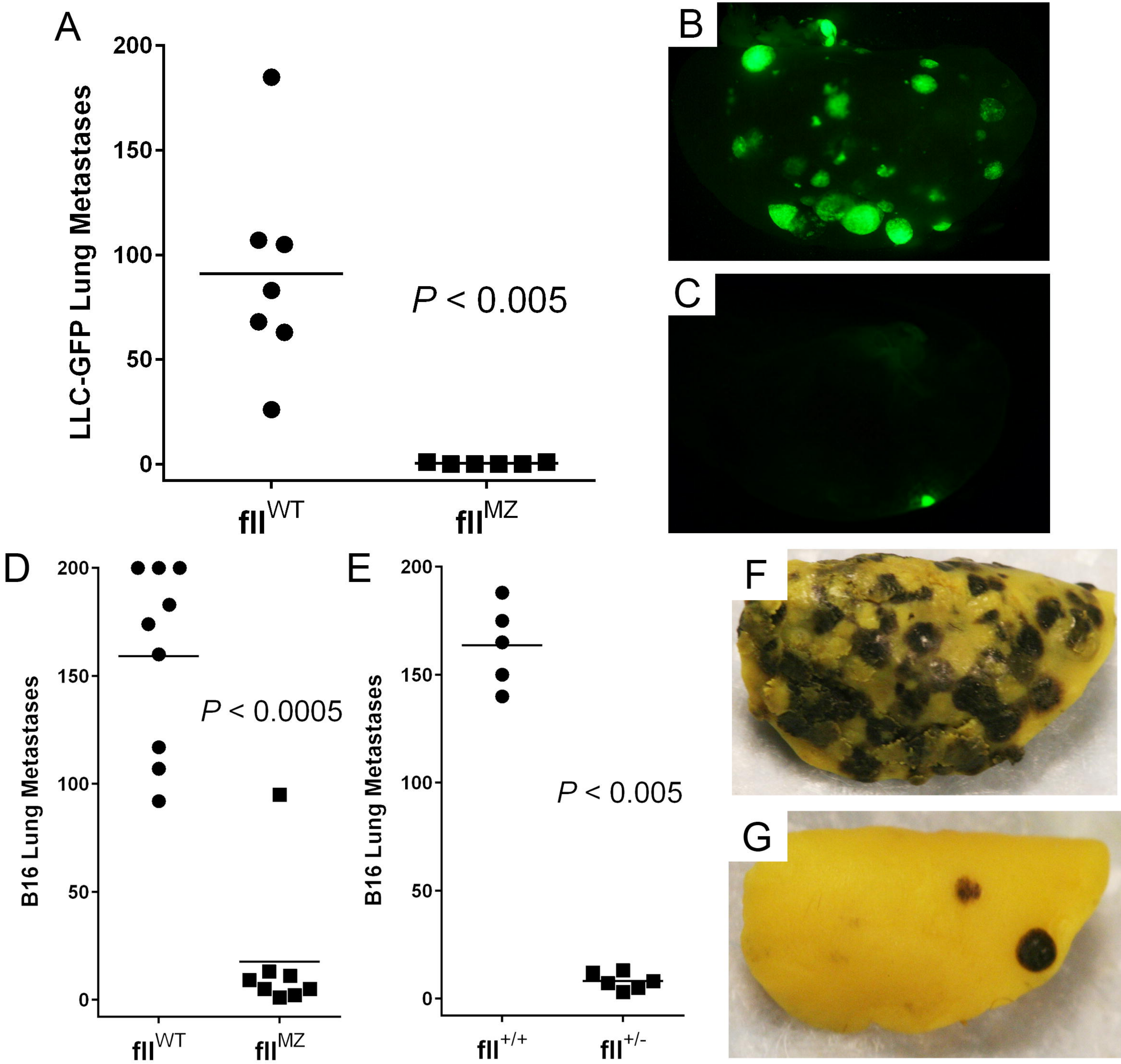

A
Tail Bleeding Times
> 600
300
200
100
0
Seconds
P < 0.001
fII^WT
fII^MZ
B
3:30:45P
fII^WT
C
1:21:39P
fII^MZ
D
Time to first thrombus (50 μm)
> 1800
1600
1200
800
400
0
Seconds
P < 0.001
fII^WT
fII^MZ
E
Time to occlusion
> 1800
1600
1200
800
400
0
Seconds
P < 0.01
fII^WT
fII^MZ

Figure 6




A
200
150
100
50
0
LLC-GFP Lung Metastases
fII^WT
fII^MZ
P < 0.005
B
C
D
200
150
100
50
0
B16 Lung Metastases
fII^WT
fII^MZ
P < 0.0005
E
200
150
100
50
0
B16 Lung Metastases
fII^+/+
fII^+/-
P < 0.005
F
G