DEFINING THE INTERACTIONS BETWEEN COAGULATION FACTOR XIII, FIBRIN(OGEN), AND RED BLOOD CELLS

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ABSTRACT

James R. Byrnes: Defining the Interactions Between Coagulation Factor XIII, Fibrin(ogen), and Red Blood Cells
(Under the direction of Alisa S. Wolberg)

Blood coagulation is the process where a cascade of enzymatic reactions generates a clot to stem the flow of blood following injury. Unfortunately, the same cascade can also form pathologic intravenous clots in a process termed venous thrombosis. Current anticoagulant therapies used to prevent thrombosis also carry a risk of bleeding. Further investigation of the basic biochemical and regulatory mechanisms underlying coagulation are required to identify new antithrombotic strategies with reduced risk of bleeding. The studies presented in this dissertation examine the interactions between three key players in the terminal stages of blood coagulation: factor XIII (FXIII), fibrin(ogen), and red blood cells (RBCs).

First, we examined the mechanism of FXIII-mediated RBC retention in clots. We found that FXIII does not promote RBC retention by crosslinking RBCs directly to the clot or by modulating fibrin network density. Instead, specific FXIII-mediated crosslinking of the fibrin α-chains is required for normal RBC retention in clots. This finding is the first to reveal a pathophysiologic role of α-chain crosslinking, and establishes this process as a crucial mediator of venous thrombosis.

Second, we investigated the nature of the FXIII-fibrinogen binding interaction to determine the domains of each protein involved in binding. We found that FXIII binds to fibrinogen γ-chain residues 390-396 via the FXIII-B subunits. These findings enhance our understanding of this important interaction in both physiologic and pathologic coagulation.

Third, we examined the regulation of the FXIII-A and -B subunits. We found that the FXIII subunits exhibit inter-tissue, reciprocal regulation whereby FXIII-B promotes FXIII-A stability in circulation, while FXIII-A increases FXIII-B stability and production. FXIII-A upregulates liver RNA-binding proteins and may modulate post-transcriptional regulation of FXIII-B synthesis. These data clarify the mechanisms governing FXIII levels in plasma and identify a unique regulatory relationship between two protein subunits synthesized in different tissues. These data also expose a new liver-regulatory function of FXIII-A.

Collectively, these studies greatly extend our understanding of the interactions between FXIII, fibrin(ogen), and RBCs, three crucial mediators of clot dynamics. Importantly, this dissertation also emphasizes the utility of basic mechanistic studies to inform translational research efforts.

"Research is what I am doing when I don't know what I am doing." – Wernher von Braun

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PREFACE

Chapter 1 contains text and figures from an article published prior to the writing of this dissertation with the following citation:

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Permission to include portions of this article in a PhD dissertation was obtained from Thieme Medical Publishers, the publisher of *Seminars in Thrombosis and Hemostasis*.

Chapter 2 represents work that was published prior to writing this dissertation with the following citation:

> Byrnes JR, Duval C, Wang Y, Hansen CE, Ahn B, Mooberry MJ, Clark MA, Johnsen JM, Lord ST, Lam W, Meijers JCM, Ni H, Ariëns RAS, Wolberg AS. Factor XIIIa-dependent red blood cell retention in clots is mediated by fibrin α-chain crosslinking. *Blood*. 2015;126(16):1940-1948.

Permission to include this article in its entirety in a PhD dissertation was obtained from The American Society of Hematology, the publisher of *Blood*.

Chapter 3 represents work that was published prior to writing this dissertation with the following citation:

> Byrnes JR, Wilson C, Boutelle AM, Brandner CB, Flick MJ, Philippou H, Wolberg AS. The interaction between fibrinogen and zymogen FXIII-A_2B_2 is mediated by fibrinogen residues γ390-396 and the FXIII-B subunits. *Blood*. 2016;128(15):1969-1978.

Permission to include this article in its entirety in a PhD dissertation was obtained from The American Society of Hematology, the publisher of *Blood*.

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LIST OF ABBREVIATIONS

3D	3-dimensional
α	Alpha
A15	Fluorescent α_2-antiplasmin-derived peptide
Aα251	Fibrinogen with the Aα-chain truncated at residue 251
α_2-AP	α_2-antiplasmin
αC	C-terminal domain of the fibrinogen α-chain
ACN	Acetonitrile
ANOVA	Analysis of variance
AU	Arbitrary unit
β	Beta
BC	Biotinylated cadaverine
BSA	Bovine serum albumin
CD11b	Cluster of differentiation 11b/integrin αM
CD18	Cluster of differentiation 18/integrin β2
CD235a	Glycophorin A
CD47	Cluster of differentiation 47
cDNA	Complimentary DNA
cm	Centimeter
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
εACA	ε-aminocaproic acid

EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal bovine serum
FC	Flow chamber
Fibrin(ogen)	Fibrinogen and fibrin, collectively
$Fib\gamma^{390\text{-}396A}$	Fibrinogen with alanine mutations in γ-chain residues 390-396
Fgn	Fibrinogen
FXIII	Factor XIII
FXIIIa	Activated factor XIII
FXIII-A	Factor XIII-A subunit
FXIII-A_2'	Thrombin-cleaved FXIII-A
FXIII-A_2*	Activated FXIII-A
FXIII-A_2B_2	Plasma FXIII heterotetramer
FXIII-A_2*I	Inhibited activated FXIII-A
FXIII-B	Factor XIII-B subunit
g	Gram
γ	Gamma
γA/γA	Fibrinogen with normally-spliced γ-chains
γ'/γ'	Fibrinogen with alternatively-spliced γ'-chains
γNNR	Fibrinogen with mutated γ-chain crosslinking residues
HBS	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMW	High molecular weight

I	Initial
IC_{50}	Half maximal inhibitory concentration
II	Prothrombin
IIa	Thrombin
K_D	Dissociation constant
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	Kilogram
L	Liter
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
M	Molar
Min	Minute
mg	Milligram
mL	Milliliter
mM	Millimolar
Mr	Relative formula mass
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
M_w	Molecular weight
ng	Nanogram
NHS	N-Hydroxysuccinimide
nL	Nanoliter

nM	Nanomolar
NPP	Normal pooled plasma
P	Pellet
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pFn	Plasma fibronectin
pM	Picomolar
PPP	Platelet-poor plasma
RBC	Red blood cell
RBP	RNA-binding protein
R_L	Signal of immobilized ligand in RU
R_{MAX}	Signal at maximal ligand binding in RU
RNA	Ribonucleic acid
RNAseq	RNA sequencing
RPM	Rotations per minute
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
RU	Response unit
rFXIII-A_2	Recombinant FXIII-A_2
rFXIII-B_2	Recombinant FXIII-B_2
S	Supernatant
SD	Standard deviation

SDS	Sodium dodecyl sulfate
SE	Standard error of the mean
siRNA	Small interfering RNA
S_M	Molecular stoichiometry of interaction
SPR	Surface plasmon resonance
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TAFI	Thrombin-activatable fibrinolysis inhibitor
TDi	Taylor dispersion injection
TF	Tissue factor
U	Units
µL	Microliter
µM	Micromolar
µm	Micrometer
VT	Venous thrombosis
WBC	White blood cell
WT	Wild-type

CHAPTER 1: INTRODUCTION

1.1 Coagulation and venous thrombosis.

Blood coagulation is a series of enzymatic reactions that converts liquid blood into a gel-like clot. This process is initiated via either the intrinsic or extrinsic pathway. The resulting enzymatic cascades merge in a common pathway that leads to the generation of thrombin. Thrombin is a serine protease that cleaves soluble fibrinogen to form fibrin monomers. Fibrin monomers polymerize into insoluble fibers that serve as the clot structural scaffold. This scaffold is then strengthened by the crosslinking activity of the transglutaminase factor XIII (FXIII). Fibrin formation and platelet activation are crucial for hemostasis (Figure 1.1). Activated platelets will pull on fibrin fibers causing the clot to contract, aiding wound closure and minimizing blood loss following trauma.

Unfortunately, dysregulation of normal coagulation mechanisms can cause pathologic clot formation, termed thrombosis (Figure 1.1). Thrombosis can occur in the arterial or venous circulation, leading to myocardial infarction, stroke, deep vein thrombosis, and pulmonary embolism. Arterial thrombosis is typically associated with atherosclerotic plaque rupture that exposes subendothelial cells and procoagulant material (e.g., tissue factor, collagen) to blood, leading to platelet activation and aggregation in high shear. Ultimately, platelet accumulation and fibrin deposition produce an occlusive platelet-rich "white thrombus" (reviewed in [4]). In contrast, venous thrombosis/thromboembolism is thought to be triggered by inappropriate expression of cell adhesion molecules and procoagulant activity on intact, but inflamed endothelium in low

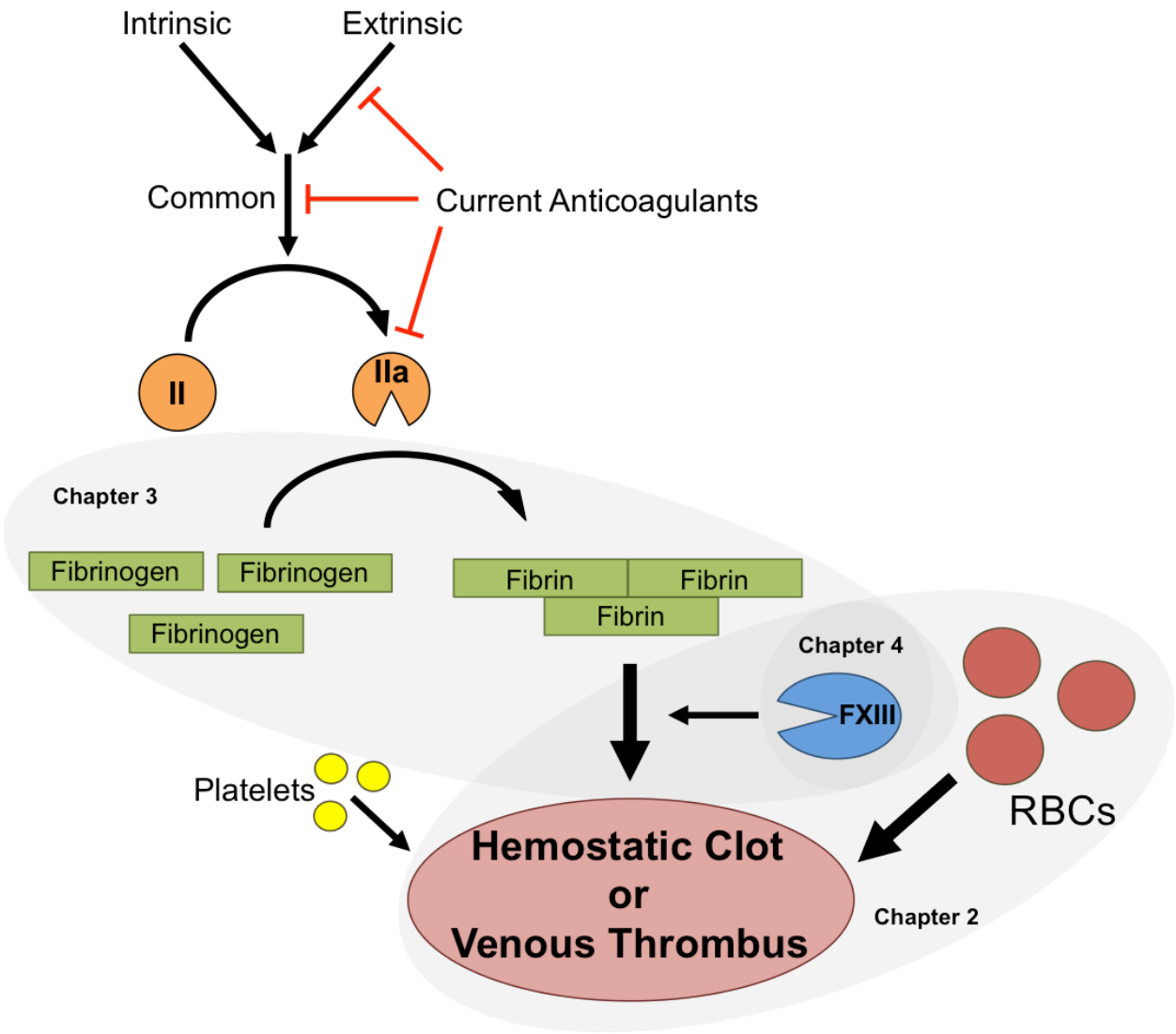

Figure 1.1. Hemostasis and venous thrombosis. Coagulation is initiated via either the intrinsic or extrinsic pathway. The two pathways meet at the common pathway that ultimately converts prothrombin (II) to thrombin (IIa). Thrombin then converts soluble fibrinogen into insoluble fibrin that polymerizes to form the structural scaffold of the clot. Coagulation factor XIII (FXIII) introduces covalent crosslinks in the fibrin network. Crosslinked fibrin, along with platelets and red blood cells (RBCs) comprise a clot. Unfortunately, the same mechanisms that generate a physiologic, hemostatic clot can also lead to pathologic venous thrombosis. Consequently, current anticoagulants target processes upstream of fibrin formation to prevent thrombosis. This dissertation examines the basic mechanisms underlying the terminal steps of coagulation. Chapter 2 investigates how FXIII promotes RBC retention in clots. Chapter 3 examines the FXIII-fibrin(ogen) binding interaction. Chapter 4 examines the regulation of FXIII subunits.

shear (reduced blood flow [stasis]). Venous thrombi are large clots primarily composed of fibrin and red blood cells (RBCs). Consequently, venous thrombi are known as "red thrombi."

Both arterial and venous thrombosis are significant public health concerns. Specifically, venous thrombosis affects over 1 in 1000 Americans annually, with each occurrence costing an average of $33,000 in the year following initial diagnosis.[5,6] 30% of venous thrombosis patients will die within 30 days of diagnosis, mostly due to pulmonary embolism. Up to one-half of surviving patients will develop recurrent venous thrombosis or suffer from post-thrombotic syndrome, which is characterized by pain, edema, and leg ulcers.[6-8]

Current anticoagulant therapies target clotting factors upstream of fibrin formation (e.g. warfarin, heparin, dabigatran, rivaroxaban, Figure 1.1). These therapies are all associated with a risk of bleeding, and further investigation of the basic biochemical mechanisms underlying coagulation is required to reveal novel antithrombotic strategies associated with minimal risk of bleeding.[8] One region of the coagulation cascade that may contain promising antithrombotic targets is the FXIII-fibrin(ogen) axis. Targeting processes after fibrin formation that mediate thrombus size and stability might reduce venous thrombosis, but persevere the body's ability to form a fibrin clot, reducing the risk of associated bleeding. However, the precise nature of interactions between FXIII, fibrin(ogen), and another key venous thrombus component, RBCs, are still unclear. This dissertation will probe the FXIII-fibrin(ogen)-RBC axis and provide insight into basic biochemical mechanisms governing these important players in hemostasis and thrombosis.

1.2 Fibrin(ogen) and factor XIII.

Fibrinogen is a 340-kDa glycoprotein that circulates in plasma at a high concentration (2-4 mg/mL).[9] Fibrinogen consists of two sets of three chains $(A\alpha B\beta\gamma)_2$ arranged in a trinodular

structure. The N-termini of these chains are located in a central E-domain with the C-termini of the Bβ- and γ-chains making two distal D-domains. The C-termini of the longer Aα-chains (αC) are largely disordered and thought to loop back and interact with the E-domain.[10-12] Following the activation of coagulation and thrombin generation, thrombin cleaves fibrinopeptides A and B from the N-termini of the Aα- and Bβ-chains, respectively, yielding a fibrin monomer. Fibrin monomers then polymerize in a half-staggered orientation to form protofibrils. Protofibrils then laterally aggregate to form the fibrin fibers that make up the insoluble fibrin network, which serves as the structural scaffold of the clot (Figure 1.2).[10]

FXIII is one of 9 members of a superfamily found in both cellular (transglutaminases 1-7 and erythrocyte band 4.2) and plasma (plasma FXIII) compartments. The cellular forms of FXIII are present in multiple cell types, including monocytes, megakaryocytes, osteoblasts, and platelets (reviewed in [13]). Plasma FXIII is unique because it circulates as two catalytic subunits (FXIII-A_2) and two non-catalytic subunits (FXIII-B_2) arranged in a non-covalent heterotetramer (FXIII-A_2B_2, M_r 325 kDa, ~70 nM, 14-28 μg/mL).[14] Mechanisms mediating the interactions between the A and B subunits are reviewed by Schroeder and Kohler[15]. Essentially all FXIII-A_2B_2 zymogen circulates in complex with fibrinogen.[16] Although older studies implicated the alternatively-spliced fibrinogen γ-chain (termed γ') in FXIII-A_2B_2 binding[17,18], a more recent study using recombinant fibrinogen suggested FXIII-A_2B_2 binds both γA- and γ'-containing molecules with similar affinity (K_D ~40 nM)[19]. This finding suggests other regions of fibrinogen mediate the interaction between FXIII-A_2B_2 and fibrinogen. Both older[20] and more recent[21] studies identified regions in the fibrinogen α-chain that bind FXIII. Smith et al.[21] found that FXIII-A_2B_2 binds a peptide containing amino acid residues 371-425 of the fibrinogen αC domain with high affinity (K_D 5-30 nM). However, they did not demonstrate binding of FXIII-A_2B_2 to

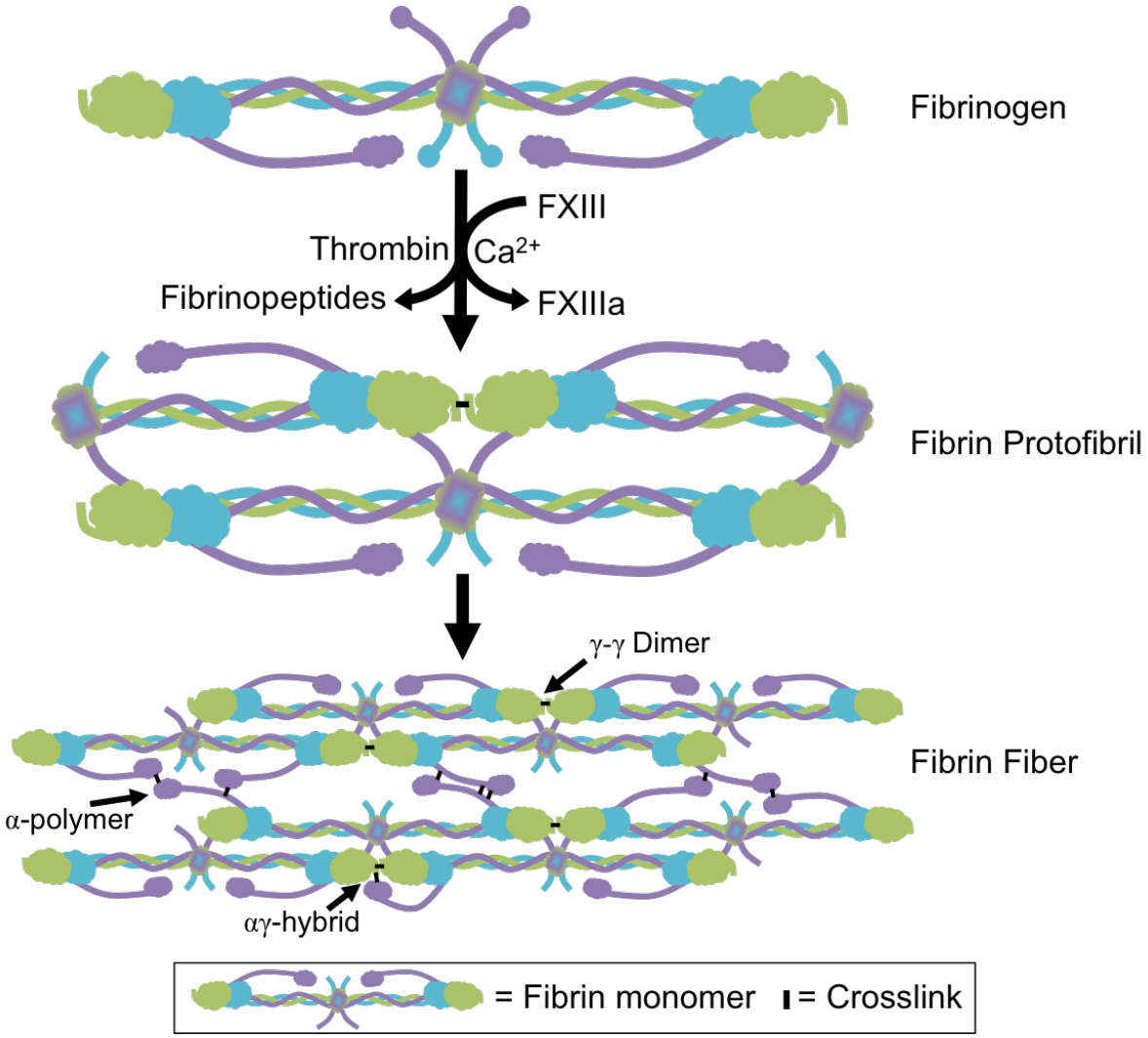

Figure 1.2. FXIIIa crosslinking during fibrin formation. Fibrinogen is a hexamer composed of 2 Aα- (purple), 2 Bβ- (blue), and 2 γ-chains (green). During coagulation, thrombin cleaves N-terminal fibrinopeptides from the Aα- and Bβ-chains, producing fibrin monomers which polymerize into protofibrils and subsequently, fibers.[10] FXIIIa increases clot stability by introducing ε-N-(γ-glutamyl)-lysyl crosslinks between residues in the γ- and α-chains of fibrin monomers within individual fibers. FXIIIa first introduces crosslinks between γ-chains (forming γ-γ dimers) and subsequently between γ- and α-chains (forming high molecular weight species [γ-multimers, α-polymers, and αγ-hybrids]).[3,22-24] Figure originally appeared in Byrnes and Wolberg.[25] Adapted from and reprinted with permission from Thieme Medical Publishers, the publisher of *Seminars in Thrombosis and Hemostasis*.

this region on full-length fibrinogen and suggested that the interaction between FXIII-A_2B_2 and the αC region may arise during FXIII activation, and that other fibrinogen residues bind zymogen FXIII-A_2B_2 in circulation.[21] We showed that murine fibrin(ogen) containing alanine mutations within γ-chain residues γ390-396 (Fib$\gamma^{390\text{-}396A}$) exhibits decreased binding of FXIII-A_2B_2, suggesting these residues mediate the carrier function in mice.[26]

During activation of plasma FXIII-A_2B_2, thrombin first catalyzes the cleavage of activation peptide(s) from the N-termini of the FXIII-A subunits (FXIII-$A_2'B_2$).[27,28] Calcium then promotes dissociation of the FXIII-B subunits from FXIII-A_2', yielding activated FXIII-A_2* (FXIIIa).[29] FXIIIa catalyzes the formation of intermolecular ε-N-(γ-glutamyl)-lysyl isopeptide bonds between glutamine residues 398/399 and lysine 406 in the fibrin γ-chain and subsequently between glutamine and lysine residues in the α-chain (Figure 1.2). Plasma FXIII activation is accelerated when it is bound to fibrinogen, which facilitates dissociation of the FXIII-B subunits.[20,30,31] This function ascribes fibrin(ogen) with an important regulatory role during FXIII(a) activation and activity. Plasma from mice that have reduced binding of FXIII-A_2B_2 to fibrin(ogen) (Fib$\gamma^{390\text{-}396A}$ mice) exhibits delayed activation of FXIII-A_2B_2 and slower formation of fibrin γ- and α-chain crosslinks.[26]

Crosslinking profoundly affects the viscoelastic properties of the fibrin network.[22,32-35] FXIIIa-mediated crosslinking significantly increases the elastic modulus (stiffness) of both individual fibrin fibers[33,34,36] and whole clot networks[32], and is required to protect the clot against premature disruption or dissolution (Figure 1.3). Using a synthetic FXIII inhibitor and a patient-derived antibody to selectively inhibit the formation of high molecular weight (HMW) crosslinked species yet permit γ-γ dimer formation, Ryan et al.[22] showed that γ-chain crosslinking, alone, is insufficient to stiffen fibrin networks, and that the increased stiffness is

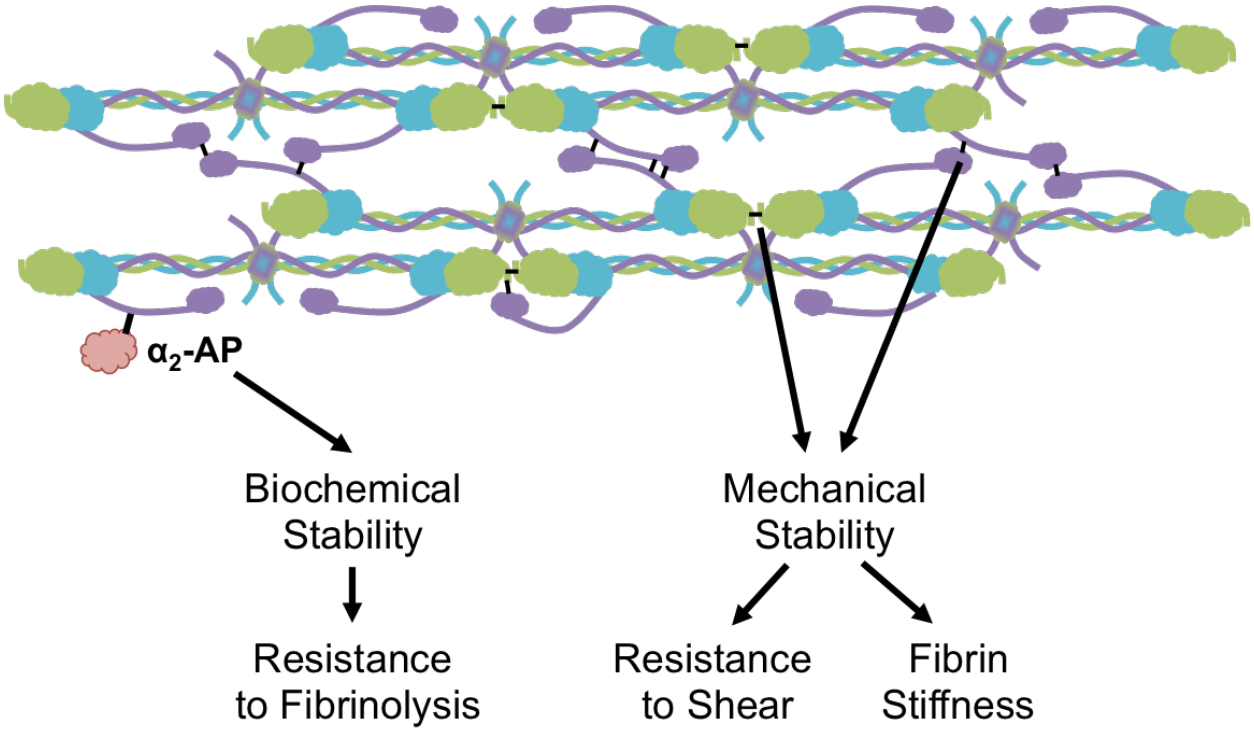

Figure 1.3. Contributions of FXIIIa to clot biochemical and mechanical stability. FXIIIa crosslinking of plasma proteins [i.e. α_2-antiplasmin, (α_2-AP)] increases the resistance of the clot to fibrinolysis. Crosslinking of the fibrin α- (purple) and γ-chains (green) increases the mechanical stability of the clot. Increased mechanical stability stiffens and renders the clot more resistant to shear forces. Adapted from a figure that originally appeared in Byrnes and Wolberg.[25] Reprinted with permission from Thieme Medical Publishers, the publisher of *Seminars in Thrombosis and Hemostasis*.

correlated with the formation of α-chain-rich, HMW crosslinked species.[22] More recent studies using a recombinant fibrinogen mutated to eliminate γ-chain crosslinking sites confirmed that α-chain crosslinking is the primary contributor to clot stiffness and elasticity.[3,23,24] Interestingly, however, fibrin crosslinking has little effect on gross fibrin network morphology, producing, at most, only a minor (~12%) increase in fibrin network density.[3,37-40]

FXIII(a) also has critical anti-fibrinolytic functions during coagulation, mediated via its ability to crosslink antifibrinolytic proteins, including α_2-antiplasmin[41,42], thrombin activatable fibrinolysis inhibitor[43], and type-2 plasminogen activator inhibitor[44] to fibrin (Figure 1.3). This activity is mediated primarily by plasma FXIII-A_2*. However, in situations in which the concentration of plasma FXIII is low (≤10%), platelet FXIII-A_2* exposed on the platelet surface also promotes fibrin stability by crosslinking α_2-antiplasmin to fibrin.[45,46]

1.3 FXIII-fibrin(ogen) axis and clot RBC content.

Previous work in our laboratory showed that FXIIIa activity is also a major determinant of thrombus RBC content.[26] Briefly, in in vitro clot contraction assays where whole blood is clotted and platelet-mediated clot contraction is allowed to occur, we observed reduced RBC retention and clot size in clots formed in the presence of a FXIIIa inhibitor or from both mouse and human FXIII-deficient plasma.[26] Similar to the in vitro assays, FXIII-deficient mice subjected to in vivo venous thrombosis models produced smaller thrombi with reduced RBC content relative to controls.[26] These data are consistent with the "excessive red cell fallout" previously reported in a family with congenital FXIII deficiency[47], and demonstrate a newly-recognized function for FXIII(a) during clot formation (Figure 1.4). This finding also suggests that FXIIIa could be a therapeutic target for the reduction of venous thrombosis. However, how FXIIIa promotes RBC retention in clots was still unclear. In a study presented in Chapter 2, we determine the

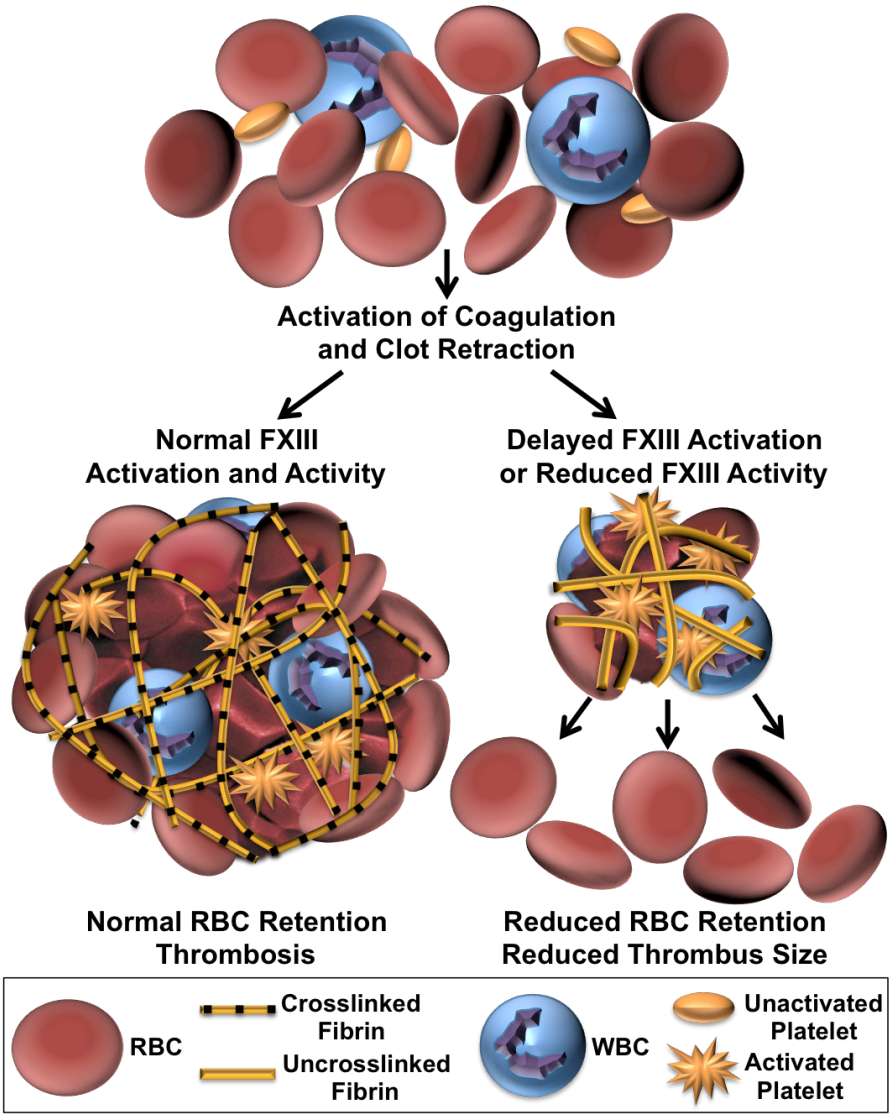

Figure 1.4. FXIIIa mediates RBC retention in clots. Under normal circumstances, FXIII is activated during clotting and promotes the inclusion of RBCs in the clot. When FXIIIa activity is deficient or delayed, RBC retention in the clot is significantly diminished, reducing clot size. Chapter 2 examines how FXIIIa promotes clot RBC retention. Chapter 3 characterizes the FXIII-fibrinogen interaction that is crucial for normal RBC retention. Figure originally appeared in Walton et al.[48] Reprinted with permission from John Wiley and Sons, the publisher of the *Journal of Thrombosis and Haemostasis*.

mechanism of FXIIIa-mediated RBC retention. Briefly, we show that FXIIIa does not directly crosslink RBCs into the clot. Instead, FXIIIa-mediated crosslinking of fibrin α-chains promotes RBC retention in forming clots.[40]

Prior work in our laboratory also showed that blood from Fibγ$^{390\text{-}396A}$ mice subjected to our in vitro clot contraction assay produces ~5-fold smaller clots with ~3.5-fold reduced RBC retention compared to clots formed from wild-type blood. Fibγ$^{390\text{-}396A}$ mice also exhibit reduced thrombus size and reduced RBC retention in in vivo venous thrombosis models.[26] Given that these mice exhibit impaired FXIII-fibrinogen binding and delayed FXIII activation[26], these results suggest that not only is FXIIIa activity important for RBC retention in clots, but the timing of FXIII activation, and therefore FXIII-fibrinogen interactions, are also crucial determinants of RBC retention in clots (Figure 1.4). This finding suggests that FXIII-fibrinogen binding, and not just FXIIIa activity, could be therapeutically targeted to reduce venous thrombosis. However, the nature of the FXIII-fibrinogen binding interaction was still unknown. In a study presented in Chapter 3, we investigate the critical regions of FXIII and fibrinogen involved in this binding interaction. Briefly, we determine that FXIII binds to fibrinogen γ-chain residues 390-396 via the FXIII-B subunits.[49]

1.4 FXIII subunit regulation.

The FXIII-A and -B subunits arise from different tissues. Transplantation studies show FXIII-A polymorphisms track with bone marrow transplantation in humans, suggesting cells of bone marrow origin produce plasma FXIII-A.[50] Consistent with this premise, recent murine Cre/lox studies implicate tissue resident macrophages in plasma FXIII-A production.[51] FXIII-B polymorphisms track with liver transplantation in humans[50], and hepatoma cell lines synthesize FXIII-B in vitro[52], suggesting that the liver synthesizes FXIII-B. However, both the mouse liver

and kidney contain *F13b* mRNA.[53] Once secreted, the FXIII subunits assemble in plasma to form FXIII-A_2B_2. Relative to FXIII-A, there is a two-fold molar excess of FXIII-B. Consequently, essentially all plasma FXIII-A circulates tightly bound to FXIII-B (K_D~10^{-10}) and there is a pool of uncomplexed FXIII-B (43-62 nM).[54]

It is well-established that the FXIII-B subunits stabilize the FXIII-A subunits.[53,55,56] In the absence of FXIII-B, FXIII-A has a half-life of <10 hours[55], whereas the FXIII-A_2B_2 complex circulates for 9-10 days.[57] Consequently, FXIII-B deficiency results in FXIII-A deficiency. Intriguingly, we[49] and others[53] have shown FXIII-A-deficient mice have significantly decreased plasma FXIII-B. Similar trends are observed in humans, with FXIII-A-deficient individuals exhibiting less than 50% normal FXIII-B.[58] Furthermore, recombinant FXIII-A_2 infusion in both monkeys[59] and humans[55] increases plasma FXIII-B. FXIII-B continues to rise even after FXIII-A_2B_2 begins to fall. Collectively, these data suggest FXIII-A and FXIII-B exhibit a unique, inter-tissue reciprocal regulation, whereby FXIII-B stabilizes FXIII-A and FXIII-A mediates FXIII-B levels.

The mechanism driving this reciprocal regulation is unclear. In an ongoing study presented in Chapter 4, we use in vivo mouse models, in vitro cell culture, and proteomic profiling to examine how FXIII-A regulates FXIII-B. Briefly, we show that FXIII-A both reduces FXIII-B clearance from plasma and induces de novo FXIII-B production in the liver (Figure 1.5). FXIII-A causes differential regulation of RNA-binding proteins in the liver, but not in the kidney, suggesting the effect of FXIII-A is liver-specific.

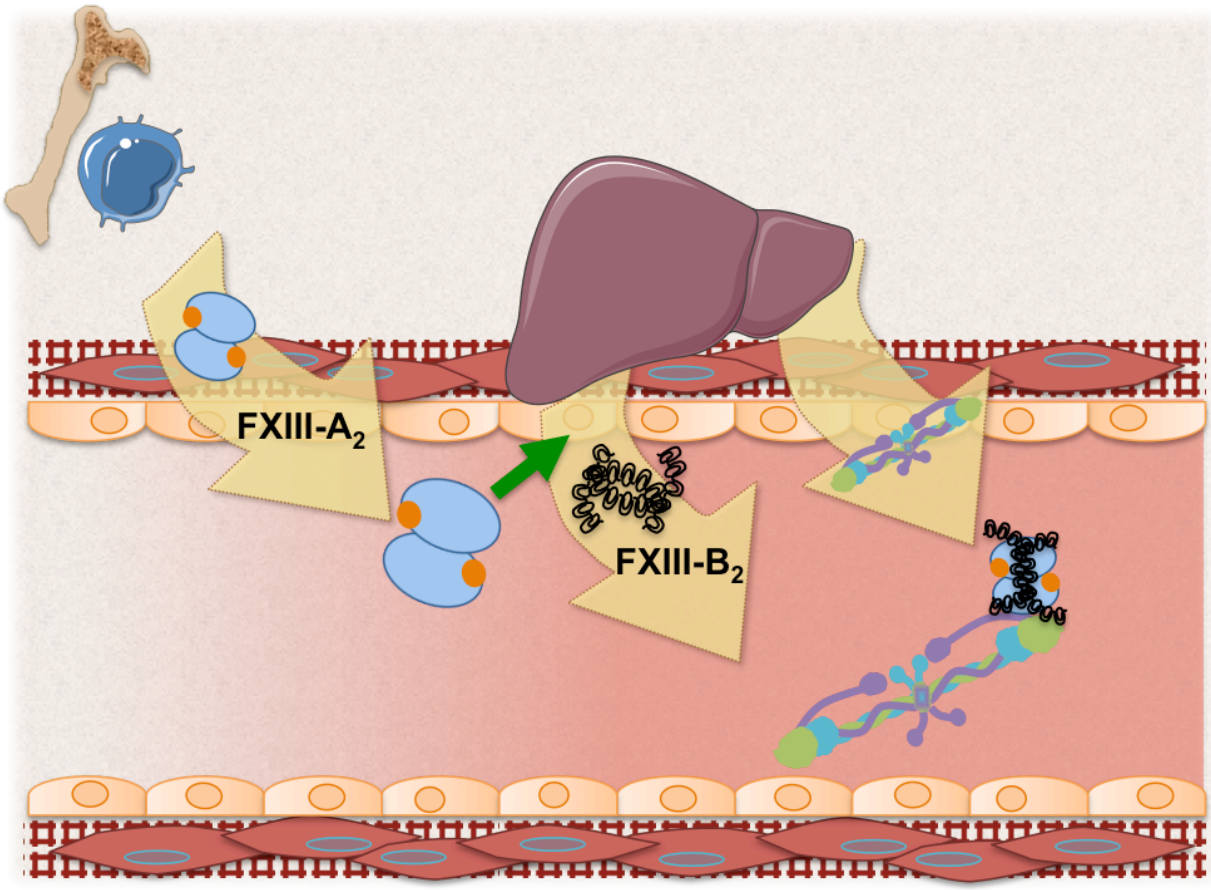

Figure 1.5. Inter-tissue, reciprocal FXIII subunit regulation. Studies presented in Chapter 4 examine the mechanisms underlying the inter-tissue, reciprocal regulation of FXIII subunits. Briefly, FXIII-A subunits are synthesized in cells of bone-marrow origin and secreted into the plasma. FXIII-A then induces de novo hepatic FXIII-B production. In plasma, FXIII-A and -B subunits assemble into the FXIII-A_2B_2 complex, which stabilizes both subunits. FXIII-A_2B_2 then circulates bound to fibrinogen.

1.5 Focus of this dissertation.

Although current anticoagulants are effective at preventing thrombosis, they also carry a risk of bleeding. Continued study of the biochemical mechanisms underlying clotting is necessary to identify therapeutic targets that will prevent pathologic thrombosis yet preserve physiologic hemostasis. The focus of this dissertation is to expand our understanding of FXIII and fibrinogen function and the regulatory processes affecting these important coagulation proteins. This dissertation blends basic biochemical approaches with in vitro and in vivo models to determine: 1) how FXIII activity mediates RBC retention in clots, 2) how FXIII and fibrin(ogen) interact, and 3) how FXIII-A regulates FXIII-B.

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CHAPTER 2: FACTOR XIIIa-DEPENDENT RETENTION OF RED BLOOD CELLS IN CLOTS IS MEDIATED BY FIBRIN α-CHAIN CROSSLINKING[1]

2.1 Overview

Factor XIII(a) [FXIII(a)] stabilizes clots and increases resistance to fibrinolysis and mechanical disruption. FXIIIa also mediates red blood cell (RBC) retention in contracting clots and determines venous thrombus size, suggesting FXIII(a) is a potential target for reducing venous thrombosis. However, the mechanism by which FXIIIa retains RBCs in clots is unknown. We determined the effect of FXIII(a) on human and murine clot weight and composition. Real-time microscopy revealed extensive RBC loss from clots formed in the absence of FXIIIa activity, and RBCs exhibited transient deformation as they exited the clots. Fibrin band-shift assays and flow cytometry did not reveal crosslinking of fibrin or FXIIIa substrates to RBCs, suggesting FXIIIa does not crosslink RBCs directly to the clot. RBCs were retained in clots from mice deficient in α_2-antiplasmin, thrombin-activatable fibrinolysis inhibitor, or fibronectin, indicating RBC retention does not depend on these FXIIIa substrates. In the presence of FXIIIa, RBC retention in clots was positively correlated with final network density; however, FXIIIa inhibition abrogated this correlation. FXIIIa inhibition reduced RBC retention in clots formed with fibrinogen that lacks γ-chain crosslinking sites, but not in clots that lack α-chain crosslinking sites. Moreover, FXIIIa inhibitor concentrations that primarily block α-, but not γ-, chain crosslinking decreased RBC retention in clots. These data indicate RBC retention in clots

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is mediated by fibrin α-chain crosslinking. These findings expose a newly-recognized, essential role for fibrin crosslinking during whole blood clot formation and consolidation, and establish FXIIIa activity as a key determinant of venous thrombus composition and size.

2.2 Introduction

Fibrinogen is a 340 kD plasma glycoprotein comprised of two sets each of three chains (Aα, Bβ, and γ) that circulates at 2-4 mg/mL. During coagulation, thrombin cleaves N-terminal peptides from the Aα- and Bβ-chains, producing fibrin monomers which polymerize into protofibrils and subsequently, fibers.[1] Fibrinogen deficiency is associated with bleeding and/or thrombosis[2], whereas elevated fibrinogen (hyperfibrinogenemia) is associated with thrombosis[3-5].

Factor XIII (FXIII) is a plasma protransglutaminase comprised of two A (FXIII-A) and two B (FXIII-B) subunits that circulate as a heterotetrameric zymogen (FXIII-A_2B_2). FXIII activation occurs via thrombin-mediated cleavage of an N-terminal activation peptide from FXIII-A, and calcium-mediated dissociation of the inhibitory, carrier FXIII-B subunits, rendering catalytically-active FXIIIa.[6] FXIII deficiency is associated with frequent bruising, hematomas, miscarriage, poor wound healing, and intracranial hemorrhage.[7]

FXIIIa increases clot stability by introducing ε-N-(γ-glutamyl)-lysyl crosslinks between residues in the γ- and α-chains of fibrin monomers within individual fibers. Although crosslinking causes only subtle-to-no changes in fibrin network morphology[8,9], crosslinking significantly decreases the extensibility and elasticity of individual fibers, and increases fibrin elastic modulus (stiffness)[10-14]. Interestingly, these effects are specifically associated with formation of high molecular weight (HMW) crosslinked fibrin species (γ-multimers, α-polymers, and αγ-hybrids).[14-17] Studies using recombinant fibrinogen that cannot undergo γ-chain crosslinking (γQ398N/Q399N/K406R [γNNR]) reveal that fiber stiffening is primarily due to α-

chain crosslinking.[15-17] Crosslinking of other plasma proteins (e.g., α_2-antiplasmin, fibronectin) to fibrin increases the resistance of the network to biochemical degradation (reviewed in [6]). Together, these studies demonstrate the multifunctional role of FXIIIa in clot mechanical and biochemical properties.

Recently, we showed that FXIIIa also mediates clot composition and size during whole blood clot formation.[18] Using an in vitro clot contraction (also called retraction) assay, we found that human and mouse whole blood clots formed in the absence of plasma FXIII or presence of a FXIIIa inhibitor have reduced retention of red blood cells (RBCs) and are significantly smaller than normal clots. Importantly, compared to wild-type controls, FXIII-deficient mice produce smaller venous thrombi that contain significantly fewer RBCs, suggesting FXIIIa-mediated retention of RBCs in clots modulates thrombus size.[18] Collectively, these results reveal a previously unrecognized function for FXIIIa activity during venous thrombosis (VT) and suggest FXIIIa is a potential therapeutic target for reducing VT. However, the mechanism by which FXIIIa mediates RBC retention in clots is not known.

Herein, we show that FXIIIa mediates RBC retention in clots specifically via its ability to crosslink fibrin α-chains. These results define new (patho)physiologic roles for FXIIIa activity and fibrin α-chain crosslinking in hemostasis and thrombosis.

2.3 Methods

Proteins and Materials. Tissue factor (TF, Innovin®) was from Siemens. T101 was from Zedira. A15 peptide was from Kerafast. Biotinylated cadaverine and Alexa Fluor® 488 streptavidin were from Life Technologies. 6X Laemmli sample buffer containing sodium dodecyl sulfate was from Boston Bioproducts. Tris-glycine gels (10%) were from Bio-Rad. Human α-thrombin, plasma FXIII-A_2B_2, and Peak 1 human fibrinogen were from Enzyme

Research Laboratories. Anti-human fibrinogen antibody was from Dako and Alexa Fluor® 488 anti-rabbit secondary was from Jackson Immunoresearch. PE-labeled anti-human CD235a was from Biolegend. Dulbecco's modified Eagle's medium/Ham's F12, 50/50 mix was from Corning. Octadecyl rhodamine B chloride was from Invitrogen and Alexa Fluor® 488 and 647 dyes were from Molecular Probes. ε-aminocaproic acid (ε-ACA) was from Sigma. Normal human pooled plasma was prepared from 34 healthy male and female donors as previously described.[19] Phlebotomy was approved by the University of North Carolina and Georgia Institute of Technology Institutional Review Boards and performed on consenting donors in accordance with the Declaration of Helsinki. Murine studies were approved by the University of Amsterdam Academic Medical Center Animal Care and Use Committee and St. Michael's Hospital Animal Care Committee.

Human blood clot contraction. Clot contraction assays were performed as described.[18] Briefly, blood was drawn from healthy donors via venipuncture and added to siliconized wells of a 96-well plate containing TF (1 pM, final), $CaCl_2$ (10 mM, final), and the FXIIIa active site inhibitor, T101 (10 μM, final) or HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4 [HBS]). Clot formation and contraction were allowed to proceed for 90 minutes at 37°C. Serum RBC content was measured by absorbance (575 nm) with a SpectraMax Plus 340 plate reader (Molecular Devices) and compared with the initial absorbance. Clots were weighed and/or prepared for microscopy.

For experiments using recombinant fibrinogen, blood was drawn from a fibrinogen-deficient individual (<40 mg/dL fibrinogen, infinite thrombin clotting time, Supplemental Figure 2.1) and processed to platelet-rich plasma (PRP) by centrifugation (150x*g*, 20 minutes). PRP was then

processed to platelet-poor plasma (PPP) by centrifugation (1500x*g*, 10 minutes). PPP was flash-frozen in liquid nitrogen and stored at -80°C. Platelets and RBCs were isolated from healthy, consenting, blood-type O-negative donors as described[18], and counted on a pocH-100*i* Hematology Analyzer (Sysmex). Platelets (200,000/μL, final) and RBCs (2 million/μL, final) were then added to thawed fibrinogen-deficient plasma and supplemented with recombinant fibrinogen (0.25 mg/mL, final in plasma fraction, unless otherwise noted) before clot contraction was initiated with TF (1 pM, final) and recalcification (10 mM, final).

Microscopy. For real-time confocal microscopy of contracting clots, RBCs were isolated from whole blood and fluorescently-labeled with octadecyl rhodamine B chloride. Alexa Fluor® 488-labeled fibrinogen (75 μg/mL, final) and labeled RBCs (10%, final) were added to whole blood and clotting was triggered with TF (1 pM, final) and recalcification (10 mM, final) in siliconized glass-bottom petri dishes at 37°C and 60% humidity. Reactions were performed in the presence of the fibrinolysis inhibitor ε-ACA (5 mM, final), in the absence and presence of T101.

For real-time difference interference contrast microscopy, clot contraction in whole blood was triggered in a polydimethylsiloxane microfluidics device with thrombin (1 U/ml [10 nM], final), $CaCl_2$ (10 mM, final), and T101 (500 μM, final).

For laser scanning confocal microscopy, normal pooled plasma clots were generated in LabTek chambered coverglass (Nalge Nunc) by recalcification in the presence of phospholipids (4 μM, final), TF (1, 10, or 100 pM, final) and Alexa Fluor 647-labeled fibrinogen (10 μg/150 μL sample) as described.[20] After 2 hours, the fibrin network was imaged using a Zeiss LSM710 laser

scanning confocal microscope with a 63X oil-immersion lens (Carl Zeiss). 30 total sections were taken at a 0.36 μm intervals in the *z*-axis. 3D deconvolution was performed using algorithms in AutoQuant X 3.0.1 software (Media Cybernetics). *Z*-projections were generated by summing individual sections in ImageJ 1.48v.

For scanning electron microscopy of retracted clots, clots were washed in HBS, fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.15 M sodium phosphate, pH 7.4, and stored at 4°C. Fixed clots were bisected longitudinally and dehydrated with a graded ethanol series (30%, 50%, 75%, 90%, and 100% [three times], each for 20 minutes). Clots were critical point-dried, sputter-coated with gold/palladium using a Hummer X sputter coater (Anatech), and viewed using a Zeiss Supra 25 Field Emission Scanning Electron Microscope (Carl Zeiss) with a 5 kV accelerating voltage and 20 μm aperture. Three random points on each clot were imaged at 5010X magnification. To determine fibrin network density, a 5X6 grid was overlaid on each image in Adobe Photoshop CS6 and the number of grid intersections that appeared over a fiber was counted (fibers/point).

Fibrin band shift assay. Citrated whole blood or PRP was clotted with thrombin (20 nM, final) and recalcification (5 mM $CaCl_2$, final) in the absence or presence of T101 (200 μM, final) for 2 hours at 37°C. Clots were then separated from serum by centrifugation (250x*g*, 15 minutes). Cells were lysed and clots washed by incubation in 1X Cell Lysis Buffer (Cell Signaling) with 1 mM phenylmethylsulfonyl fluoride (MP Biomedicals), followed by homogenization and washing with distilled water. Clots were subsequently dissolved in 50 mM dithiothreitol/12.5 mM EDTA in 8 M urea (DTT/EDTA/urea) at 60°C for 1 hour. Samples were

boiled in SDS-containing sample buffer and separated using SDS-PAGE on 10% Tris-glycine gels.

FXIIIa substrate crosslinking to RBCs. RBCs were isolated from citrated whole blood by centrifugation (150x*g*, 20 minutes), washed in citrate-glucose-saline, and packed as described.[18] Packed RBCs (10 μL) were incubated with a Cy5-labeled peptide derived from the amino-terminus of α_2-antiplasmin (A15, Ac-GNQEQVSPLTLLKWC[Cy5]-NH_2, 3 μM, final)[22] or biotinylated cadaverine (BC, 5 mM, final) in the presence of FXIII-A_2B_2 (20 μg/mL, final), thrombin (5 nM, final), and $CaCl_2$ (10 mM, final), rotating for 1 hour (40 μL, final volume). Control samples contained 0.5 mg/mL Peak 1 fibrinogen, and no RBCs. For SDS-PAGE, reactions were quenched and dissolved in DTT/EDTA/urea, boiled, and separated on 10% Tris-glycine gels. A15-crosslinked species were directly visualized on a GE Typhoon FLA-9000 Imager (GE Healthcare). Gels of BC samples were transferred to PVDF membranes and probed with 0.2 μg/mL Alexa Fluor® 488-labeled streptavidin before visualization on the Typhoon Imager. For flow cytometry, samples were diluted 1:200 in flow buffer (21 mM Tris [pH 7.4], 140 mM NaCl, 11.1 mM dextrose, 4.7 mM KCl, 1.2 mM $MgSO_4$, and 0.1% PEG 8000) and labeled with phycoerythrin-labeled anti-CD235a. Samples containing BC were also labeled with Alexa Fluor® 488-labeled streptavidin. Samples were diluted in flow buffer and analyzed using a Stratedigm S1000Ex flow cytometer.

Murine blood clot contraction. Blood was drawn into citrate from the inferior vena cava of anesthetized wild-type, α_2-antiplasmin-deficient[23], and thrombin-activatable fibrinolysis inhibitor (TAFI)-deficient[24] mice, or *Fn*$^{fl/fl}$ *Mx Cre*$^{-}$ (pFn^{+}) and *Fn*$^{fl/fl}$ *Mx Cre*$^{+}$(pFn^{-}) mice[25]. Plasma

fibronectin (pFn) depletion was induced by three intraperitoneal injections of 250 μg of polyinonic-polycytidylic acid (Sigma) into *Cre*$^{+}$ and *Cre*$^{-}$ littermates at two-day intervals. Depletion of pFn in plasma and platelets was confirmed by Western blotting. For wild-type, α_2-antiplasmin-deficient, and TAFI-deficient mice, blood was pooled from 6-9 mice before clotting. For pFn^{+} and pFn^{-} mice, blood from 6-9 separate mice was studied. Blood was added whole or diluted 1:3 in HBS to siliconized tubes containing TF (1 pM, final) and $CaCl_2$ (10 mM, final), as described.[18] FXIIIa was inhibited with T101 (5 μM, final). Serum RBC content was measured by absorbance (575 nm).

Isolation of differentially-aged RBC populations. RBCs were separated into three fractions using density gradient centrifugation, as described.[26] Briefly, blood was collected into acid-citrate-dextrose and separated by centrifugation (800x*g*, 15 minutes). Packed RBCs were resuspended in RPMI to 50% hematocrit and lymphocytes were removed using a 2:1 (w/w) α-cellulose/microcrystalline cellulose column. RBCs were then washed twice with 10 mM HEPES, 12 mM NaCl, 115 mM KCl, 5% BSA. After washing, RBCs were centrifuged (1075x*g*, 25 minutes) against a discontinuous Percoll gradient (65, 60, 55, and 50%). Following isolation, each fraction was washed twice and stored at 4°C for up to 24 hours before reconstitution in PRP for use in clot retraction experiments.

Recombinant fibrinogen generation. Recombinant fibrinogen variants were expressed and purified as described.[15,27,28] Briefly, Chinese hamster ovary cells engineered to express wild-type fibrinogen (γA/γA)[27], or fibrinogen with mutations that remove γ-chain crosslinking sites (γQ398N/Q399N/K406R, γNNR[15]) or truncate the α-chain (Aα251[28]), were grown in roller

bottles at 37 °C in serum-free DMEM/F12 50/50 supplemented with insulin-transferrin-sodium selenite (10 μg/mL, Roche Basel, Switzerland) and aprotinin (10 U/mL, Sigma). Conditioned media was harvested, treated with phenylmethylsulfonyl fluoride (150 μM) and stored at -20 °C until purification. Fibrinogen was purified by ammonium sulfate precipitation and immunoaffinity chromatography as previously described.[15,28] Compared to γA/γA fibrinogen, recombinant fibrinogen variants clotted with similar onset times and rates, as expected.[17,28]

Western blotting. Clots were dissolved in DTT/EDTA/urea (60°C, 1 hour), boiled in SDS-containing sample buffer, and separated using SDS-PAGE on 10% Tris-glycine gels before transfer to PVDF membranes. Densitometry was performed using ImageJ 1.48v. Band intensity of HMW species and γ-γ dimers was normalized to the β-chain before normalization to untreated controls.

Statistical methods. Serum RBC content and clot weights were compared using paired or one-tailed Student's *t*-tests, as appropriate. Significance of correlations was determined by Pearson's correlation coefficient. Percent changes in clot weight, network density, fiber thickness, probe crosslinking to RBCs, and IC_{50} values were compared using an unpaired, two-tailed Student's *t*-test. $P<0.05$ was considered statistically significant.

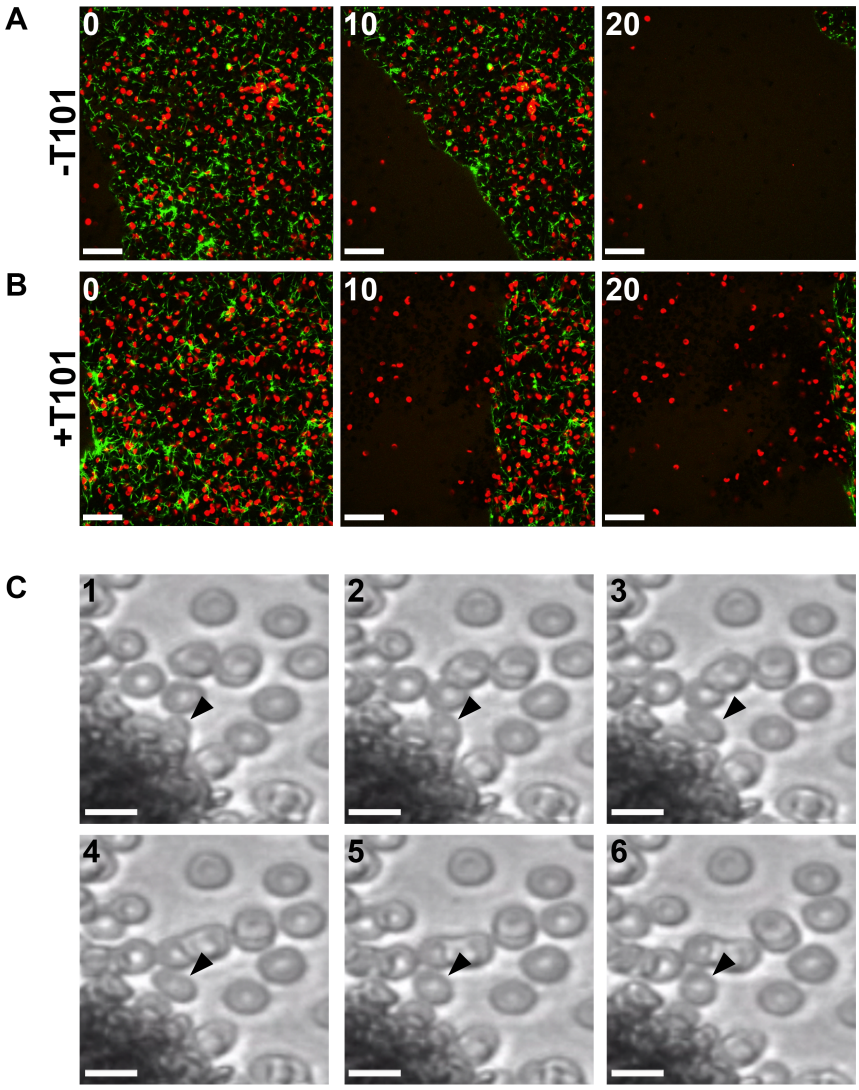


Figure 2.1. In the absence of FXIIIa activity, RBCs are extruded from the clot during clot contraction. (A-B) Clot formation and contraction was triggered by recalcification (10 mM, final) and addition of TF (1 pM, final) to whole blood spiked with octadecyl rhodamine B-labeled RBCs, Alexa Fluor 488®-labeled fibrinogen, and ε-ACA (to inhibit fibrinolysis). Clot contraction was visualized on a Zeiss 710 NLO confocal laser scanning microscope with an incubation chamber and temperature stage (Carl Zeiss, Thornwood, NY), and monitored at 40x magnification. Representative frames of contracting clots formed in the absence (A) and presence (B) of T101 (10 μM, final). Times (in seconds) are indicated in each panel. Scale bar is 50 μm. (C) Clot formation and contraction was triggered by addition of thrombin (1U/mL [10 nM], final) and recalcification (10 mM, final) to whole blood. Clot contraction was visualized at 10X with digital zoom on a Nikon Eclipse TE2000-U inverted microscope (Nikon Instruments, Melville, NY). Frames depict RBC extrusion from a contracting clot. Black arrowheads highlight a deforming RBC as it exits the clot. Numbers indicate successive frames. Scale bar is 10 μm.

2.4 Results

In the absence of FXIIIa activity, RBCs are extruded from the clot during clot contraction. We previously showed that FXIIIa promotes RBC retention in contracting clots.[18] To visualize this process, we generated whole blood clots in the presence of Alexa Fluor® 488-labeled fibrinogen, octadecyl rhodamine B chloride-labeled RBCs, and ε-ACA (to inhibit fibrinolysis), in the absence and presence of the FXIIIa inhibitor, T101. We then visualized clot contraction using real-time confocal and differential interference contrast microscopy (Figure 2.1). In clots with normal FXIIIa activity, most RBCs were retained within the clot (Figure 2.1A). In contrast, inhibiting FXIIIa with T101 increased RBC mobility within the contracting clot and increased RBC extrusion from the clot (Figure 2.1B). In some clots, we observed "plumes" of RBCs being ejected from the clot. In both cases, RBCs exiting the clots exhibited substantial, but transient, deformation, consistent with their highly deformable nature (Figure 2.1C). Notably, RBCs resumed their characteristic discoid shape once free of the clot (Figure 2.1C). Together with previous findings[18], these images demonstrate a critical role for FXIIIa activity in RBC retention in clots.

FXIIIa does not crosslink RBCs to fibrin. We first tested the hypothesis that FXIIIa directly crosslinks RBCs to fibrin. Although RBCs noncovalently bind fibrin(ogen)[29-31], a covalent interaction between RBCs and fibrin has not been identified. To determine if FXIIIa crosslinks a RBC surface protein to fibrin, we analyzed fibrin in clots generated in the absence and presence of RBCs and FXIIIa activity. We anticipated that if FXIIIa crosslinks RBCs to fibrin, crosslinked fibrin formed in the presence of RBCs would migrate more slowly than fibrin formed in the absence of RBCs. Figure 2.2 shows that clots formed in the presence of RBCs did

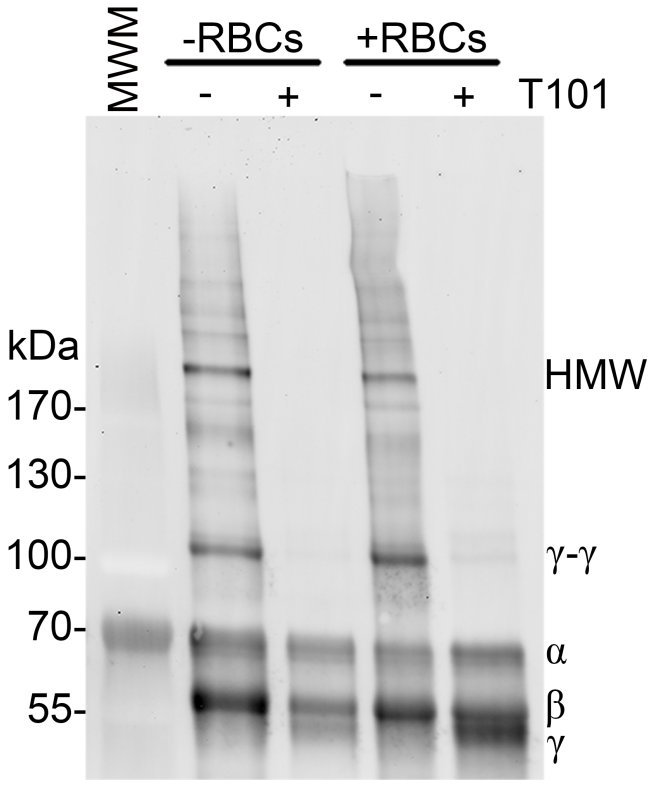


Figure 2.2. FXIIIa does not crosslink fibrin to RBCs. Clotting was initiated in recalcified (5 mM, final) PRP (-RBCs) or whole blood (+RBCs) with thrombin (20 nM, final), in the absence or presence of T101 (200 μM, final). Clots were then dissolved and fibrin crosslinking patterns were analyzed by Western blotting using a polyclonal anti-human fibrinogen antibody. Representative blot of N=4 experiments.

not exhibit fibrin band shifts or new bands relative to clots formed in the absence of RBCs. These data are consistent with prior studies that identified transglutaminase-2 substrates on the cytoplasmic, but not outer, surface of the RBC membrane[32,33], and suggest FXIIIa does not crosslink RBCs to fibrin.

FXIIIa does not crosslink glutamine- or lysine-reactive substrates to RBCs. To identify any fibrin-*independent* FXIIIa substrates on RBCs, we utilized two probes for FXIIIa activity: A15 peptide and biotinylated cadaverine (BC). A15 peptide contains a glutamine residue recognized by FXIIIa[22] and BC mimics a reactive lysine substrate of FXIIIa[34]. Whereas FXIIIa efficiently crosslinked A15 (Figure 2.3A) and BC (Figure 2.3B) to fibrin, analysis of cell lysates of washed RBCs incubated with FXIIIa and A15 or BC did not reveal any labeled proteins (Figure 2.3A-B, lower limit of detection, ~300 molecules/cell, data not shown).

Since RBC interactions with plasma proteins can be mediated by cell membrane lipids, including sulfatide[35], we also incubated washed RBCs with FXIIIa and A15 peptide or BC and analyzed intact cells using flow cytometry. Similar to findings with SDS-PAGE, we did not detect A15 peptide or BC on intact CD235a-positive cells (RBCs) in the absence or presence of FXIIIa, indicating RBCs do not have ligands for these established FXIIIa substrates (Figure 2.3C-D). Collectively, these findings indicate FXIIIa does not promote RBC retention by crosslinking RBCs into the clot.

α_2-antiplasmin, TAFI, and fibronectin are not required for FXIIIa-mediated RBC retention. Three established non-fibrin substrates of FXIIIa are α_2-antiplasmin, TAFI, and fibronectin. We therefore tested whether these proteins are required for RBC retention by

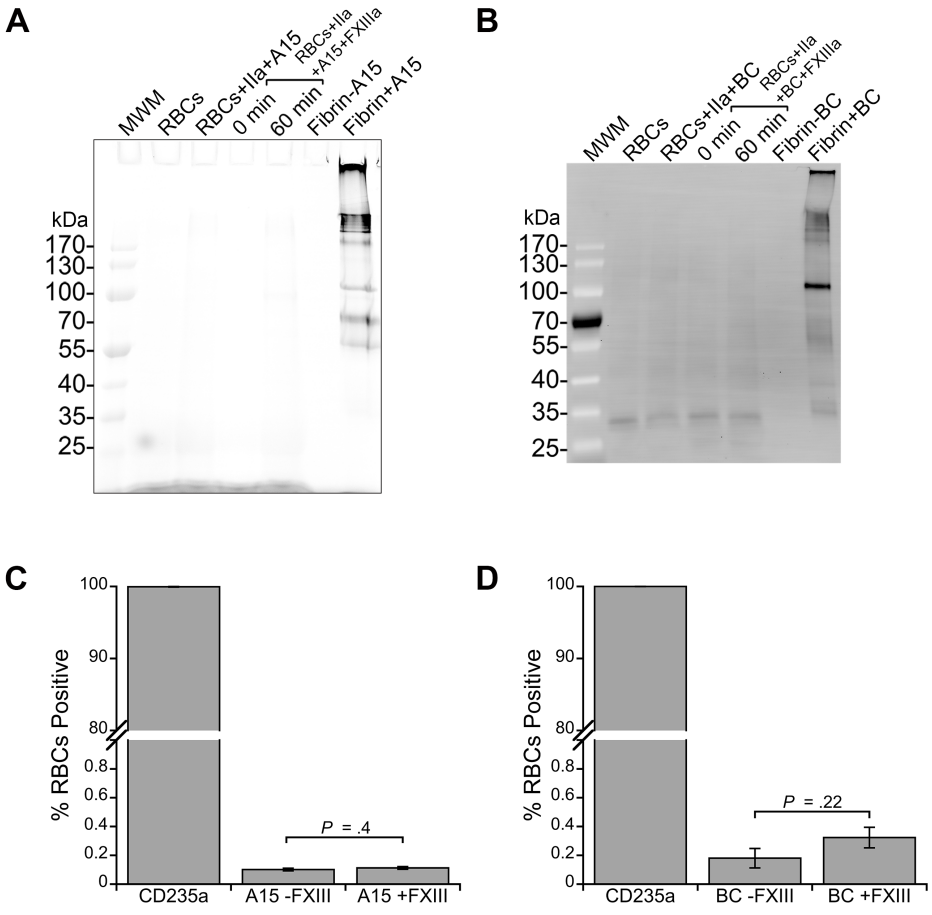


Figure 2.3. FXIIIa does not crosslink FXIIIa substrates to RBCs. (A) Cy5-labeled A15 peptide (glutamine donor, 3 μM, final) or (B) biotinylated cadaverine (BC, lysine acceptor, 5 mM, final) were incubated with FXIII (20 μg/mL, final) and washed RBCs in the presence of thrombin (IIa, 5 nM, final) and $CaCl_2$ (10 mM, final) to probe for reactive lysine and glutamine residues, respectively, on the RBC surface. RBCs were then lysed, proteins separated by SDS-PAGE, and labeling visualized using Cy5 fluorescence (A15) or by transfer to a PVDF membrane and probing with Alexa Fluor® 488-labeled streptavidin. Control reactions contained fibrinogen (0.5 mg/mL, final), FXIII, thrombin, $CaCl_2$, and A15 or BC. (C-D) For flow cytometry, intact cells were incubated with a phycoerythrin-labeled anti-human CD235a antibody and analyzed for A15 (C) and BC (D) labeling. Bars are means ± standard error (SE).

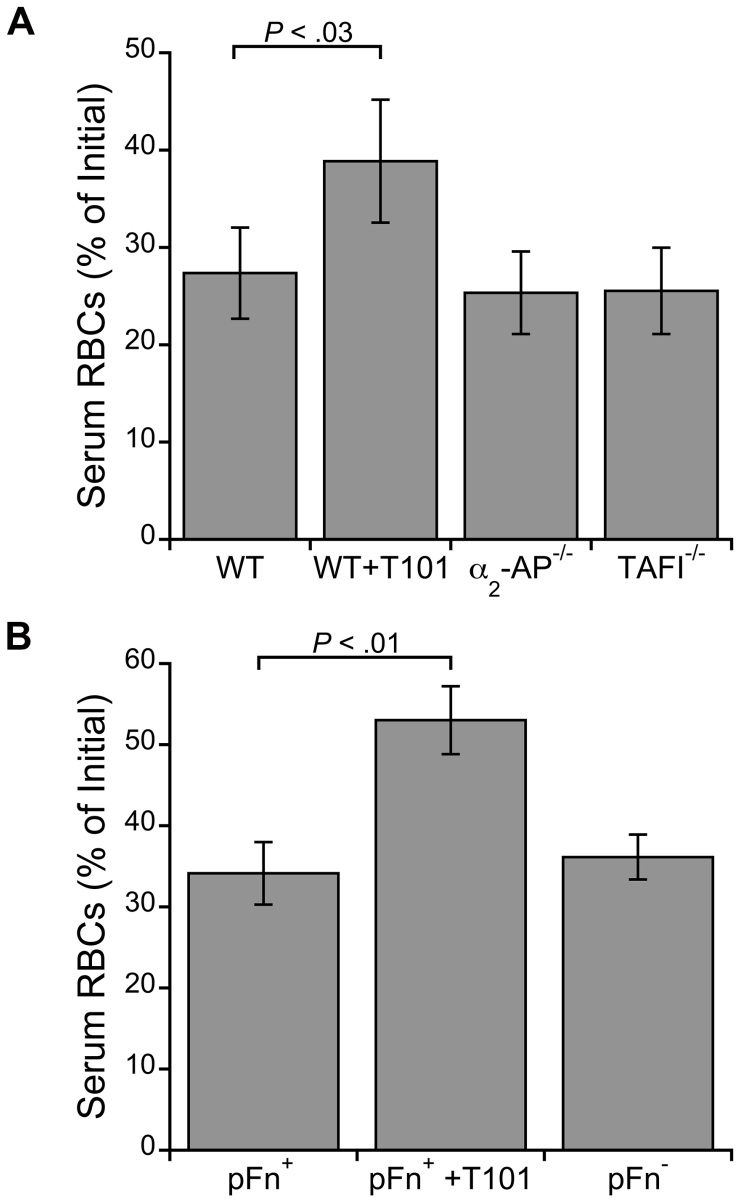


Figure 2.4. FXIIIa does not require α_2-antiplasmin, TAFI, or fibronectin to promote RBC retention in clots. Serum RBC content from ex vivo clot contraction assays using whole blood from (A) wild-type (WT), α_2-antiplasmin-deficient (α_2-AP$^{-/-}$), and TAFI-deficient (TAFI$^{-/-}$) mice (N=3) or (B) fibronectin-sufficient (pFn^{+}) and -deficient (pFn^{-}) mice (N=6-9). Clotting was initiated with TF (1 pM, final) and recalcification (10 mM, final). WT and pFn^{+} blood was treated with T101 (5 μM, final) as positive controls. Bars are means ± SE.

analyzing clot contraction of whole blood from mice deficient in these proteins. While FXIIIa inhibition increased RBC extrusion from wild-type mouse clots (P<0.03, Figure 2.4A-B), deficiency in α_2-antiplasmin, TAFI, or fibronectin did not increase RBC loss (Figure 2.4A-B). Moreover, RBC loss was similar in wild-type blood clotted in the absence and presence of the fibrinolysis inhibitor ε-ACA (serum RBC content was 31.6 and 32.5% of initial, respectively). Together, these results show that these canonical FXIIIa substrates are not required for RBC retention in clots, and that the effect of FXIIIa is not due to its antifibrinolytic function.

Fibrin network density mediates RBC retention in clots only in the presence of FXIII activity. RBC retention in clots has traditionally been attributed to steric effects of the fibrin network. Therefore, we examined the effect of fibrin network density on RBC retention by triggering clot formation using a range of TF concentrations (1, 10, and 100 pM, final) to produce clots with different initial network densities[20] in the absence and presence of FXIIIa activity (±T101) (Figure 2.5A-B). As expected, increasing the TF concentration increased fibrin network density in both the absence and presence of FXIIIa activity, and at each TF concentration, clots formed in the absence and presence of FXIIIa activity had similar network densities. We then correlated the TF concentration with the serum RBC content and weight of fully contracted whole blood clots. As predicted, increasing the concentration of TF decreased serum RBC content and increased clot weight (Figure 2.5C-D). However, at all TF concentrations tested, FXIIIa inhibition increased RBC extrusion from clots 2-4-fold (P<0.002, Figure 2.5C) and decreased clot weight 16-48% (P<0.0002, 2.5D), indicating an independent effect of FXIIIa. We also measured final fibrin network density in fully contracted whole blood clots. As seen in studies of plasma clots[8,9], the absence or presence of FXIIIa activity (±T101)

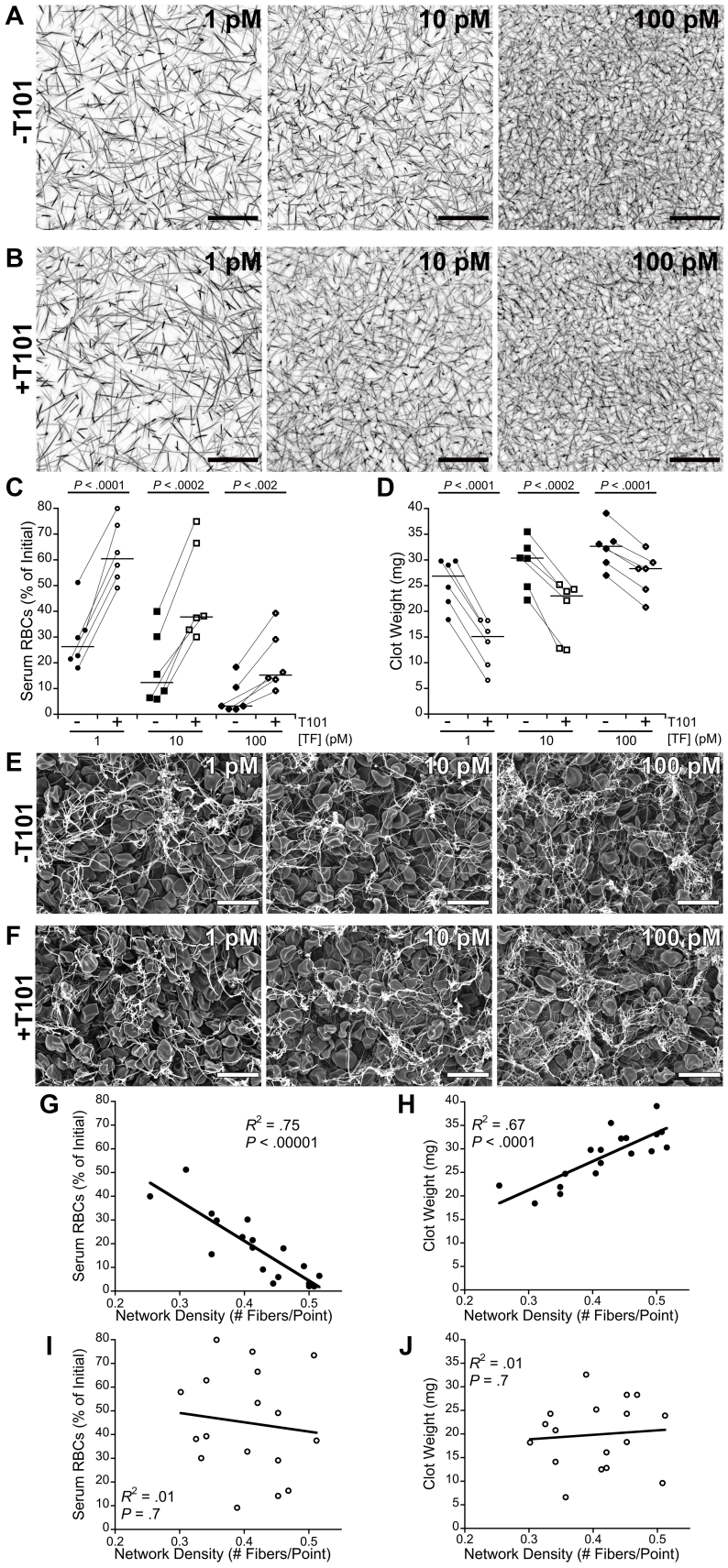


Figure 2.5. FXIIIa mediates RBC retention independent of fibrin network density. (A-B) Recalcified (10 mM, final) plasma spiked with Alexa Fluor® 647-labeled fibrinogen was clotted with the indicated TF concentrations in the absence (A) or presence (B) of T101 (200 μM, final). Images are representative confocal micrographs (z-projections of 30 individual slices) of clots visualized on a Zeiss LSM710 laser scanning confocal microscope with a 63X oil-immersion lens (Carl Zeiss, Thornwood, NY). Scale bar is 30 μm. (C) Serum RBC content and (D) clot weight following clot contraction. Each dot represents an individual clot. Lines connect clots formed from the same blood donor. Horizontal, dark lines indicate medians. (E-F) Representative scanning electron micrographs of clots formed in recalcified (10 mM, final) whole blood with the indicated TF concentrations in the absence (E) and presence (F) of T101 (10 μM, final). Clots were visualized at 5010X on a Zeiss Supra 25 Field Emission Scanning Electron Microscope (Carl Zeiss, Thornwood, NY). Scale bar is 10 μm. Micrographs were used to measure fibrin network density, which was compared to the serum RBC content (G, I) or clot weight (H, J) following clot contraction in the absence (G**,** H) and presence (I, J) of T101.

did not significantly alter fibrin network density (P>0.07) or fiber thickness (P>0.10) at any TF concentration tested (Figure 2.5E-F). In the presence of FXIIIa activity, final fibrin network density was strongly, negatively correlated with serum RBC content (Figure 2.5G) and strongly, positively correlated with final clot weight (Figure 2.5H). Interestingly, however, these correlations were lost in the presence of T101 (Figure 2.5I, J), suggesting substantial structural heterogeneity arises in the fibrin network during the extensive RBC loss and clot contraction that occur in the absence of FXIIIa. These data suggest FXIIIa does not promote RBC retention in clots simply by increasing fibrin network density, and show independent effects of FXIIIa on RBC retention in contracted clots.

FXIIIa inhibition reduces RBC retention in clots lacking γ-, but not α-chain, crosslinking. Finally, we tested the hypothesis that FXIIIa promotes RBC retention in clots via its ability to crosslink fibrin. FXIIIa crosslinks residues between γ- and α-chains within fibrin fibers, and studies have revealed distinct effects of each type of crosslinking on the fibrin network.[14-17,36] Therefore, we specifically interrogated the individual contributions of γ- and α-chain crosslinks to RBC retention.

We first performed clot contraction assays using recombinant fibrinogens mutated to eliminate either γ- or α-chain crosslinking (Supplemental Figure 2.2). To test the role of γ-chain crosslinking, we used a fibrinogen variant (γQ398N/Q399N/K406R, γNNR[15]) that lacks the three γ-chain crosslinking residues. To test the role of α-chain crosslinking, we used fibrinogen with an Aα-chain truncation at residue 251 (Aα251[28]), which eliminates most of the α-chain crosslinking residues. We reconstituted fibrinogen-deficient plasma with RBCs, platelets, and either normal (wild-type) recombinant fibrinogen (γA/γA), γNNR, or Aα251 in the absence and

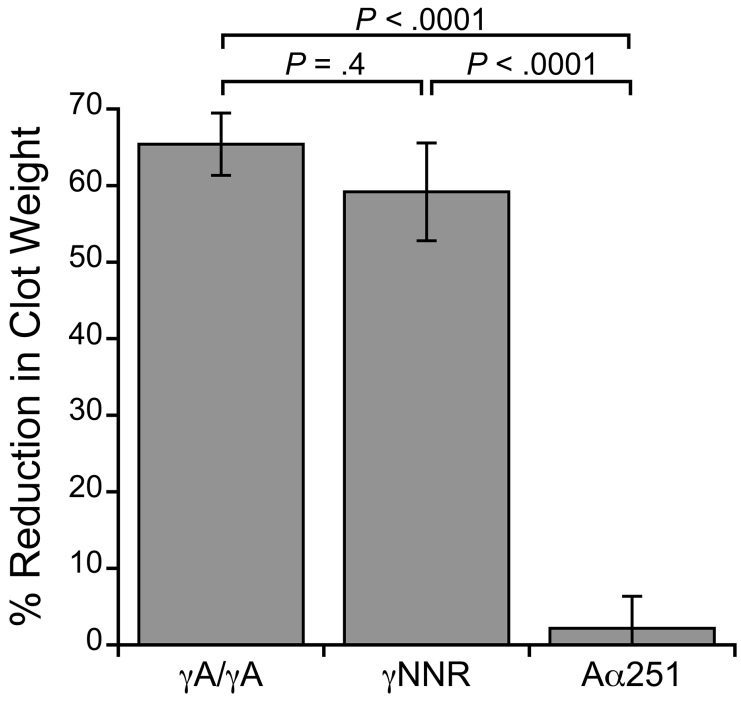


Figure 2.6. FXIIIa inhibition does not reduce RBC retention in clots formed with Aα251 fibrinogen. Percent reduction in clot weight of contracted clots formed from TF-treated, recalcified fibrinogen-deficient plasma reconstituted with RBCs and platelets (2 million/μL and 200,000/μL, respectively), and γA/γA, γNNR, or Aα251 fibrinogen (0.25 mg/mL, final), in the absence or presence of 10 μM (final) T101 (N=3-7 per fibrinogen). Bars are means ± SE.

presence of T101. Figure 2.6 shows the percent reduction in clot weight caused by the presence of T101 for each variant. FXIIIa inhibition significantly reduced the size of clots formed with γA/γA fibrinogen (3.3±0.5 versus 1.2±0.3 mg, respectively, $P<0.001$) and γNNR (3.7±0.5 versus 1.6±0.4 mg, respectively, $P<0.01$). These data indicate γ-chain crosslinking is not required for RBC retention in contracted clots. In contrast, inhibiting FXIIIa in clots formed with Aα251 did not reduce clot size (7.9±0.6 versus 7.7±0.6 mg, respectively, $P=0.3$). Since clots formed from Aα251 were larger than those formed from γA/γA and γNNR fibrinogens, likely due to the abnormal fibrin network structure previously reported for this variant[36], we also measured RBC retention in clots formed from lower concentrations of Aα251 fibrinogen (0.125 mg/mL, final). While reducing fibrin concentration reduced clot weight, these clots still failed to show an effect of FXIIIa inhibition on clot weight (data not shown).

To further test the contributions of γ- and α-chain crosslinking in RBC retention, we exploited previous observations that low concentrations of FXIIIa inhibitors selectively inhibit formation of HMW α-chain-rich crosslinked species without reducing γ-γ dimer formation.[14,37] We generated plasma clots in the presence of a range of T101 concentrations (0-200 μM), dissolved the clots, and probed for fibrin crosslinking via Western blotting (Figure 2.7A). Consistent with the previous studies[14,37], T101 inhibited HMW species formation at ~40-fold lower concentrations than it inhibited γ-γ dimer formation (IC_{50} = 0.52±0.12 *versus* 21.1±5.8 μM, respectively, $P<0.03$, Figure 2.7B). Importantly, when we superimposed the effects of T101 on fibrin crosslinking, clot weight, and RBC retention, we found that T101 reduced clot weight (IC_{50} = 0.60±0.09 μM, Figure 2.7B) and RBC retention (not shown) at the same concentrations at which it inhibited HMW species formation (Figure 2.7B). Together, these data show FXIIIa promotes RBC retention in clots by crosslinking fibrin α-chains.

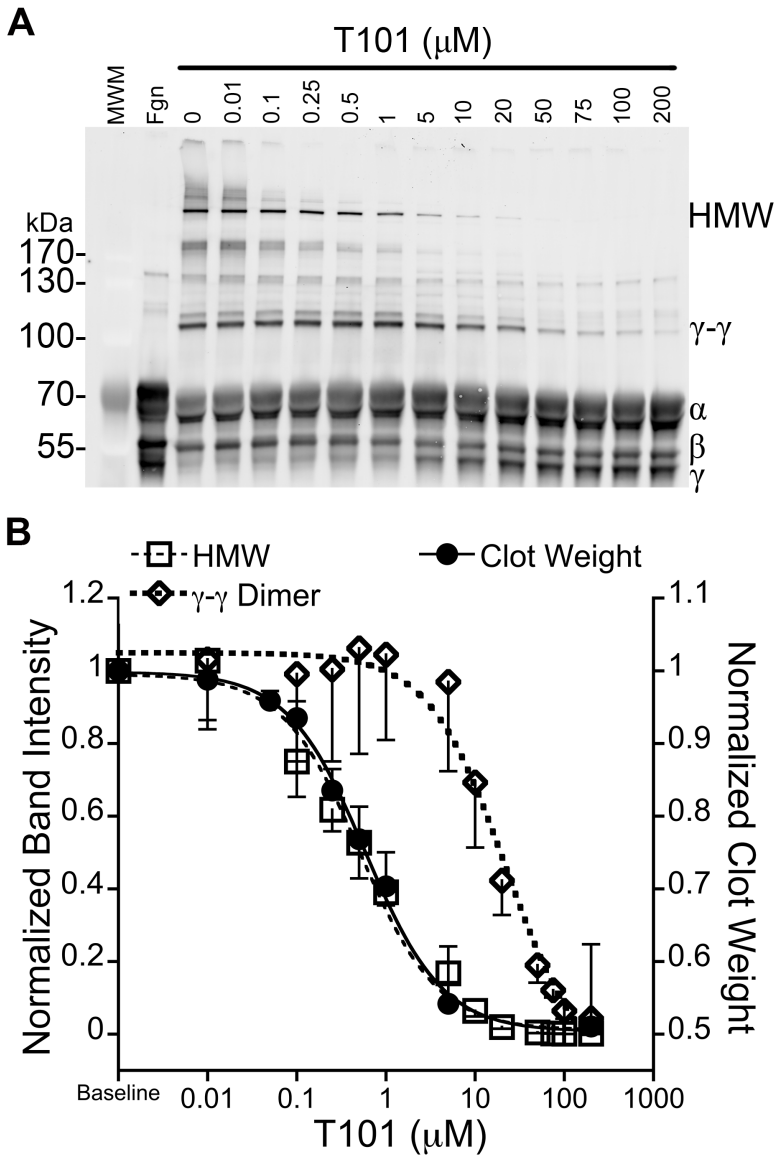


Figure 2.7. RBC retention is reduced at concentrations of T101 that inhibit α-chain crosslinking. (A) Recalcified (10 mM, final) plasma was clotted with TF (1 pM) in the presence of increasing concentrations of T101. Clots were dissolved and analyzed by Western blotting with polyclonal anti-human fibrinogen antibody. (B) Normalized intensity of γ-γ dimer (open diamonds, short dashed line, N=3) or HMW bands (open squares, long dashed line, N=3) superimposed on normalized clot weight following clot contraction in the presence of T101 (closed circles, solid line, N=5-7). Data are means ± SE.

2.5 Discussion

The importance of FXIII activity in stabilizing clots is well-established. However, the recent recognition of FXIII's essential role in determining thrombus composition and size[18] raises important questions about its contributions to clot formation in vivo. Our study to determine the mechanism mediating RBC retention in clots reveals previously unrecognized functions for FXIIIa activity and fibrin crosslinking. We showed RBC retention is not mediated by direct crosslinking of RBCs to the clot, but is increased by increased fibrin network density in the presence, but not absence, of FXIIIa activity. This finding demonstrates an essential role of crosslinked fibrin in determining thrombus cellular content. Importantly, we found that FXIIIa crosslinking of fibrin α-chains promoted RBC retention in clots. Although previous studies have documented unique, independent contributions of α- and γ-chain crosslinking to fibrin biophysical characteristics[14-17,36], our findings are the first to associate these characteristics with a specific function during thrombus formation in vivo. The findings presented here define the mechanism by which FXIIIa mediates thrombus composition and expose a newly-recognized, essential (patho)physiologic function for fibrin crosslinking.

It has traditionally been thought that the fibrin network functions like a net that traps and retains RBCs flowing by the developing clot. Our findings support, but substantially refine, this concept. We observed that in a normally-crosslinked clot, RBC retention is strongly associated with fibrin network density. Fibrin network density is mediated by several factors, including the concentrations of fibrinogen and thrombin present during fibrin formation (reviewed in [38]). We and others have shown that plasma hypercoagulability results in the formation of clots with high fibrin network density.[39-44] It is tempting to speculate that these dense networks trap more RBCs, resulting in large, occlusive venous thrombi. Indeed, we previously observed that

hyperprothrombinemia leads to the formation of clots with increased network density[45] and that mice with elevated prothrombin produce significantly larger venous thrombi[39] that have higher RBC content (Aleman and Wolberg, unpublished observation). Together, these findings suggest an abnormally high density of crosslinked fibrin promotes venous occlusion by increasing RBC retention in the thrombus, and therefore, thrombus size.

The relationship between network density and RBC retention was lost in un-crosslinked clots. Since we and others have observed little-to-no effect of fibrin crosslinking on network density[8,9], increased RBC extrusion from un-crosslinked clots is unlikely to be due to abnormal network density. Rather, these data support the premise that FXIIIa's effects on clot biophysical properties mediate RBC retention in the clots. Previous studies have demonstrated distinct contributions of γ- and α-chain crosslinking to fibrin viscoelastic properties. Using a synthetic inhibitor and patient-derived anti-FXIII antibody, Ryan et al. showed that the FXIIIa-mediated increase in clot stiffness correlates with the generation of α-chain-rich HMW species and not with γ-γ dimers.[14] Furthermore, only partial α-chain crosslinking was required to reach near-maximal clot stiffness[14], consistent with our observation that even partial crosslinking of α-monomers increased RBC retention. More recently, Collet et al. used recombinant Aα251 fibrinogen to show that clots with only γ-chain crosslinking are nearly 4-times less stiff than γ- and α-chain crosslinked clots.[36] Similarly, studies using γNNR fibrin (only α-chain crosslinking) showed the majority of the stiffness of fully-crosslinked clots is provided by α-chain crosslinking.[15-17] Consequently, our observation that α-chain crosslinking mediates FXIIIa-mediated RBC retention in clots identifies a potential (patho)physiologic consequence of α-chain crosslinking and fibrin fiber stiffening during clot formation in vivo.

The observation that γ- and α-chain crosslinking is inhibited by different concentrations of inhibitor[14,37] may suggest a conformational change in FXIIIa facilitates γ- versus α-chain crosslinking, and that the α-chain-specific conformation is more susceptible to inhibition than the γ-specific conformation. Alternately, the spatial relationship between FXIIIa and the γ- and α-chains during FXIII activation may contribute to differential timing and inhibition of γ- and α-chain crosslinking. We recently localized FXIII zymogen binding to the homologous murine γ-chain residues 390-396[18], which would conveniently position activated FXIIIa near the γ-chain crosslinking sites (γ398/399/406). Thus, the rate of γ-chain crosslinking may exceed α-chain crosslinking due to the proximity of FXIII(a) to the γ-chain after activation. Whether these differences can be exploited to evaluate γ- versus α-chain crosslinking in vivo remains to be determined.

Although previous studies demonstrated specific interactions between fibrin and RBCs[29-31], and implicate CD47[46] and a β3-like molecule[47] on the RBC surface, we were unable to detect any FXIIIa substrates on the RBC surface. We were also unable to detect differences in retention of young *versus* old RBCs in clots (data not shown), indicating age-dependent changes in RBC protein expression or morphology[48] do not influence their presence in clots. These data suggest FXIIIa does not covalently ligate RBCs to the clot. This finding is somewhat surprising, since a recent report suggested FXIIIa is highly promiscuous, with over 147 potential substrates in plasma.[49] However, our findings are consistent with a previous study that also did not identify any transglutaminase substrates on the RBC surface.[32] Together, these results indicate FXIIIa retains considerable substrate specificity during coagulation in whole blood.

The consequences of RBC retention in clots is an area of active investigation. Clot contraction induces the appearance of compacted RBCs (so-called "polyhedrocytes").[50] This

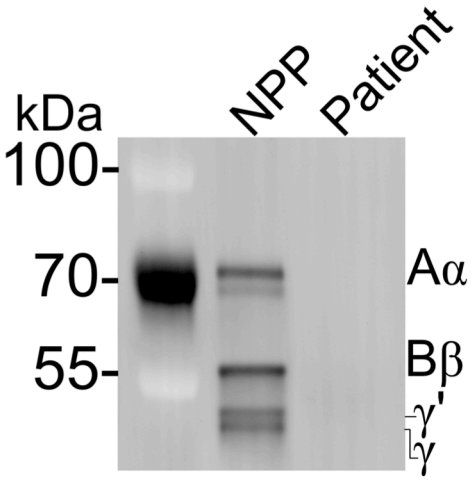
observation demonstrates substantial platelet-mediated forces are transmitted through the clot during contraction[51,52] and suggests the fibrin network must be sufficiently dense and stiff to retain RBCs during this process. The contribution of crosslinking to fibrin elasticity, extensibility, and stiffness occurs at both whole-clot and single-fiber scales.[10-14] In addition to clot contraction, shear stress from blood flow in vivo also exerts considerable force onto the fibrin network. Thus, in the absence of FXIIIa-mediated crosslinking, fibrin may deform or even break, permitting RBCs to escape. Further studies examining the contributions of crosslinked fibrin, as well as platelet contractile force and RBC deformability, to clot formation are needed to fully define the how α-chain crosslinking mediates RBC retention in clots.

This study has potential limitations. First, although we did not identify FXIIIa substrates on the RBC surface, our lower limit of detection was ~300 molecules/cell. However, it would be surprising if a protein present at lower copy number could effectively promote RBC retention within the clot. Second, fibrin crosslinking could generate a neoepitope that promotes RBC binding and retention; however, we did not observe substantial differences in RBC binding to fibrin in the absence and presence of FXIIIa. Third, Aα251 exhibits slightly delayed crosslinking[36], which may have reduced the effect of FXIIIa. However, results from experiments with recombinant fibrinogens were consistent with findings using full-length fibrinogen in plasma, and support the conclusion that α-chain crosslinking mediates RBC retention. Finally, our experiments were performed under static conditions. Although stasis recapitulates aspects of VT[53], other mechanisms may mediate RBC content in clots formed under higher (arterial) shear.

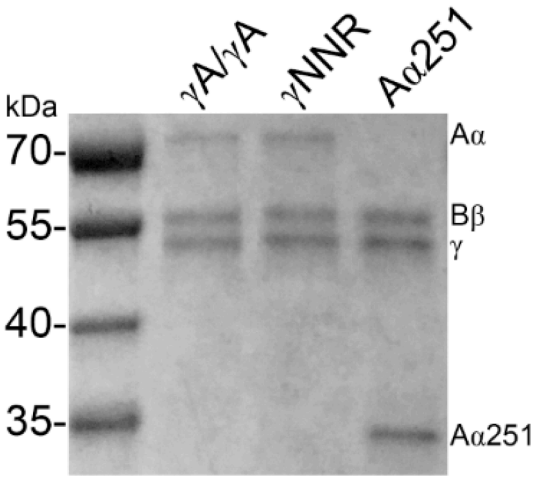
In summary, our results show FXIIIa promotes RBC retention in clots by crosslinking fibrin. Formation of α-chain-rich, HMW crosslinked fibrin species correlated strongly with RBC retention in clots, suggesting biophysical strengthening of the fibrin network secondary to α-

chain crosslinking determines clot composition. Overall, these findings refine our understanding of the FXIII-fibrinogen axis and establish α-chain crosslinking as a potential therapeutic target for reducing VT.

2.6. Supplemental figures.



Supplemental Figure 2.1. Plasma from the fibrinogen-deficient patient lacks all three fibrinogen chains. Representative Western blot of fibrinogen content in normal pooled plasma (NPP) and fibrinogen-deficient patient plasma. Fibrinogen chains were visualized using a polyclonal anti-human fibrinogen antibody.



Supplemental Figure 2.2. Recombinant γA/γA, γNNR, and Aα251 fibrinogen preparations are pure and contain intact Aα-, Bβ-, and γ-chains. Recombinant fibrinogens were expressed and purified as described.[1-3] Samples were boiled in 6X Laemmli sample buffer containing sodium dodecyl sulfate and β-mercaptoethanol, separated by SDS-PAGE, and stained with Coomassie brilliant blue. The α-, β- and γ-chains migrate as expected.

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CHAPTER 3: THE INTERACTION BETWEEN FIBRINOGEN AND ZYMOGEN FXIII-A_2B_2 IS MEDIATED BY FIBRINOGEN RESIDUES γ390-396 AND THE FXIII-B SUBUNITS[2]

3.1 Overview

The coagulation transglutaminase factor XIII (FXIII) exists in circulation as the heterotetrameric proenzyme FXIII-A_2B_2. Effectively all FXIII-A_2B_2 circulates bound to fibrinogen, and excess FXIII-B_2 circulates in plasma. The motifs that mediate interaction of FXIII-A_2B_2 with fibrinogen have been elusive. We recently detected reduced binding of FXIII-A_2B_2 to murine fibrinogen that has γ-chain residues 390-396 mutated to alanines (Fibγ$^{390\text{-}396A}$). Here, we evaluated binding features using human components, including recombinant fibrinogen variants, FXIII-A_2B_2, and isolated FXIII-A_2 and -B_2 homodimers. FXIII-A_2B_2 co-precipitated with wild-type (γA/γA), alternatively-spliced (γ′/γ′), and αC-truncated (Aα251) fibrinogens, whereas co-precipitation with human Fibγ$^{390\text{-}396A}$ was reduced by 75% (P<0.0001). Surface plasmon resonance showed γA/γA, γ′/γ′, and Aα251 fibrinogens bound FXIII-A_2B_2 with high affinity (nM); however, Fibγ$^{390\text{-}396A}$ did not bind FXIII-A_2B_2. These data indicate fibrinogen residues γ390-396 comprise the major binding motif for FXIII-A_2B_2. Compared to γA/γA clots, FXIII-A_2B_2 activation peptide release was 2.7-fold slower in Fibγ$^{390\text{-}396A}$ clots (P<0.02). Conversely, activation of recombinant FXIII-A_2 (lacking FXIII-B_2) was similar in γA/γA and Fibγ$^{390\text{-}396A}$ clots, suggesting fibrinogen residues γ390-396 accelerate FXIII-A_2B_2 activation in a

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FXIII-B_2-dependent mechanism. Recombinant FXIII-B_2 bound γA/γA, γ′/γ′, and Aα251 with similar affinities as FXIII-A_2B_2, but did not bind or co-precipitate with Fibγ$^{390\text{-}396A}$. FXIII-B_2 also co-precipitated with fibrinogen from FXIII-A-deficient mouse and human plasmas. Collectively, these data indicate that FXIII-A_2B_2 binds fibrinogen residues γ390-396 via the B subunits, and that excess plasma FXIII-B_2 is not free, but rather, circulates bound to fibrinogen. These findings provide insight into assembly of the fibrinogen/FXIII-A_2B_2 complex in both physiologic and therapeutic situations.

3.2 Introduction

Factor XIII (FXIII) is a plasma protransglutaminase that circulates at 14-28 μg/mL (43-86 nM, reviewed in [1]). Zymogen FXIII is composed of two A subunits (FXIII-A_2) and two carrier B subunits (FXIII-B_2) assembled as a non-covalent heterotetramer (FXIII-A_2B_2). In plasma, FXIII-A_2 is tightly-associated (K_D ~100 pM)[2] with FXIII-B_2. Excess FXIII-B_2 (43-62 nM) is present in circulation.[2,3] During coagulation, FXIII-A_2B_2 is activated by thrombin-mediated cleavage of an N-terminal, 37-amino acid activation peptide from the FXIII-A subunits (FXIII-A_2′). After activation peptide release, calcium promotes dissociation of the inhibitory FXIII-B subunits, yielding fully activated FXIII-A_2* (FXIIIa). Once activated, FXIIIa catalyzes the formation of ε-N-(γ-glutamyl)-lysyl crosslinks between γ- and α-chains of fibrin and between fibrin and other plasma proteins. Crosslinking is essential for clot mechanical and biochemical stability (reviewed in [1]). Fibrin α-chain crosslinking also promotes red blood cell retention in venous thrombi and consequently, mediates thrombus composition and size.[4,5]

FXIII-A_2B_2 circulates in complex with fibrinogen (K_D ~10 nM)[6], and these proteins are readily co-precipitated from plasma.[7] However, the fibrinogen residues that mediate binding to FXIII-A_2B_2 in humans have not been defined. Early studies suggested the alternatively-spliced

fibrinogen γ′-chain contained the FXIII-A_2B_2 binding site.[8,9] However, studies using recombinant fibrinogen showed that FXIII-A_2B_2 binds to γ- and γ′-containing fibrinogen with similar affinity[10], suggesting the γ′-extension is not necessary for FXIII-A_2B_2 binding. More recently, Smith et al. observed high affinity binding of FXIII-A_2B_2 to a glutathione-S-transferase-fused peptide containing amino acid residues 371-425 of the fibrinogen αC domain.[11] However, whether the fibrinogen αC domain fulfills the carrier function of FXIII-A_2B_2 remains unclear.

We recently observed decreased co-precipitation of FXIII-A_2B_2 with murine fibrinogen that has alanine substitutions within residues γ390-396 (NRLSIGE to AAAAAAA, Fib$\gamma^{390\text{-}396A}$), suggesting these γ-chain residues mediate the FXIII-A_2B_2 carrier function in mice.[4] Accordingly, Souri et al. subsequently detected binding of FXIII-A_2B_2 to the human fibrinogen γ-chain at residues C-terminal of γLys356.[12] Notably, fibrinogen residues γ390-396 are highly conserved in mammals (NRLTIGE [human, gorilla, dog], NRLSIGD [rat], and NRLAIGE [giant panda]), suggesting these residues fulfill this function across species.

Herein, we used entirely human components, including recombinant human fibrinogen variants and human FXIII heterotetramers and homodimers, as well as FXIII-deficient mice, to define the interaction between these proteins. Our data reveal a direct interaction between human fibrinogen residues γ390-396 and the FXIII-B subunits, and uncover a fundamental mechanism mediating FXIII-fibrinogen complex assembly in blood. These data have important implications for both physiologic assembly of this complex in healthy individuals, and assembly in FXIII-A_2-deficient patients receiving therapeutic recombinant FXIII-A_2.

3.3 Methods

Proteins and Materials. Anti-human fibrinogen antibody was from Dako (Carpinteria, CA) and AlexaFluor 488 anti-rabbit and anti-sheep secondary antibodies were from Jackson

Immunoresearch (West Grove, PA). Two rabbit polyclonal anti-FXIII-B antibodies were used: HPA003827 (Sigma Aldrich, St. Louis, MO) and A074 (Zedira, Darmstadt, Germany), as indicated. Plasma FXIII-A_2B_2, anti-human FXIII-A antibody, and peak 1 human fibrinogen (FXIII-depleted) were from Enzyme Research Laboratories (ERL, South Bend, IN). Recombinant FXIII-A_2 (rFXIII-A_2) was a generous gift of Novo Nordisk (Bagsværd, Denmark). FXIII-A_2B_2 (plasma-derived) used for surface plasmon resonance and recombinant FXIII-B_2 (rFXIII-B_2, produced in insect cells) were from Zedira. Insect cell-derived FXIII-B_2 undergoes different post-translational modification than human plasma-derived FXIII-B_2 and migrates slightly faster on SDS-PAGE. *Taq* polymerase was from New England Biolabs (Ipswich, MA). Dulbecco's modification of Eagle's medium/Ham's F12, 50/50 mix was from Corning (Manassas, VA). Glycine was from Sigma (St. Louis, MO). Polyvinylidene fluoride membranes were from Millipore (Billerica, MA), 6X SDS loading dye was from Boston Bioproducts (Ashland, MA), and 10% Tris-glycine gels were from Bio-Rad (Hercules, CA). Murine studies were approved by the Institutional Animal Care and Use Committees of the Cincinnati Children's Hospital Medical Center and the University of North Carolina at Chapel Hill.

Expression of recombinant fibrinogen variants. To generate recombinant Fib$\gamma^{390\text{-}396A}$, the fibrinogen γ-chain expression vector pMLP-$\gamma^{13,14}$ was used as a template to generate two overlapping fragments of cDNA corresponding to the C-terminal region of the γ-chain containing γ390-396A. The 5′ fragment was amplified using a forward primer containing a BstXI site (5′-GCTGGCCATCTCAATGGAGT-3′) and a reverse mutagenesis primer (5′-TTGCTGTCCAGCTGCAGCCGCAGCTGCGGCGAATGGGAT-3′). The 3′ fragment was amplified using a forward mutagenesis primer (5′-

ATCCCATTCGCCGCAGCTGCGGCTGCAGCTGGACAGCAA-3′) and a reverse primer containing a NotI site (5′-GGGGCGGCCGCCCATTATAT-3′). These two fragments were gel-purified, thermally-denatured, and reannealed. The resulting hybrid fragment was extended and amplified using the BstXI and NotI primers before digestion with BstXI and NotI. This digested fragment was then ligated into BstXI- and NotI-digested pMLP-γ with T4 DNA Ligase (Thermo Fisher, Waltham, MA). Competent DH5α *Escherichia coli* were transformed with the ligated DNA and the γ390-396A mutation was confirmed by Sanger sequencing (Eton Bioscience, Research Triangle Park, NC). The pMLP-γ390-396A expression vector was then transfected into Chinese Hamster Ovary cells containing expression vectors for normal fibrinogen Aα- and Bβ-chains, as previously described.[14] Positive clones were identified by dot blotting with anti-human fibrinogen antibody. Recombinant human wild-type (γA/γA), alternatively-spliced (γ′/γ′), αC-truncated (Aα251), and Fibγ$^{390\text{-}396A}$ (Fibγ$^{390\text{-}396A}$) fibrinogen variants were then expressed in Chinese Hamster Ovary cells and affinity-purified as previously described.[14-16]

Fibrinogen precipitation experiments. Fibrinogen (1 mg/mL [2.9 μM], final) was incubated with FXIII-A_2B_2 (20 μg/mL [60 nM], final), rFXIII-A_2 (10 μg/mL [60 nM], final), or rFXIII-B_2 (10 μg/mL [63 nM], final) at room temperature for 15 minutes. Glycine (165 mg/mL) was then added and samples were rotated for 1 hour, after which the precipitate was pelleted by centrifugation (7000x*g*, 15 minutes) and resuspended in HEPES-buffered saline (HBS; 20 mM HEPES [pH 7.4], 150 mM NaCl). Fibrinogen, FXIII-A, and FXIII-B content of the initial sample, pellet, and supernatant were assessed by Western blotting under reducing conditions, unless otherwise specified. Briefly, samples were separated by SDS-PAGE on 10% Tris-Glycine gels, transferred to polyvinylidene fluoride membranes, and probed with primary antibodies

(rabbit anti-human fibrinogen [1:7000], sheep anti-human FXIII-A [1:1000], or rabbit anti-human FXIII-B [HPA003827, 1:500]) overnight at 4°C before incubation with fluorescently-labeled secondary antibodies for 1 hour at room temperature. Blots were visualized using a Typhoon FLA9000 Imager (GE Healthcare, Little Chalfont, UK). Densitometry was performed with ImageJ 1.48v. Percentage of FXIII-A or FXIII-B in the pellet was determined by dividing the intensity of the subunit band in the pellet by the sum of the band intensities in both the pellet and the supernatant.

For experiments with mouse plasma, blood was drawn via the inferior vena cava and processed to platelet-poor plasma by centrifugation (5000x*g*, 10 minutes). Fibrinogen was isolated from wild-type (*F13a*$^{+/+}$), heterozygous (*F13a*$^{+/-}$), FXIII-deficient (*F13a*$^{-/-}$)[4,17], or afibrinogenemic[18] mouse plasma by glycine precipitation[4]. For experiments with immunodepleted human plasma (Affinity Biologicals, Ancaster, ON, lacks both FXIII-A and FXIII-B), plasma was first reconstituted with 10 μg/mL rFXIII-B_2 and diluted three-fold prior to glycine precipitation. Precipitated fibrinogen and FXIII-A were detected as above. Both murine and human FXIII-B were detected using anti-human FXIII-B antibody (A074, 1:1000 overnight at room temperature).

Surface plasmon resonance (SPR) using Taylor dispersion injections. SPR was performed using a OneStep® titration function based on Taylor dispersion injection (TDi) theory.[19,20] Compared to traditional (fixed concentration) injections that record analyte binding with respect to injection time of a series of samples of different concentrations, TDi analyzes a continuous analyte concentration gradient formed after a single injection in a capillary tube before the sample enters the SPR detector. This approach encodes a second, independent time

domain into an analyte gradient, which permits the use of a single binding curve that does not need to reach steady-state to obtain binding affinities. Thus, compared to fixed concentration injections, OneStep® titration reduces the time required to analyze a given analyte, lowers the dependence of parameters on one another and on experimental variability, increases resolving power, and enables analysis of interactions from a fewer number of injections. This approach was particularly important for analyzing high concentrations of limited amounts of recombinant fibrinogen variants.

Preparation of SPR analytes. FXIII ligands and fibrinogen analytes were dialyzed into running buffer at 4°C for 16 hours with rotation and one buffer change. Dialysis was performed in a volume 10,000-times that of the ligand volume using a Slide-A-Lyzer® MINI dialysis unit (molecular weight cutoff 7,000 Daltons, Life Technologies, Grand Island, NY). Protein concentration was determined using a Nanodrop1000 (Thermo Fisher, Waltham, MA).

A COOH-V chip (SensiQ Technologies, Oklahoma City, OK) was installed into a SensiQ Pioneer platform per the manufacturer's instructions and the *prime* function was performed three times using filter-sterilized, degassed running buffer (10 mM HEPES, 140 mM NaCl, 0.05% Tween-20, pH 7.4). The chip surface was preconditioned for adsorption by injecting 2 bursts each of 10 mM HCl, 50 mM NaOH and 0.1% SDS (100 µL/min, 10 seconds). The chip was primed a further 3 times using running buffer. All chips were checked to ensure they were aligned for each flow channel; if they were not well aligned a *normalize* function was performed using 100% DMSO, followed by 3 *prime* steps. The chip surface was then activated by injecting a 50:50 solution of EDC/NHS (25 µL/min, 4 minutes), resulting in o-acylisourea active ester groups. The dialyzed FXIII ligands were flowed across the activated chip surface at 5 µL/min

flow rate at minimum concentration of 50 µg/mL, diluted into 10 mM sodium acetate with a pH one order of magnitude lower than the pI of the protein. The target ligands were allowed to couple to the active esters via primary amine groups until an R_{MAX} of at least 50 was reached according to Equation 1. Any uncoupled ester groups were blocked by injecting ethanolamine (20 µL/minute, 5 minutes). Each protein target was immobilized on either flow-channel (FC) 1 or 3, and FC2 was always used as a reference channel.

Equation 1.

$$R_{MAX} = \frac{M_w\ Analyte}{M_w\ Ligand} \times R_L \times S_M$$

Where:
R_{MAX} = The maximum binding capacity assigned between the immobilized ligand and the analyte, in resonance units (RU)
M_w Analyte = Molecular mass of the molecule in solution in Daltons
M_w Ligand = Molecular mass of the immobilized ligand in Daltons
R_L = Immobilized ligand, in RU
S_M = Predicted molecular stoichiometry of analyte to ligand

Prior to analyte injections, the chip matrix was conditioned for regeneration using 3 bursts each of 1, 2, and 3 M NaCl (30 µL/min for 2 minutes), interspersed with 3 bursts of running buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 1.5 mM $CaCl_2$) at the same rate. Salt was washed from the chip using 3 bursts of running buffer (30 µL/min for 2 minutes), and the loops were purged 3 times. All kinetic binding assays were performed with both the analysis and sample rack temperatures at 20°C. Three *prime* functions were performed prior to analysis using running buffer.

SPR of FXIII-fibrinogen interactions. Fibrinogen analytes were diluted to 50 nM or 1 μM using the same batch of running buffer for blanks and the SensiQ Pioneer OneStep® titration function. Fibrinogen analytes were injected into the sensor chamber at a flow-rate of 30 μL/min using the OneStep® titration function[19,20] with a loop-inject of 75% following 5 leadoff blanks and 3 bulk standard injections of 3% sucrose in running buffer. The 50 nM and 1 μM samples had dissociation times of 500 and 1000 seconds, respectively. The chip surface was regenerated with 2 M NaCl (30 μL/min, 60 seconds), followed by 3 M NaCl (30 μL/min, 60 seconds).

Data were analyzed with Qdat data analysis software (SensiQ Technologies Inc., Oklahoma City, OK). Binding data were fit using a simple k_a/k_d model and aggregation/retention parameters adjusted per binding curve according to goodness of fit and curve type. Sensorgrams from experiments with 50 nM fibrinogen analytes (maximum) recognized one binding site and were fit using a one-site model. Sensorgrams with 1 μM fibrinogen analyte (maximum) identified two binding sites for some FXIII ligands, so these data were fit using a two-site binding model; however, given the plasma concentrations of FXIII-A_2B_2 and fibrinogen, affinities for the second binding sites (0.7-21 μM) were considered too weak to be physiologically-relevant and are not reported.

FXIII activation and fibrin crosslinking. FXIII-A_2B_2 (20 μg/mL [60 nM], final) or rFXIII-A_2 (10 μg/mL [60 nM], final) were incubated with fibrinogen (0.15 mg/mL [440 nM], final) at room temperature for 15 minutes. Reactions were triggered with thrombin (2 nM, final) and calcium (10 mM, final). This low thrombin concentration enabled us to detect early FXIII activation and fibrin crosslinking. Reactions were quenched and clots dissolved with 50 mM dithiothreitol/12.5 mM EDTA in 8 M urea (60°C, 1 hour). Samples were boiled in SDS-

containing sample buffer and separated using SDS-PAGE on 10% Tris-glycine gels before transfer to polyvinylidene fluoride membranes. Membranes were probed with primary antibodies (sheep anti-human FXIII-A [1:1000] or rabbit anti-human fibrinogen [1:7000]; overnight, 4°C) before incubation with fluorescently-labeled secondary antibodies (1 hour, room temperature). Blots were visualized using a Typhoon FLA9000 Imager. Densitometry was performed with ImageJ 1.48v. FXIII activation was determined by dividing the intensity of the FXIII-A′ band by the sum of the FXIII-A and FXIII-A′ bands to obtain % of FXIII-A′. Crosslinking of fibrin γ-chains was determined as previously described.[12,21]

Statistics. Descriptive statistics (mean, standard deviation [SD], standard error of the mean [SE]) were calculated and Lilliefors test was used to assess normality. FXIII-A_2 and FXIII-B_2 co-precipitation with each fibrinogen variant was compared to γA/γA using ANOVA with Dunnett's post-hoc testing (Kaleidagraph, Synergy Software, v4.5). FXIII activation and fibrin crosslinking rates were compared using 2-tailed Student's *t*-tests for equal or unequal variances, as appropriate. $P<0.05$ was considered statistically significant.

3.4 Results

Fibrinogen residues γ390-396 mediate FXIII-A_2B_2 binding to soluble human fibrinogen. Previous studies have suggested FXIII-A_2B_2 binds to fibrinogen at the alternatively-spliced γ′-chain[8,9], residues in the αC domain[11], or residues in the γ-chain[4,12]. To compare FXIII-A_2B_2 binding to these regions of fibrinogen, we expressed and purified recombinant human fibrinogen proteins: wild-type (γA/γA), alternatively-spliced (γ′/γ′), αC-truncated (Aα251), and Fibγ$^{390\text{-}396A}$. As expected[14-16,22], each of these fibrinogen variants contained all three chains, polymerized normally, and were >95% clottable (Figure 3.1 and data not shown). We pre-incubated these

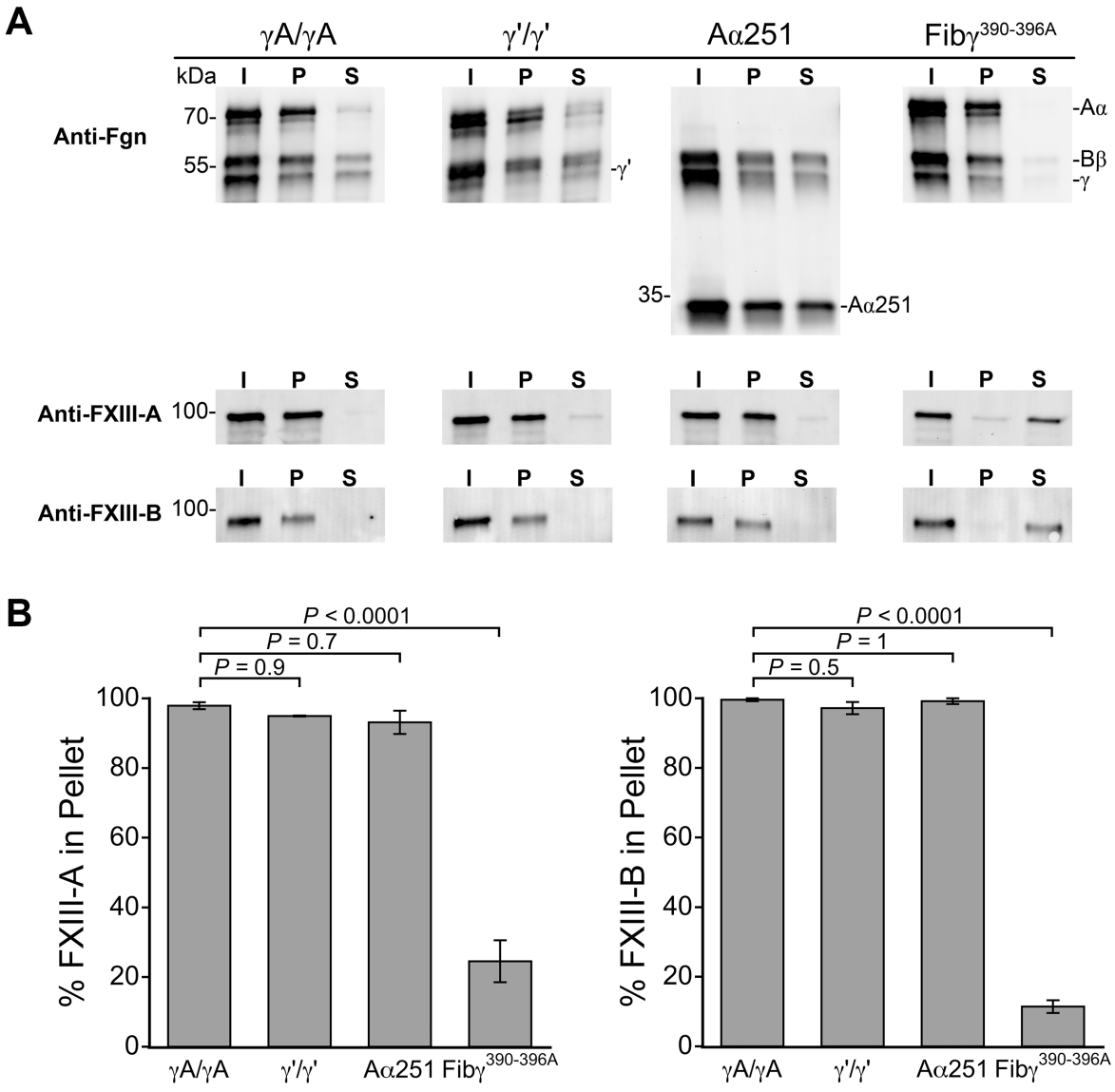


Figure 3.1. Fibrinogen residues γ390-396 are necessary for FXIII-A_2B_2 binding. Recombinant human fibrinogen variants (γA/γA, γ′/γ′, Aα251, and Fibγ$^{390\text{-}396A}$) were mixed with FXIII-A_2B_2 (1 mg/mL [2.9 μM] and 20 μg/mL [60 nM], final, respectively) and precipitated with glycine. (A) Representative Western blots for fibrinogen (Fgn), FXIII-A, and FXIII-B in the initial sample (I), pellet (P), or supernatant (S). Note that P and S samples were prepared after the addition of glycine and are therefore diluted relative to the I sample. (B) Quantitation of all blots, indicating percent of FXIII-A or FXIII-B in the pellet, relative to total FXIII in the pellet and supernatant. Bars are means ± SE, N=3.

fibrinogen variants with FXIII-A_2B_2, precipitated fibrinogen with glycine, and performed SDS-PAGE and Western blotting to identify FXIII-A_2B_2 present in the pellets and supernatant. FXIII-A_2B_2 co-precipitated with γA/γA, γ′/γ′, and Aα251 fibrinogen constructs (Figure 3.1). However, co-precipitation of FXIII-A_2B_2 with Fibγ$^{390\text{-}396A}$ was reduced by 75% (P<0.0001, Figure 3.1).

We also quantified binding of the fibrinogen variants to FXIII-A_2B_2 using SPR. Consistent with the co-precipitation data, γA/γA and γ′/γ′ fibrinogens bound FXIII-A_2B_2 with similar, high affinity (Table 3.1, Supplemental Figure 3.1). Compared to γA/γA and γ′/γ′ fibrinogens, Aα251 fibrinogen binding to adherent FXIII-A_2B_2 was slightly reduced, consistent with the finding that the αC domain contributes to FXIII-A_2B_2-binding function[11]. Regardless, given these affinity constants (Table 3.1), greater than 99% of circulating FXIII-A_2B_2 (plasma concentration ~43-86 nM[2,3]) would bind each of these variants. In contrast, Fibγ$^{390\text{-}396A}$ fibrinogen did not bind FXIII-A_2B_2 (Table 3.1, Supplemental Figure 3.1). Collectively, these data indicate the primary binding site for zymogen FXIII-A_2B_2 is present in γA/γA, γ′/γ′, and Aα251 fibrinogens, but absent in Fibγ$^{390\text{-}396A}$ fibrinogen. Together with previous findings[4], these results suggest the highly-conserved fibrinogen residues γ390-396 mediate binding of zymogen FXIII-A_2B_2 to fibrinogen in both mice and humans.

Human fibrin(ogen) residues γ390-396 mediate the ability of fibrin to accelerate FXIII-A_2B_2 activation. During fibrin formation, FXIII-A_2B_2 binding to the fibrin D:E:D/thrombin complex accelerates FXIII activation peptide cleavage and FXIII activation.[12,23-29] Using mice expressing murine Fibγ$^{390\text{-}396A}$ fibrinogen, we previously detected delayed FXIII activation and fibrin crosslinking in plasma.[4] To directly determine the contribution of fibrin(ogen) residues γ390-396 to these functions, we now assessed the ability of human Fibγ$^{390\text{-}396A}$ fibrin(ogen) to

Immobilized Ligand	Analyte	N	Equilibrium dissociation constant (K_D), nM
FXIII-A_2B_2	γA/γA	6	3.8 ± 2.4
	γ′/γ′	6	10.4 ± 11.1
	Aα251	3	71.0 ± 16.2
	Fibγ$^{390\text{-}396A}$	6	No Binding
rFXIII-B_2	γA/γA	4	0.4 ± 0.3
	γ′/γ′	6	53.0 ± 75.1
	Aα251	4	58.6 ± 26.5
	Fibγ$^{390\text{-}396A}$	6	No Binding

Table 3.1. SPR analysis of fibrinogen variant binding to immobilized FXIII-A_2B_2 and rFXIII-B_2. Equilibrium dissociation constant values are mean ± SD for the number of experiments indicated (N).

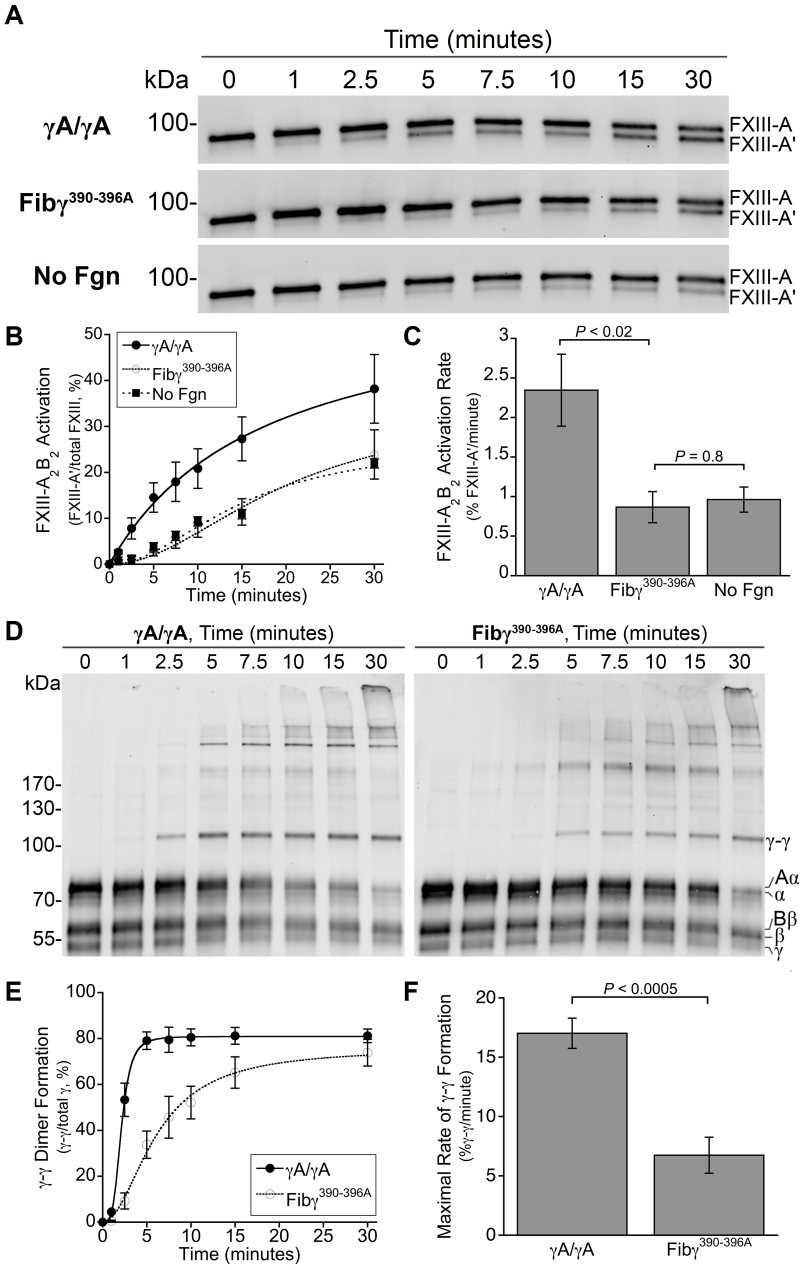


Figure 3.2. Fibrin(ogen) residues γ390-396 mediate the acceleratory effect of fibrin(ogen) on FXIII-A_2B_2 activation. FXIII-A_2B_2 (20 µg/mL [60 nM], final) was mixed with recombinant fibrinogens (γA/γA or Fibγ$^{390-396A}$, 150 µg/mL [440 nM], final) or buffer (No Fgn). Reactions were triggered by addition of thrombin (2 nM, final) and $CaCl_2$ (10 mM, final), quenched at the indicated time points, and analyzed by SDS-PAGE with Western blotting and densitometry. Activation peptide cleavage was detected using anti-FXIII-A antibody. Fibrin crosslinking was detected using anti-fibrinogen antibody. (A) Representative Western blots and (B) quantitation of FXIII-A_2B_2 activation over time from all blots. (C) Maximal rates of FXIII-A_2B_2 activation calculated from panel B. (D) Representative Western blots of fibrin crosslinking, and quantitation of (E) γ-γ dimer formation and (F) γ-γ dimer formation rate. Data are means ± SE, N=3-6 replicates per time point.

support FXIII-A_2B_2 activation in a purified system. Compared to reactions with human γA/γA fibrin(ogen), reactions with Fibγ$^{390\text{-}396A}$ fibrin(ogen) showed 2.7-fold slower release of the FXIII activation peptide (2.35±0.46% versus 0.87±0.20% FXIII-A′/minute, respectively, P<0.02, Figure 3.2A-C), and was similar to that observed in the absence of fibrinogen (0.96±0.16% FXIII-A′/minute, P=0.8, Figure 3.2A-C).

The rate of fibrin formation was similar for γA/γA and Fibγ$^{390\text{-}396A}$ (Figure 3.2D and data not shown). However, the rate of fibrin crosslinking was slower for Fibγ$^{390\text{-}396A}$. Specifically, γ-γ dimer formation was 2.5-fold slower (6.7±1.5 versus 17.0±1.3% γ-γ/minute, for Fibγ$^{390\text{-}396A}$ versus γA/γA, respectively, Figure 3.2D-F). The delays in both FXIII-A_2B_2 activation and crosslinking activity are consistent with a lack of binding of zymogen FXIII-A_2B_2 to Fibγ$^{390\text{-}396A}$ fibrinogen.

The ability of fibrin(ogen) residues γ390-396 to accelerate FXIII activation is FXIII-B subunit-dependent. Souri et al. previously suggested the acceleratory effect of fibrin(ogen) on FXIII-A_2B_2 activation is FXIII-B subunit-dependent.[12] We therefore also measured activation of FXIII-A_2 (rFXIII-A_2) in the absence of FXIII-B_2 and compared these rates in the presence of γA/γA and Fibγ$^{390\text{-}396A}$ fibrin(ogen). Compared to activation of FXIII-A_2B_2, activation of rFXIII-A_2 was slower (Figure 3.2A-C and Figure 3.3A-C, P<0.02), consistent with a critical role for the FXIII-B subunits in this reaction. Interestingly, however, in contrast to that seen with FXIII-A_2B_2, activation of rFXIII-A_2 was similar in the presence of γA/γA and Fibγ$^{390\text{-}396A}$ fibrin(ogen) (0.60±0.06 versus 0.46±0.06% FXIII-A′/minute, respectively, Figure 3.3A-C). Moreover, in the presence of rFXIII-A_2, the formation rate of γ-γ dimers was more similar for γA/γA and Fibγ$^{390\text{-}396A}$ (15.6±0.5 versus 12.6±0.5% γ-γ/minute, respectively, Figure 3.3D-F), relative to reactions in

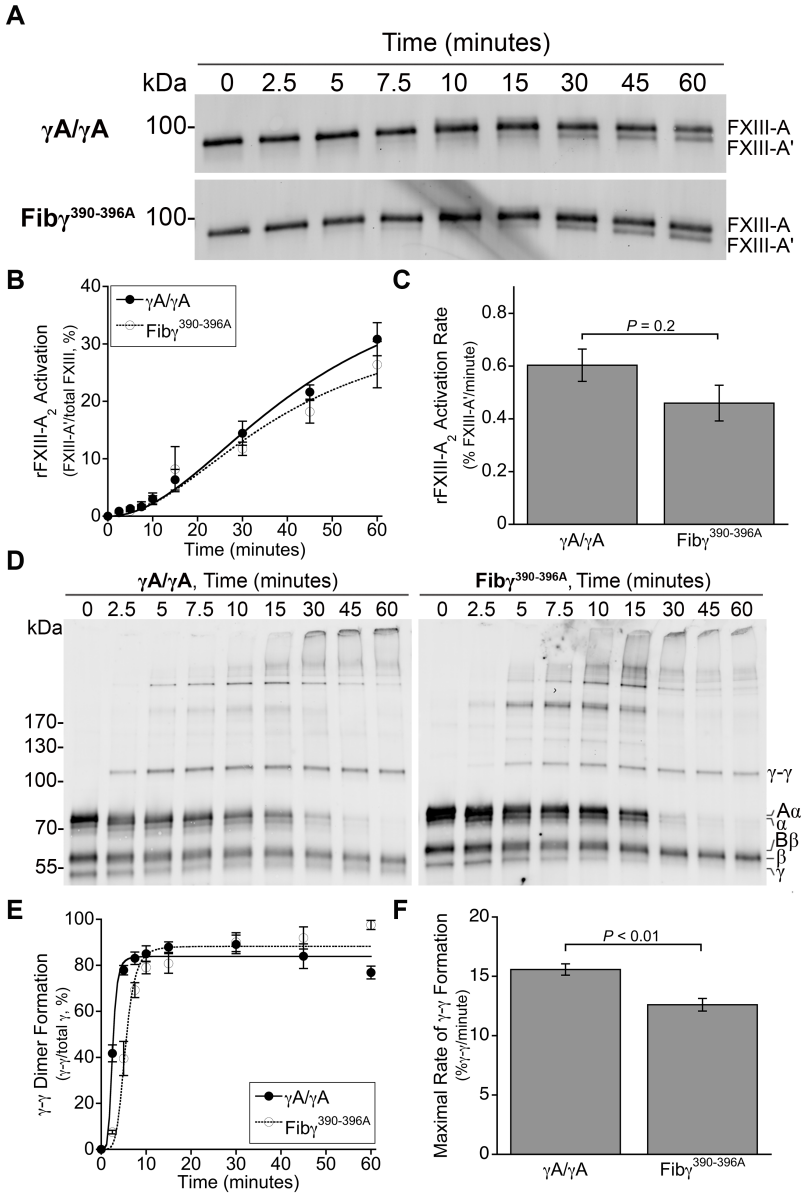


Figure 3.3. Fibrin(ogen) residues γ390-396 do not accelerate FXIII-A_2 activation. Recombinant FXIII-A_2 (10 μg/mL [60 nM], final) was mixed with γA/γA or Fibγ$^{390\text{-}396A}$ fibrinogen (150 μg/mL [440 nM], final). Reactions were triggered by addition of thrombin (2 nM, final) and $CaCl_2$ (10 mM, final), quenched at the indicated time points, and analyzed by SDS-PAGE with Western blotting and densitometry. Activation peptide cleavage was detected using anti-FXIII-A antibody. Fibrin crosslinking was detected using anti-fibrinogen antibody. (A) Representative Western blots and (B) quantitation of rFXIII-A_2 activation over time from all blots. (C) Maximal rates of rFXIII-A_2 activation were calculated from quantified Western blots. (D) Representative Western blots of fibrin crosslinking and quantification of (E) γ-γ dimer formation and (F) formation rate. Data are means ± SE, N=4 experiments.

the presence of FXIII-A_2B_2. These findings show Fib$\gamma^{390\text{-}396A}$ can be crosslinked, indicating the delayed crosslinking seen with FXIII-A_2B_2 (Figure 3.2D-F) was not due to a substantial disruption of structure in this region. Rather, these data attribute the delay seen with FXIII-A_2B_2 (Figure 3.2D-F) to decreased interaction between fibrin(ogen) residues γ390-396 and the FXIII-B subunit. Together, these data suggest fibrin(ogen) residues γ390-396 accelerate FXIII activation, and do so in a FXIII-B subunit-dependent mechanism.

The FXIII-B subunit binds fibrinogen residues γ390-396. We then directly tested the hypothesis that FXIII binding to fibrinogen residues γ390-396 is mediated by the B subunits using both precipitation and SPR assays. First, we precipitated recombinant γA/γA or Fib$\gamma^{390\text{-}396A}$ fibrinogen in the presence of rFXIII-A_2, rFXIII-B_2, or rFXIII-A_2 plus rFXIII-B_2. rFXIII-A_2 did not co-precipitate with either γA/γA or Fib$\gamma^{390\text{-}396A}$ fibrinogen (Figure 3.4A, B). However, rFXIII-B_2 readily co-precipitated with γA/γA fibrinogen (Figure 3.4A), but not Fib$\gamma^{390\text{-}396A}$ fibrinogen (Figure 3.4B). Addition of rFXIII-B_2 to rFXIII-A_2 rescued co-precipitation of rFXIII-A_2 with γA/γA fibrinogen (Figure 3.4A), likely through the formation of rFXIII-A_2B_2 heterotetramers. Conversely, rFXIII-B_2 did not rescue rFXIII-A_2 co-precipitation with Fib$\gamma^{390\text{-}396A}$ (Figure 3.4B). Experiments using purified, plasma-derived γA/γA (peak 1) fibrinogen fully recapitulated findings with recombinant γA/γA (data not shown).

Second, we examined binding of the fibrinogen variants to surface-bound rFXIII-B_2. These data revealed that γA/γA bound rFXIII-B_2 with similar affinity as seen with FXIII-A_2B_2 (Table 3.1, Supplemental Figure 3.2). γ′/γ′ and Aα251 fibrinogens also bound rFXIII-B_2 (Table 3.1, Supplemental Figure 3.2), indicating both that the binding motif for FXIII-B_2 is present on each of these fibrinogen molecules and that the presence of FXIII-A_2 does not enhance FXIII-B_2

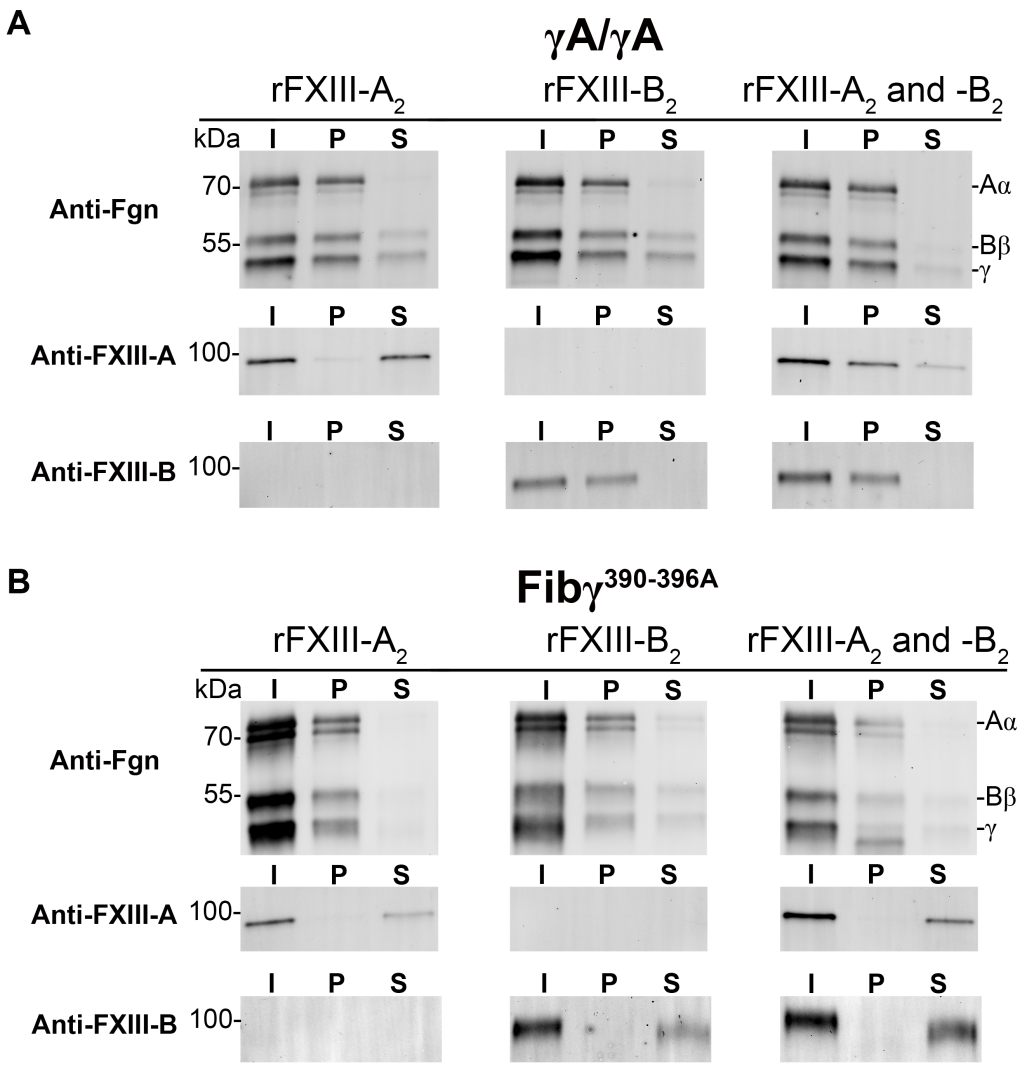


Figure 3.4. FXIII-A2B2 binds fibrinogen residues γ390-396 via the FXIII-B subunits. Recombinant FXIII-A2 (10 µg/mL [60 nM], final), rFXIII-B2 (10 µg/mL [62 nM], final), or both, were mixed with (A) γA/γA or (B) Fibγ390-396A fibrinogen (1 mg/mL [2.9 µM], final) and precipitated with glycine. (A-B) Representative Western blots for fibrinogen (Fgn), FXIII-A, and FXIII-B in the initial sample (I), pellet (P), or supernatant (S). Note that P and S samples were prepared after the addition of glycine and are therefore diluted relative to the I sample. Blots are representative of N=3 experiments.

binding to fibrinogen. These data also confirm that rFXIII-B_2 co-precipitation with fibrinogen in the previous experiments was not due to the presence of glycine. Notably, however, we were unable to detect binding of Fib$\gamma^{390\text{-}396A}$ fibrinogen to rFXIII-B_2 (Table 3.1, Supplemental Figure 3.2). Collectively, these co-precipitation and SPR data indicate that the FXIII-B subunit(s) of FXIII-A_2B_2 mediate binding to fibrinogen residues γ390-396.

Excess FXIII-B_2 in plasma circulates bound to fibrinogen. FXIII-B_2 is present in ~2-fold molar excess over FXIII-A_2 in plasma and is reported to circulate as free (unbound) FXIII-B_2 homodimer.[2,3] However, the co-precipitation and SPR data indicating FXIII-B_2 can bind fibrinogen in the absence of FXIII-A_2 (Figure 3.4A, Table 3.1) raise the interesting possibility that "free" FXIII-B_2 in plasma actually circulates bound to fibrinogen. Indeed, given the measured affinity of FXIII-B_2 to fibrinogen (0.4 nM, Table 3.1) and the estimated plasma concentration of "free" FXIII-B_2 (~43-62 nM)[2,3], greater than 99% of circulating FXIII-B_2 should be bound to fibrinogen. Therefore, to determine whether FXIII-B_2 circulates with fibrinogen in plasma, we precipitated fibrinogen from plasma from FXIII-A_2-sufficient (*F13a*$^{+/+}$) and FXIII-A_2-deficient (*F13a*$^{+/-}$ and *F13a*$^{-/-}$) mice[4,17] and used SDS-PAGE and Western blotting to detect FXIII-B in the precipitate. Consistent with previous observations[30], there was a FXIII-A subunit dose effect on the total amount of FXIII-B present in the initial plasma sample (Figure 3.5A), suggesting FXIII-A_2 influences the circulating level of FXIII-B_2. Regardless, Figure 3.5A shows that FXIII-B_2 co-precipitated with fibrinogen from FXIII-A-deficient plasma. Experiments using FXIII-A-deficient human plasma fully recapitulated findings with FXIII-A-deficient mouse plasma (Figure 3.5B). To test the specificity of the precipitation protocol, we subjected afibrinogenemic mouse plasma to glycine precipitation. As expected, no fibrinogen

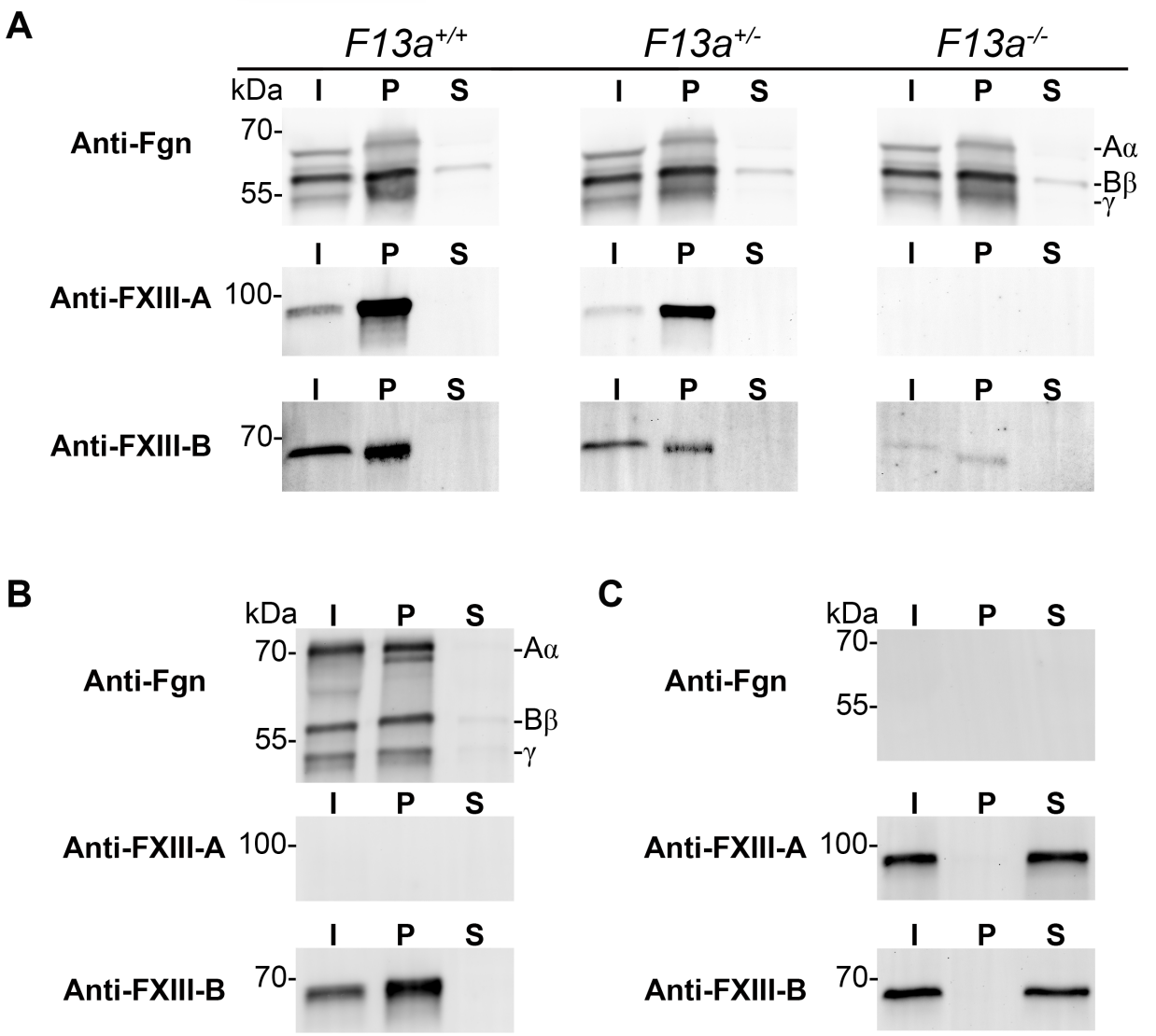


Figure 3.5. In the absence of FXIII-A_2, FXIII-B_2 co-precipitates with plasma fibrinogen. Fibrinogen was precipitated from (A) *F13a*$^{+/+}$, *F13a*$^{+/-}$, or *F13a*$^{-/-}$ mouse plasma, (B) FXIII-depleted human plasma, or (C) afibrinogenemic mouse plasma using glycine. Panels show representative Western blots for fibrinogen (Fgn), FXIII-A, and FXIII-B in the initial plasma (I), pellet (P), or supernatant (S) under reducing (Fgn) or non-reducing (FXIII-A, FXIII-B) conditions. Note in panel A, plasma albumin in I and S samples causes the Aα-chain to migrate faster than in the P samples. Blots are representative of N=3 experiments with mouse plasmas, and N=2 experiments with human plasma.

was precipitated (Figure 3.5C). Importantly, glycine did not precipitate FXIII-B_2 from afibrinogenemic mouse plasma (Figure 3.5C), indicating that FXIII precipitation in these experiments is fibrinogen-dependent. Thus, these data suggest excess FXIII-B_2 in plasma does not circulate in a "free" state, but instead circulates bound to fibrinogen.

3.5 Discussion

The observation that FXIII-A_2B_2 circulates in complex with fibrinogen is well-established; however, the motifs on fibrinogen and FXIII-A_2B_2 that mediate this interaction have been controversial. Our study that integrates both solid- and solution-phase binding experiments and functional assays reveals critical components of both fibrinogen and FXIII-A_2B_2 necessary for binding. First, our data indicate that the primary binding site for zymogen FXIII-A_2B_2 on human fibrinogen is not found in the alternatively-spliced γ′-extension or the αC region, but instead lies within γ-chain residues 390-396. Together with data from mice[4], this finding suggests these highly-conserved fibrinogen residues mediate this interaction in multiple species. Second, we showed that FXIII-A_2B_2 binds fibrinogen residues γ390-396 via the FXIII-B subunits. These data support previous studies suggesting an interaction between the FXIII-B subunit and fibrinogen[8,12], and extend these findings by defining the FXIII-B binding motif on fibrinogen. Third, we showed that FXIII-B_2 can bind fibrinogen in the absence of FXIII-A_2. This intriguing finding suggests "free" FXIII-B_2 in plasma actually circulates bound to fibrinogen, and has important implications for understanding assembly of the fibrinogen/FXIII-A_2B_2 complex in both physiologic and therapeutic situations.

Our finding that fibrinogen residues γ390-396 support FXIII-A_2B_2 binding is consistent with several prior studies implicating the D-domain[31], residues C-terminal to residue Lys356 of the γ-chain[12], and residues γ390-396 of murine fibrinogen[4] in this interaction. However, our findings

are discordant with studies implicating the alternatively-spliced γ′-chain[8,9] as the primary mediator of this interaction. The reasons for this discord may relate to differences in the assay systems used. For example, although experiments using anion exchange chromatography suggested FXIII-A_2B_2 elutes in the same fraction as γA/γ′, this study did not directly compare binding of FXIII-A_2B_2 to these fibrinogen variants.[8] In contrast we tested this interaction by both co-precipitation and direct binding assays that enabled us to maintain fibrinogen in solution during the binding events. This assay design may be particularly important because the same residues we have implicated in FXIII-A_2B_2 binding have previously been shown to support fibrin(ogen) binding to the CD11b (α_M) subunit of CD11b/CD18 (Mac-1) integrin present on monocytes, macrophages, and neutrophils.[22,32] The observation from those studies that CD11b binds to residues γ390-396 in insoluble fibrin and adherent fibrin(ogen), but not soluble fibrinogen, has led to the hypothesis that fibrin formation or fibrinogen adherence to a surface induces structural changes within these residues. Thus, previous experimental designs that used surface (or resin)-bound fibrin(ogen) or fibrin[6,8,27,33,34] may not have recapitulated the conformation of residues γ390-396 that would bind zymogen FXIII when fibrinogen is in solution. This possibility is also interesting when considering the role of the nearby fibrin(ogen) γ′ extension (residues 407-427) in FXIII-A_2B_2 binding. If residues in the γ′-extension influence structure within residues γ390-396, data from assays using surface- or resin-bound fibrin(ogen) or fibrin may have indicated the alternatively-spliced γ′-chain has different affinity for FXIII-A_2B_2. Similarly, ultracentrifugation experiments with γA/γ′ fibrinogen[9] may have been confounded by the presence of gel-like fibrin(ogen) dimers[35,36]. Thus, these conditions may have led to the conclusion that the zymogen binding motif is contained within the γ′-extension.

More recent findings that FXIII-A_2B_2 binds to a peptide derived from the fibrinogen αC

domain (residues α371-425) suggested FXIII-A_2B_2 binds to the fibrinogen αC region.[11] In that study[11] it was not possible to distinguish the relative contributions of the αC region versus the D-region to this interaction in full-length fibrinogen. Our SPR data indicating Aα251 fibrinogen has weaker binding than γA/γA fibrinogen to FXIII-A_2B_2 suggest some FXIII-binding character is derived from the αC domain. Future studies using variant fibrinogens with combined mutations in the γ- and α-chains may resolve the relative contribution of the αC region in this interaction.

The high degree of inter-species homology within fibrinogen residues γ390-396 has traditionally been attributed to their other essential function in supporting fibrin(ogen) binding to CD11b.[22,32] Interestingly, the earliest FXIII-fibrinogen system[37] and the α_M I-domain that binds fibrin[38,39] appeared together with the rise of vertebrates over 400 million years ago. No homozygous mutations have been identified in this region, emphasizing the physiologic importance of this fibrin(ogen) sequence. Therefore, the high homology in this region may result from strong evolutionary pressure to maintain both of these functions. Although the same fibrinogen residues mediate binding to both FXIII-A_2B_2 and CD11b, it is unlikely that these binding events compete. First, there is a vast excess of fibrinogen relative to FXIII-A_2B_2. Second, FXIII-A_2B_2 circulates with soluble fibrinogen[6], whereas CD11b binds insoluble fibrin[40]. Thus, these interactions likely occur in distinct physiologic settings.

Based on our and published findings, we propose the following model (Figure 3.6). FXIII-A_2B_2 circulates bound to fibrinogen at a site comprised of residues γ390-396 and supported by the αC region.[11] During coagulation, fibrinogen transports FXIII-A_2B_2 into the nascent clot via its interaction with the FXIII-B subunits. As fibrin polymerizes, FXIII-A_2B_2 bound to the D-domain of one fibrin monomer contacts thrombin bound to the E-domain of another fibrin monomer at the D:E:D interface.[41] Formation of this complex promotes FXIII activation peptide

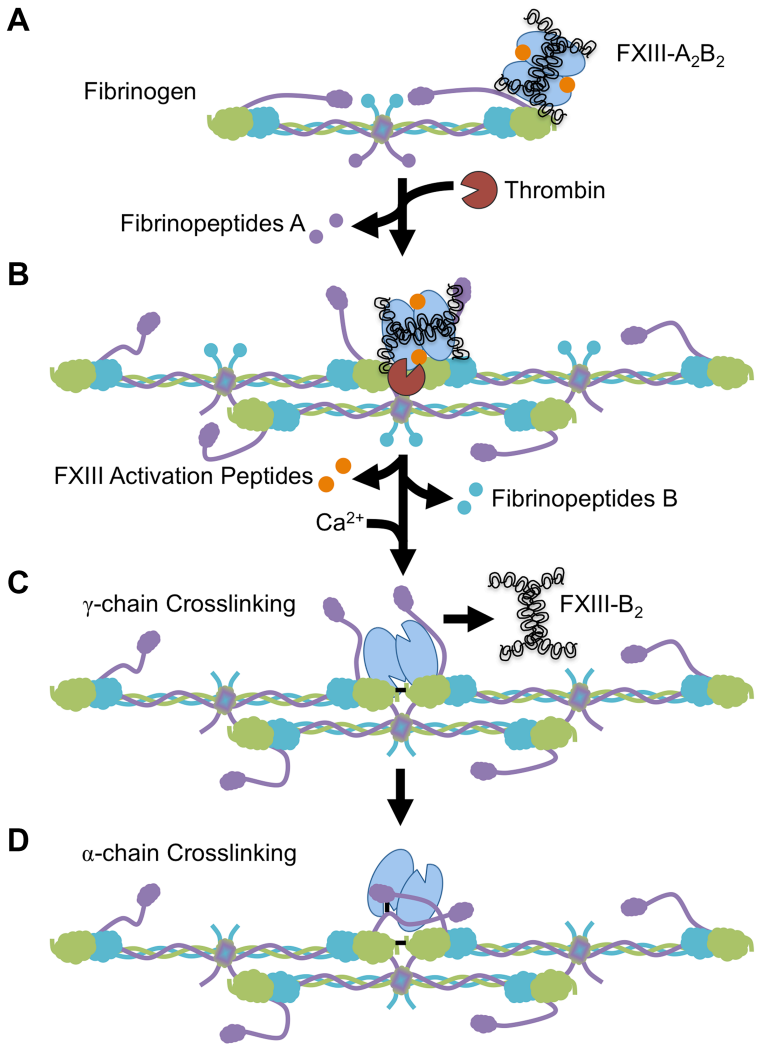


Figure 3.6. FXIII-A_2B_2 binding to fibrinogen residues γ390-396 promotes FXIII-A_2B_2 activation and activity. (A) Fibrinogen is comprised of two Aα- (medium gray), two Bβ- (dark gray), and two γ-chains (light gray) arranged in a trinodular structure with two distal D-domains and a central E-domain. The Aα-chains have a C-terminal domain (αC) that extends beyond the D-domain. FXIII-A_2B_2 circulates bound to fibrinogen γ-chain residues 390-396 via the FXIII-B subunits. (B) Once coagulation is initiated, thrombin interacts with the fibrinogen E-domain and cleaves fibrinopeptides A from the Aα-chains. As fibrin monomers polymerize, FXIII-A_2B_2 associated with γ390-396 is brought into contact with thrombin at the D:E:D interface to form a ternary complex.[41] This complex facilitates thrombin-mediated activation peptide cleavage from the FXIII-A subunits.[23-29,41] (C) Following activation peptide cleavage, fibrin promotes the calcium-mediated FXIII-B subunit dissociation from the FXIII-A subunits to yield FXIIIa (active sites indicated by stars).[12,23] FXIIIa then crosslinks (black line) the nearby γ-chains yielding γ-γ dimers.[21] This γ-chain crosslinking also promotes dissociation of FXIII-B_2 from the fibrin clot.[12] (D) FXIIIa translocates from the γ-chain to the αC region, binding at or near α-chain residue E396[11,45,46], and catalyzes the formation of crosslinks between fibrin α-chains and between fibrin and other plasma proteins.

cleavage and release from the FXIII-A subunits[23-29,41], followed by dissociation of the FXIII-B subunits from the FXIII-A subunits[12,23]. These sequential steps yield fully-activated FXIII-A_2*. Generation of FXIII-A_2* at residues γ390-396 conveniently localizes FXIIIa near the γ-chain crosslinking sites (residues γQ398/399 and γK406), which are the first fibrin residues to undergo crosslinking.[21,42-44] This γ-chain crosslinking also promotes FXIII-B_2 dissociation from the fibrin clot.[12] The FXIII-A_2* interaction with αC residue E396[11,45,46] then facilitates the translocation of its active site to the fibrin α-chain, where it catalyzes the formation of crosslinks between fibrin α-chains and between α-chains and other plasma proteins. Formation of these crosslinks is critical for the ability of FXIII(a) to promote resistance of clots to biomechanical and biochemical disruption. Moreover, we recently showed that the spatio-temporal regulation of FXIII activation kinetics during coagulation is also critical for determining red blood cell retention in contracted clots.[4] Thus, this model reconciles data from studies on the binding, activation, and activity of FXIII and reveals the importance of the FXIII-B subunits in fibrinogen/FXIII-A_2B_2 interactions, in FXIII-A_2B_2 activation, and consequently, in fibrin crosslinking and clot composition and stability. Although we did not identify the specific FXIII-B residues that mediate this interaction, previous studies have implicated sushi domains 1 and/or 10 in this interaction.[12] Further studies are ongoing to localize the FXIII-B residues that support binding to fibrinogen residues γ390-396.

A major finding from this work is the observation that FXIII-B_2 can bind fibrinogen in the absence of FXIII-A_2. Notably, the tight affinity of FXIII-B_2 binding to fibrinogen, together with the plasma concentrations of FXIII-B_2 and fibrinogen, suggests essentially all FXIII-B_2 in plasma is bound to fibrinogen. This observation appears to contradict the tenet that "free B" circulates in plasma.[3] However, FXIII-B_2 used in the previous report[3] was prepared by

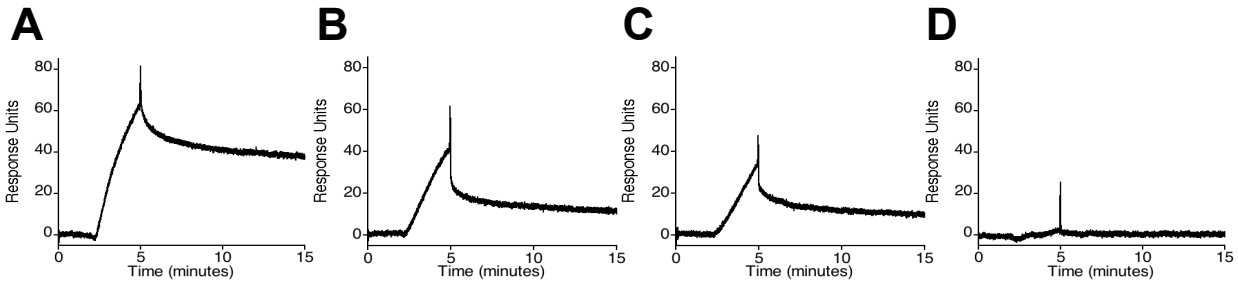
ammonium sulfate precipitation and heat denaturation to specifically remove fibrinogen. Thus, that study was not designed to characterize FXIII binding to other plasma proteins, and "free B" was likely only meant to imply "not bound to FXIII-A_2." This nomenclature has been interpreted overly-broadly since that time. Since both FXIII-B_2 and fibrinogen are synthesized by hepatocytes, these proteins may associate during or immediately following their secretion. Subsequent association of the FXIII-A_2 subunits, which are synthesized by cells of bone marrow origin, with the fibrinogen/FXIII-B_2 complex would then result in formation of the complete fibrinogen/FXIII-A_2B_2 complex. Consequently, our data that suggest FXIII-B_2 circulates bound to fibrinogen may reveal part of a step-wise mechanism that leads to production of fibrinogen/FXIII-A_2B_2 complexes. In addition, these data may have important implications for understanding the mechanism of action of therapeutic rFXIII-A_2 infusion for FXIII-A-deficiency. Binding of infused rFXIII-A_2 to fibrinogen-bound FXIII-B_2 (versus "free" FXIII-B_2) ensures that rFXIII-A_2 becomes incorporated into a functional, fibrinogen-bound complex that is crucial for normal FXIII activation and function.

Our study has potential limitations. First, loss of FXIII binding to Fib$\gamma^{390\text{-}396A}$ may reflect disrupted structure within the γ-domain. However, this possibility seems unlikely because crystallographic studies suggest this region is disordered even in the native molecule.[47-49] Moreover, although the pattern of fibrin crosslinking is subtly altered in both mouse[4] and human Fib$\gamma^{390\text{-}396A}$ clots, both murine[4,22] and human Fib$\gamma^{390\text{-}396A}$ can be fully crosslinked at the canonical residues located immediately C-terminal to γ390-396 (γQ398/399 and γK406). Thus, mutations within these residues do not appear to catastrophically alter structure within this domain. Second, the protocol used to precipitate fibrinogen may also promote FXIII precipitation and/or FXIII interaction with fibrinogen, and we and others[8] have observed spontaneous precipitation of

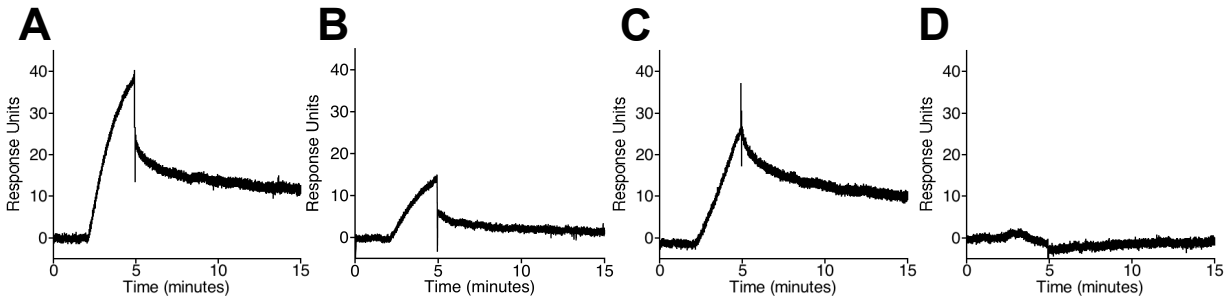
isolated FXIII-B subunits in certain experiments. However, glycine did not precipitate FXIII from afibrinogenemic plasma or in the presence of non-binding Fibγ$^{390\text{-}396A}$ fibrinogen. Moreover, data from the precipitation experiments were supported by both SPR analyses and functional FXIII activation assays performed in the absence of glycine.

In summary, our data expose critical molecular interactions mediating FXIII binding to fibrinogen. Identification of these motifs advances our understanding of this interaction in both physiologic and pathophysiologic situations.

3.6 Supplemental figures



Supplemental Figure 3.1. Recombinant fibrinogen binding to FXIII-A_2B_2. Surface plasmon resonance (SPR) was performed using OneStep® titration as described in the Methods and Supplemental Methods. Representative SPR binding curves using 1 μM fibrinogen (maximum) for (A) γA/γA, (B) γ′/γ′, (C) Aα251, and (D) Fibγ$^{390\text{-}396}$ binding to FXIII-A_2B_2. Curves representative of *N*=3-6 experiments as indicated in Table 3.1.



Supplemental Figure 3.2. Recombinant fibrinogen binding to FXIII-B_2. SPR was performed as described in the Methods and Supplemental Methods. Representative SPR binding curves using 1 μM fibrinogen (maximum) for (A) γA/γA, (B) γ′/γ′, (C) Aα251, and (D) Fibγ$^{390\text{-}396A}$ binding to recombinant FXIII-B_2. Curves representative of *N*=4-6 experiments as indicated in Table 3.1.

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CHAPTER 4: RECIPROCAL, INTER-TISSUE REGULATION OF FACTOR XIII-A AND -B SUBUNITS DETERMINES FACTOR XIII LEVELS IN PLASMA

4.1 Overview

Plasma coagulation factor XIII (FXIII) is composed of two FXIII-A and two FXIII-B subunits (FXIII-A_2B_2). Bone marrow-derived cells produce FXIII-A, whereas *F13b* mRNA is found in the liver and kidney. Interestingly, FXIII-A-deficient mice and humans have decreased FXIII-B. In humans, therapeutic infusion of recombinant FXIII-A_2 (rFXIII-A_2) increases FXIII-B. The mechanism mediating this inter-tissue reciprocal regulation has not been defined. To examine this relationship, we developed a mouse model of rFXIII-A_2 infusion. Infused *F13a*$^{-/-}$ mice showed 8-fold increased FXIII-B 30 hours post-infusion. FXIII-B clearance was slightly reduced when complexed with FXIII-A, suggesting FXIII-A contributes to FXIII-B stability. However, the pharmacokinetic profile of FXIII-B after rFXIII-A_2 infusion also indicated de novo FXIII-B production. FXIII-B was not released from intracellular stores, and rFXIII-A_2 infusion did not elevate liver or kidney *F13b* mRNA relative to untreated controls. Proteomic profiling of liver lysates from *F13a*$^{+/+}$, *F13a*$^{-/-}$, and rFXIII-A_2-infused *F13a*$^{-/-}$ mice revealed significant enrichment of RNA-binding proteins in both *F13a*$^{+/+}$ and rFXIII-A_2-infused *F13a*$^{-/-}$ mice relative to *F13a*$^{-/-}$ mice. In contrast, proteomic profiling of kidney tissue revealed evidence of cell stress in rFXIII-A_2-infused mice, but no increase in RNA-binding proteins. These data suggest a liver-specific effect of FXIII-A. Accordingly, rFXIII-A_2 treatment increased FXIII-B production in two human hepatocellular carcinoma lines. In conclusion, FXIII-B promotes FXIII-A stability, while FXIII-A increases FXIII-B stability and production. FXIII-A specifically increased liver

RNA-binding proteins, which may post-transcriptionally regulate *F13b*. Identification of this unique regulatory mechanism exposes inter-tissue crosstalk critical for hemostasis and identifies a newly-recognized function of FXIII-A in modulating liver function.

4.2 Introduction

Plasma coagulation Factor XIII (FXIII) is a protransglutaminase composed of two A (FXIII-A) and two B (FXIII-B) subunits arranged as a non-covalent heterotetramer (FXIII-A_2B_2). FXIII-A and -B arise from distinct tissues. FXIII-A polymorphisms track with bone marrow transplantation in humans, indicating cells of bone marrow origin are the source of plasma FXIII-A.[1] Recent murine Cre/lox studies revealed tissue resident macrophages produce plasma FXIII-A.[2] Hepatoma cell lines synthesize FXIII-B in vitro[3] and FXIII-B polymorphisms track with human liver transplantation, suggesting that the liver is the site of FXIII-B synthesis.[1] However, *F13b* mRNA is found in both the mouse liver and kidney[4]. FXIII-A_2B_2 assembly occurs in plasma, and essentially all plasma FXIII-A circulates tightly bound to FXIII-B. There is also an excess of uncomplexed FXIII-B in circulation (43-62 nM).[5]

Relative to FXIII-A_2B_2, FXIII-A has a short plasma half-life ($t_{1/2}$ ~9-10 days and <10 hours, respectively)[6,7], indicating FXIII-B stabilizes FXIII-A. Interestingly, several lines of evidence suggest FXIII-A regulates FXIII-B. First, there is a *F13A* gene-dose-dependent effect on plasma FXIII-B levels: FXIII-A-deficient individuals exhibit over 2-fold reduced FXIII-B.[8] Similarly, *F13a*$^{-/-}$ mice exhibit a ~5-fold reduction in FXIII-B.[4,9] Second, recombinant FXIII-A_2 (rFXIII-A_2) infusion in both monkeys[10] and humans[7] increases plasma FXIII-B. FXIII-B continues to rise even after FXIII-A_2B_2 begins to fall. The mechanism underlying this unique, inter-tissue, reciprocal regulation is unclear. Instances of reciprocal protein regulation are common in intracellular feedback loops[11], and natural killer and autoreactive T cell populations exhibit

systemic, cellular reciprocal regulation[12]. However, an inter-tissue, reciprocal regulatory mechanism where two protein subunits regulate their respective levels systemically has, to our knowledge, not previously been described.

Herein, we use in vitro, in vivo, and proteomics approaches to define the mechanism of FXIII-A-mediated FXIII-B regulation. Our findings demonstrate that FXIII-A upregulates RNA-binding proteins in the murine liver, which may be involved in post-transcriptional regulation of *F13b* and other mRNA transcripts. These findings are the first to implicate FXIII-A as a regulator of liver function.

4.3 Methods

Materials and Proteins. Recombinant human FXIII-A_2 (rFXIII-A_2) was a gift of Novo Nordisk (Bagsvaerd, Denmark). Anti-human FXIII-A antibody (SAF13A-AP) and human thrombin was from Enzyme Research Laboratories (South Bend, IN). T101, recombinant FXIII-B_2 (rFXIII-B_2), and anti-human FXIII-B antibody (A074) were from Zedira (Darmstadt, Germany). Anti-human fibrinogen antibody (A0080) was from Dako (Carpinteria, CA). Plasma-derived FXIII-A_2B_2 was a gift of CSL-Behring (King of Prussia, PA). Recombinant hirudin was from Hyphen Biomed (Neuville-sur-Oise, France). Cell culture media and supplements were from Gibco (Montgomery County, MD). Fetal bovine serum (FBS) was from Sigma (St. Louis, MO).

Western blot determination of plasma fibrinogen, FXIII-A and -B levels. The University of North Carolina at Chapel Hill Institution of Animal Care and Use Committee approved all murine studies. Murine blood was harvested from the inferior vena cava (IVC) into 3.2% sodium citrate (10% vol/vol) and processed to platelet-poor plasma (PPP) by centrifugation (5000*g*, 10

minutes). PPP was diluted 1:6 (FXIII-B blots) or 1:120 (fibrinogen, FXIII-A blots) in water and 6X sodium dodecyl sulfate (SDS) loading buffer (Boston Bioproducts, Ashland, MA) before boiling under nonreducing (FXIII-B) or reducing (2.5% β-mercaptoethanol, fibrinogen, FXIII-A) conditions. Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% Tris-Glycine gels (Bio-Rad, Hercules, CA) before transfer to polyvinylidene fluoride membranes (Millipore, Burlington, MA). Membranes were probed with primary antibodies (rabbit anti-fibrinogen [1:7000, 4°C overnight], sheep anti-FXIII-A [1:1000, 4°C overnight] or rabbit anti-FXIII-B [1:500, room temperature overnight]). Blots were visualized using the appropriate fluorescent secondary antibody (1:10000, 1 hour, room temperature) and a GE Typhoon FLA9000 Imager (GE Healthcare, Little Chalfont, United Kingdom). Densitometry was performed using ImageJ 1.48v and a standard sample was used to normalize between blots.

rFXIII-A_2 infusions. 8-12 week old FXIII-A-deficient (*F13a*$^{-/-}$)[13] mice were anesthetized using 3% isoflurane in oxygen. rFXIII-A_2 (0.875 mg/mL in sterile saline) was infused via the tail vein using a 30G sterile needle and syringe. Mice received 4 mg/kg rFXIII-A_2 as previously described.[14] Blood was harvested over time from the retro-orbital sinus or the IVC into 3.2% sodium citrate (10% vol/vol, final). Blood for the 0 hour timepoint was collected immediately post-infusion. At the end of the time course, mice were sacrificed. Organs were harvested and flash frozen for subsequent analysis. Isolated blood was processed to PPP as described above and flash frozen.

In vivo FXIII-B clearance. FXIII-A_2B_2 (0.5 mg/mL, final) was pre-activated in sterile N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES)-buffered saline (HBS, 20 mM

HEPES, 150 mM NaCl, pH 7.4) using thrombin (10 nM, final) and calcium ($CaCl_2$, 10 mM, final) to separate the FXIII-A and -B subunits. The reaction proceeded for 1 hour at 37°C in the presence of the FXIII inhibitor T101 (250 μM, final) to inhibit active FXIII-A subunits (FXIII-A_2*I). Reactions were quenched (30 minutes at 37°C) with a 6-fold molar excess of recombinant hirudin to inhibit thrombin. Unactivated FXIII-A_2B_2 was prepared by adding FXIII-A_2B_2 to reactions following thrombin inhibition. Pre-activated or unactivated FXIII-A_2B_2 was infused into *F13a*$^{-/-}$ mice as above, except the dose was reduced to 2 mg/kg. Plasma was harvested at the times indicated.

Real-time quantitative reverse polymerase chain reaction (RT-qPCR) of *F13b* transcripts. Tissue from *F13a*$^{+/+}$, *F13a*$^{-/-}$, and rFXIII-A_2-treated *F13a*$^{-/-}$ mice was harvested (48 hours post-infusion for rFXIII-A_2-treated mice) as above and immediately flash frozen. Tissue was cryogenically pulverized and RNA isolated from tissue powder using QiaShredder and RNeasy Mini Kits (Qiagen, Hilden, Germany) per the manufacturer's instructions. RNA was quantified on a Spectramax Plus 384 microplate reader with a SpectraDrop micro volume plate (Molecular Devices, Sunnyvale, CA). 1 μg of RNA was converted to cDNA using the QuantiTect reverse transcription kit (Qiagen) and RT-qPCR was performed performed using the QuantiFast SYBR Green kit (Qiagen) with a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). *F13b* transcript levels (forward primer: CGCTGAGACACTTGCCATTTA, reverse: GTCTACGCTCATGGGGAAGTA) were compared using the $\Delta\Delta C_T$ method, relative to *F13a*$^{-/-}$. *Gapdh* was used as a housekeeping gene (forward primer: TGGCCTTCCGTGTTCCTAC, reverse: GAGTTGCTGTTGAAGTCGCA).

Sample preparation for LC-MS/MS analysis. Livers and kidneys from *F13a*$^{+/+}$, *F13a*$^{-/-}$, and rFXIII-A_2-treated *F13a*$^{-/-}$ mice were harvested processed as above. At least three biological replicates were used for each condition. Tissue powder was lysed using tPer lysis buffer (Thermo Scientific, Rockford, IL) supplemented with protease inhibitors (Cell Signaling, Danvers, MA) and homogenized using a sterile 25G needle and syringe. Protein was quantified with bicinchoninic acid assay (Thermo) and total protein concentrations normalized to the same value. Samples were acetone precipitated by adding four times the sample volume with cold acetone overnight, followed by centrifugation at 16,000 RPM for 15 minutes. Each sample (50 µg) was reduced with 5 mM dithiothreitol for 30 minutes at 56ºC, and then alkylated with 15 mM iodoacetamide in the dark at room temperature. Samples were digested with trypsin (Promega, Madison, WI) overnight at 37ºC, and the peptide samples were desalted using C18 spin columns (Pierce, Rockford IL). Peptide samples were dried down via vacuum centrifugation and reconstituted in 5% acetonitrile/0.1% formic acid. Peptides were quantified using the Pierce Quantitative Colorimetric Peptide Assay.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The peptide samples (1 µg) were analyzed by LC-MS/MS using an Easy nanoLC 1000 coupled to a QExactive HF mass spectrometer (Thermo). Samples were injected onto an Easy Spray PepMap C18 column (75 µm inner diameter × 25 cm, 2 µm particle size, Thermo) and separated using a 2 hour method. The gradient for separation consisted of 5–32% mobile phase B at a 250 nL/min flow rate, where mobile phase A was 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The QExactive HF was operated in data-dependent mode where the 15 most intense precursors were selected for subsequent fragmentation. Resolution for the

precursor scan (m/z 400–1600) was set to 120,000 with a target value of 3×10^6 ions. MS/MS scan resolution was set to 15,000 with a target value of 5×10^4 ions. The normalized collision energy was set to 27% for higher-energy collisional dissociation. Peptide match was set to preferred, and precursors with unknown charge or a charge state of 1 and ≥ 7 were excluded.

Data analysis. Raw data files were processed using MaxQuant version 1.5.3.17 and searched against a Uniprot mouse database (downloaded April 2017, containing 50,936 entries), using Andromeda within MaxQuant. Enzyme specificity was set to trypsin, up to two missed cleavage sites were allowed, carbamidomethylation of cysteine was set as a fixed modification and oxidation of Met was set as a variable modification. A 1% false discovery rate was used to filter all data. Label-free quantification using razor + unique peptides and match between runs (2 minute time window) were enabled. Quantified protein data were analyzed using Perseus v1.51 (Max Planc Institute of Biochemistry). Briefly, contaminants and reverse sequences were removed, as were proteins that were identified in less than 60% of the samples. Data were imputed from a normal distribution of protein intensities and missing values replaced. Over-imputed samples and outliers by principal component analysis were removed. Data were then log2 transformed and converted into normalized Z-scores for ANOVA. Proteins with a significance of $P<0.05$ were used in subsequent hierarchical clustering in Perseus. Enrichment analysis was performed using the STRING database.[15] Enrichments with a $P<0.01$ were considered significant.

Cell culture models. HEK293 cells were cultured in Eagle's minimum essential medium supplemented 1X penicillin/streptomycin and 10% FBS. HepG2 cells were cultured in Eagle's

minimum essential medium supplemented with 1X non-essential amino acids, 1 mM sodium pyruvate, 1X penicillin/streptomycin, and 10% FBS. Huh7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1X antibiotic/antimycotic. For FXIII-B production studies, cells were maintained in serum-free media supplemented with 10 μg/mL insulin-transferrin-selenite (Roche, Basel, Switzerland) and 10 U/mL bovine lung aprotinin (Calbiochem, San Diego, CA). Conditioned media from cells cultured in the presence of vehicle or rFXIII-A_2 was collected after 3-5 days, concentrated 10-fold using 3,000 dalton molecular weight cutoff centrifugal concentrator columns (Millipore), and FXIII-B visualized by Western blot as above. For siRNA knockdown in Huh7 cells, cells were transfected with 100 nM siRNA using RNAiMAX Lipofectamine in OptiMEM media (Thermo) per the manufacturer's protocol for reverse transfection. Cells were transfected with 100 nM SilencerSelect Negative Control #1, SilencerSelect *Gapdh* Positive Control, or *F13b* siRNA (s4958, Thermo). 24 hours after transfection, cells were switched to serum-free media and media harvested after 3 days for FXIII-B analysis. Cell proliferation was measured with by MTT viability assay (Thermo).

4.4 Results

FXIII-A increases FXIII-B in mice. Both FXIII-A-deficient humans[8] and mice[4] exhibit reduced levels of plasma FXIII-B. Relative to $F13a^{+/+}$ mice, $F13a^{+/-}$ mice have ~60% reduced plasma FXIII-A and $F13a^{-/-}$ mice are completely FXIII-A-deficient.[16] Compared to $F13a^{+/+}$, $F13a^{+/-}$ and $F13a^{-/-}$ mice had 33% and 90% reduced levels of FXIII-B, respectively (Figure 4.1A-B). FXIII-B levels in $F13a^{-/-}$ mice did not vary with gender or time of day (data not shown). FXIII-B binds both FXIII-A and fibrinogen in plasma[9], so we first tested the role of

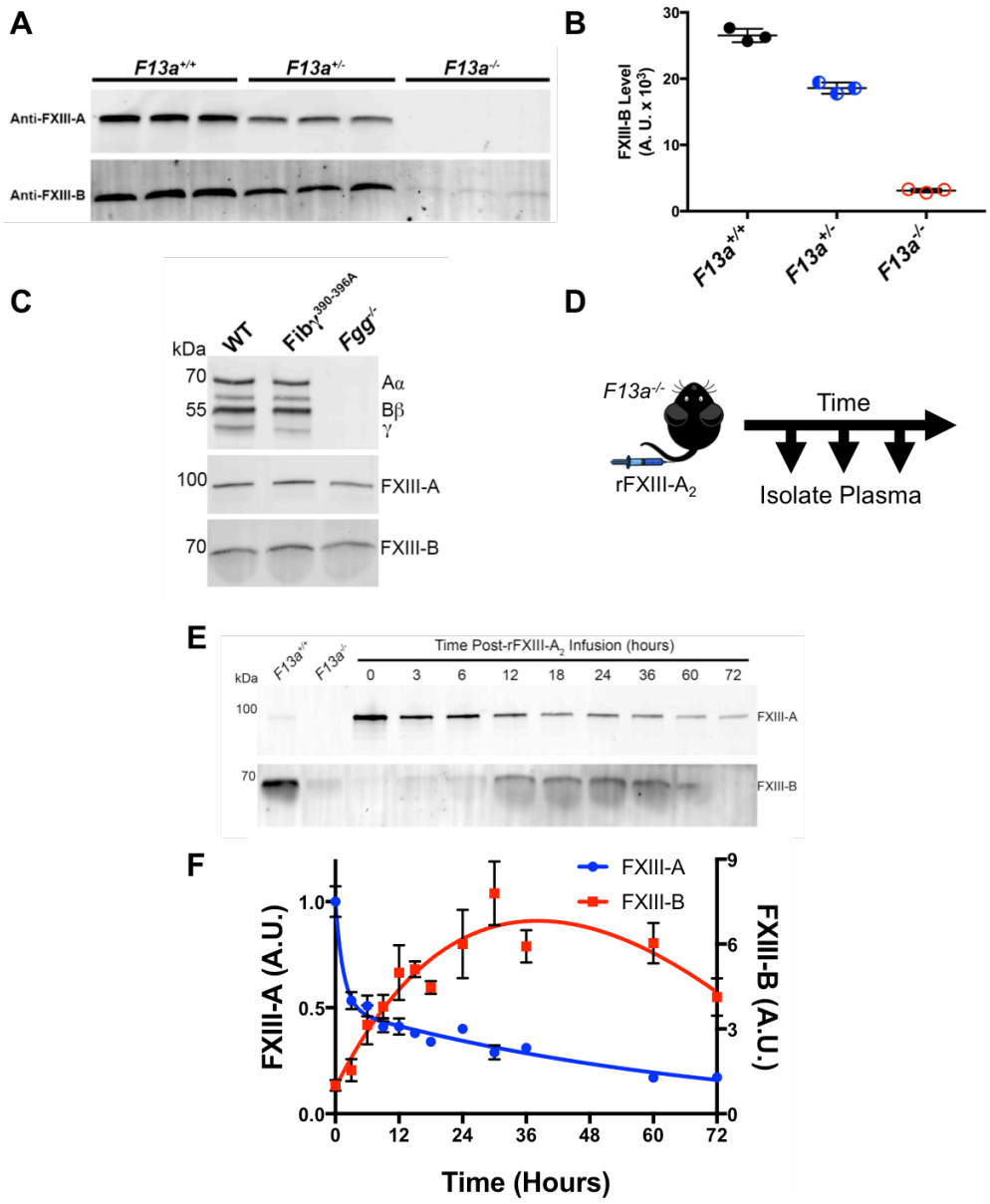

Figure 4.1. FXIII-A increases FXIII-B in vivo. (A) FXIII-A and -B in *F13a*$^{+/+}$, *F13a*$^{+/-}$ or *F13a*$^{-/-}$ mouse plasma was visualized using Western blot with anti-FXIII-A or -B antibodies. Each lane represents plasma from one mouse. (B) FXIII-B band intensity was quantified using densitometry. Each dot represents one mouse and lines represent mean -/+ standard deviation (SD). (C) Fibrinogen, FXIII-A, and FXIII-B were visualized in plasma from wild-type (WT), Fibγ$^{390-396A}$, or afibrinogenemic (*Fgg*$^{-/-}$) mice with Western blot using anti-fibrinogen, anti-FXIII-A, and anti-FXIII-B antibodies. (D) Schematic of in vivo rFXIII-A_2 infusion model. 8-12 week old *F13a*$^{-/-}$ mice were infused with rFXIII-A_2 (4 mg/kg) via the tail vein and blood was collected at various times post-infusion. (E) Plasma FXIII-A and -B levels at the indicated times post-infusion were visualized by Western blot with anti-FXIII-A and FXIII-B antibodies, respectively. Representative blots are shown. (F) FXIII-A and -B were quantified using densitometry and compared to levels at the 0 hour timepoint. Dots represent means -/+ standard error (SE), N=3-18.

fibrinogen in mediating FXIII-B levels. Western blotting revealed normal FXIII-A and -B subunit levels in plasma from afibrinogenemic (*Fgg*$^{-/-}$) mice and mice with disrupted FXIII-fibrinogen binding (Fib$\gamma^{390\text{-}396A}$)[17] (Figure 4.1C). These data indicate that fibrinogen is not required to maintain FXIII in circulation, and suggest FXIII-A mediates plasma FXIII-B.

To investigate the role of FXIII-A in modulating FXIII-B, we developed an in vivo model of FXIII-B production using *F13a*$^{-/-}$ mice. We infused *F13a*$^{-/-}$ mice with human rFXIII-A_2 (4 mg/kg, IV, Figure 4.1D) and measured plasma FXIII over time. FXIII-A exhibited biphasic clearance (Figure 4.1E-F). These two clearance phases likely represent initial, rapid clearance of uncomplexed FXIII-A and later, slow clearance of FXIII-A in complex with FXIII-B (FXIII-A_2B_2). FXIII-B levels rose after a brief (~3 hour) lag-time, and maximal FXIII-B occurred 30 hours post-infusion (7.8-fold increased, Figure 4.1E-F). After 30 hours, FXIII-B slowly decreased (Figure 4.1E-F). We observed similar trends in another line of FXIII-A-deficient mice (data not shown). These findings indicate FXIII-A regulates plasma FXIII-B in mice.

FXIII-A reduces FXIII-B clearance. We first hypothesized that FXIII-A stabilizes FXIII-B in plasma. To evaluate FXIII-B clearance in vivo, we infused *F13a*$^{-/-}$ mice with FXIII-B in its FXIII-A-complexed (FXIII-A_2B_2) or -uncomplexed (FXIII-B_2) form (Figure 4.2A). Western blotting of plasmas harvested post-infusion revealed both subunits were more rapidly cleared when uncomplexed (Figure 4.2B-C). To determine if FXIII-A protects FXIII-B from proteolytic degradation in the plasma compartment, we incubated rFXIII-B_2 in human plasma for 24 hours at 37°C in the absence or presence of rFXIII-A_2. Western blotting showed that rFXIII-B_2 did not degrade, even in the absence of rFXIII-A_2 (Figure 4.2D). Together, these findings suggest that FXIII-A reduces FXIII-B clearance from plasma.

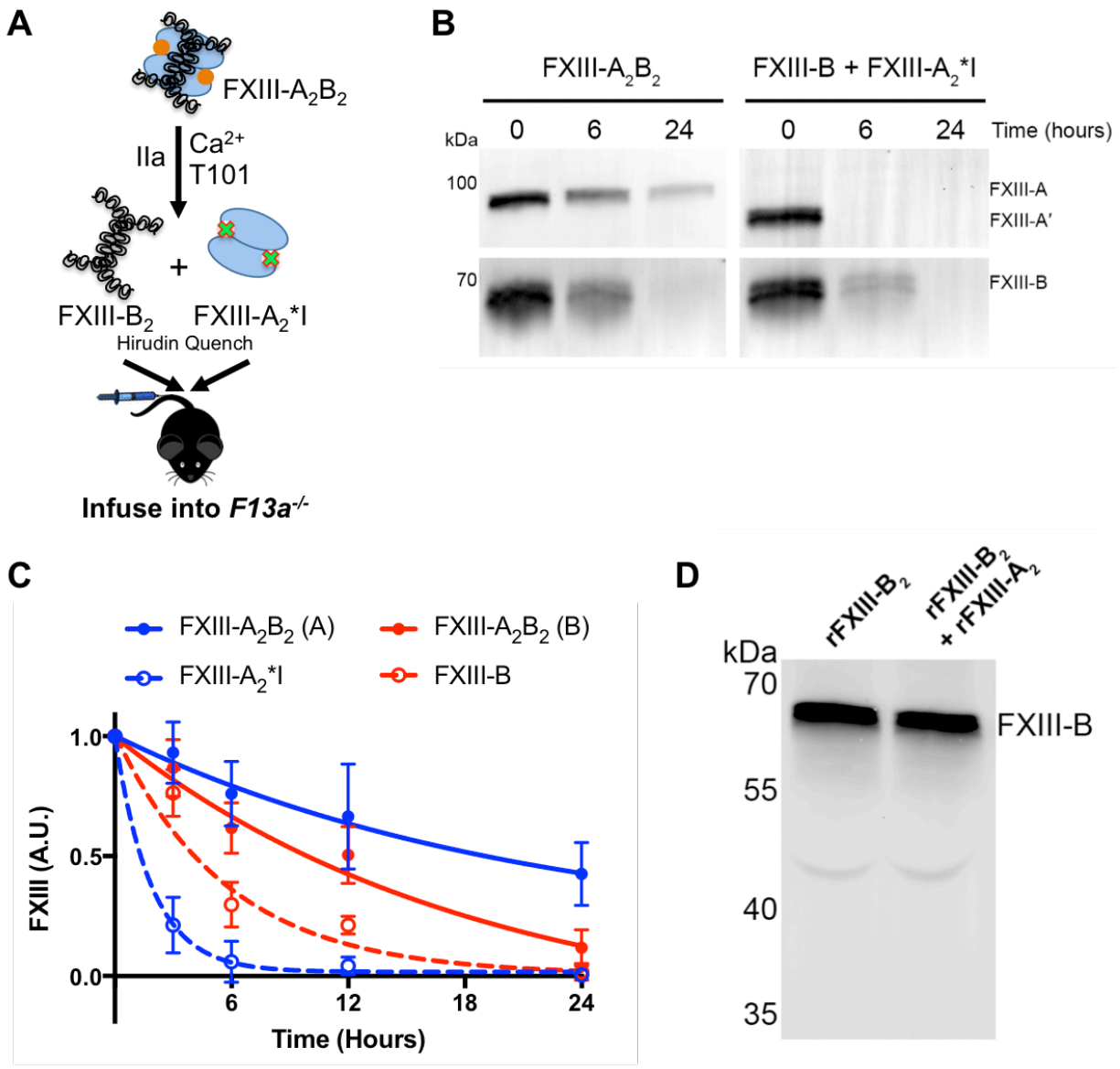

Figure 4.2. FXIII-A stabilizes FXIII-B in plasma. (A) Schematic of in vitro FXIII-A_2B_2 activation with thrombin (10 nM) and $CaCl_2$ (10 mM) to separate FXIII-A and -B subunits prior to infusion. Reactions were performed in the presence of the transglutaminase inhibitor T101 (250 μM) and thrombin was inhibited with a 6-fold molar excess of hirudin prior to infusion in *F13a*$^{-/-}$ mice via the tail vein. (B) Representative Western blot of FXIII-A and -B levels in mice infused with complexed (FXIII-A_2B_2) or uncomplexed (FXIII-B + FXIII-A_2*I) at the indicated times post-infusion using anti-FXIII-A and -B antibodies. (C) FXIII-A (blue) and -B (red) levels were quantified for each condition (solid line, complexed; dashed line, uncomplexed) using densitometry and compared to levels at the 0 hour timepoint. Data represent means -/+ SD, N=2-9. (D) rFXIII-B_2 was incubated in human plasma for 24 hours at 37°C in the absence or presence of rFXIII-A_2 and visualized using Western blot with anti-FXIII-B antibody. Representative blot of N=2 experiments.

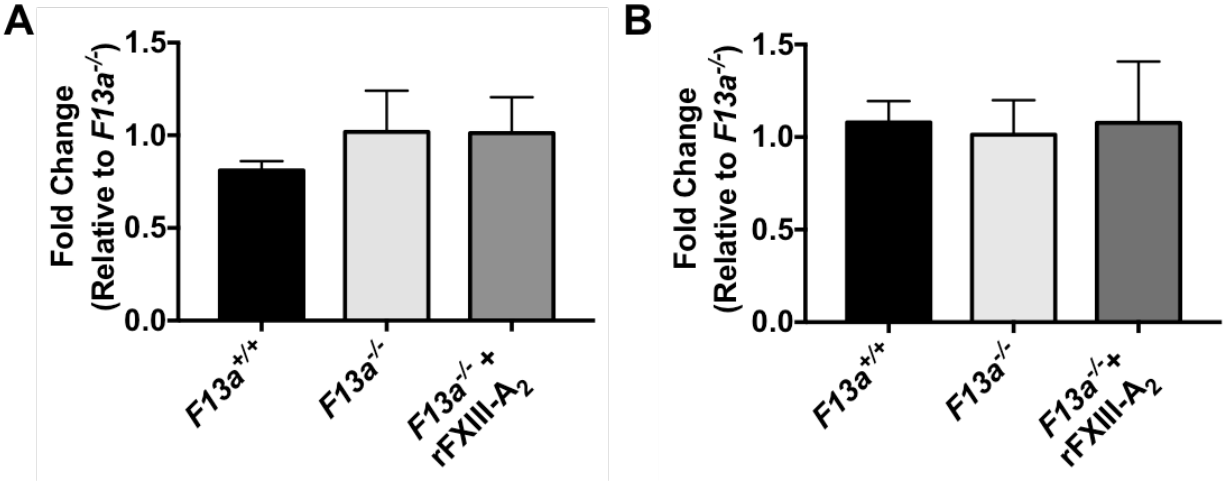

Figure 4.3. FXIII-A does not alter *F13b* transcription. *F13b* mRNA was quantified in liver (A) and kidney (B) tissue using RT-qPCR and compared to levels in *F13a*$^{-/-}$ mice. Bars represent mean fold change -/+ SD. N=3-4.

FXIII-A does not increase *F13b* transcription. Previous studies[7,10] and our in vivo data (Figure 4.1), show that FXIII-B levels continue to rise even after all infused FXIII-A is bound to endogenous FXIII-B. These findings suggest that FXIII-A also induces de novo FXIII-B production. We first hypothesized that FXIII-A increases *F13b* gene expression. *F13b* mRNA was previously detected in only the mouse liver and kidney[4]. Recent RiboTag pulldown/RNAseq experiments also detected *F13b* mRNA in mouse liver and kidney (Audrey C. A. Cleuren, personal communication). Therefore, to determine the effect of FXIII-A on *F13b* expression, we measured *F13b* mRNA in both mouse liver and kidney. RT-qPCR revealed no differences in liver or kidney *F13b* transcript levels in $F13a^{+/+}$, $F13a^{-/-}$, and rFXIII-A_2-treated $F13a^{-/-}$ mice (Figure 4.3). These findings indicate that FXIII-A does not increase *F13b* gene expression.

FXIII-A increases liver RNA-binding proteins. We next hypothesized that FXIII-A regulates FXIII-B via a post-transcriptional mechanism. To identify proteins FXIII-A alters in the liver, we performed label-free quantitative proteomic profiling of $F13a^{+/+}$, $F13a^{-/-}$, and rFXIII-A_2-treated $F13a^{-/-}$ liver lysates (Figure 4.4A). Analysis of liver proteomes revealed 96 differentially regulated proteins (P<0.05). Both principal component analysis (Figure 4.4B) and hierarchical clustering (Figure 4.4C) clearly separated the three groups of mice. First, we analyzed differences between $F13a^{+/+}$ and $F13a^{-/-}$ livers. Whereas STRING database functional gene ontology term analysis revealed no enrichments in the 26 proteins downregulated in $F13a^{+/+}$ mice, the 43 proteins upregulated in $F13a^{+/+}$ mice contained 13 proteins implicated in RNA-binding (RNA-binding proteins, RBPs). KEGG pathway analysis also revealed that proteins involved in glycolysis/gluconeogenesis and metabolism were significantly (P=3.25e-05) enriched in $F13a^{+/+}$ mouse livers, suggesting FXIII-A affects liver metabolic pathways.

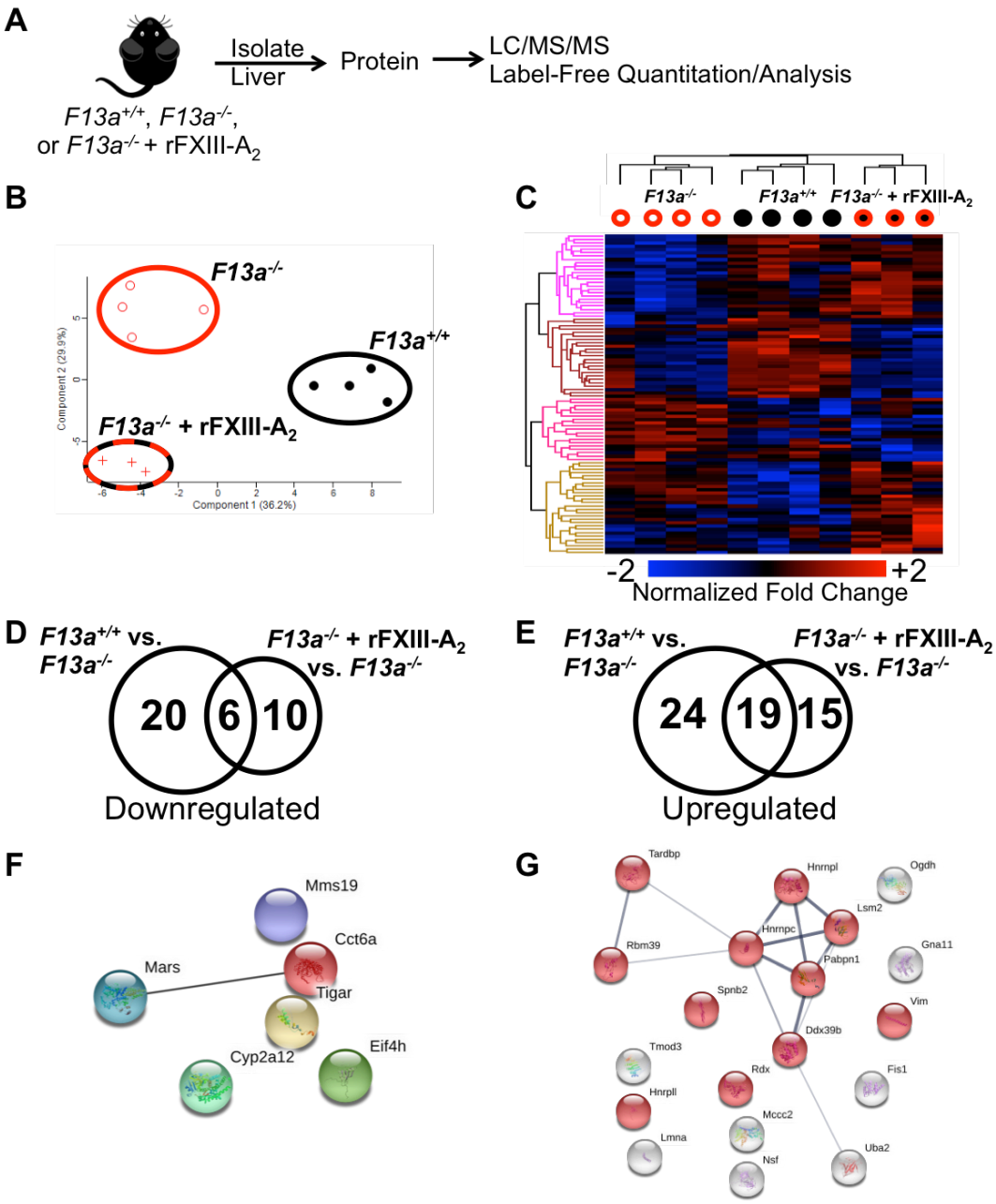

Figure 4.4. FXIII-A increases liver RNA-binding proteins. (A) Schematic of label-free, quantitative proteomic profiling of *F13a*$^{+/+}$, *F13a*$^{-/-}$, and rFXIII-A$_2$-treated *F13a*$^{-/-}$ liver lysates (N=3-4 mice per group). Data was analyzed using MaxQuant and Perseus. (B) Principal component analysis separates the three groups of mice, each dot representing one mouse. (C) Heat map of significantly altered (ANOVA P<0.05) proteins between mouse groups following hierarchical clustering (blue=downregulated, red=upregulated). Venn diagrams show the number of downregulated (D) and upregulated (E) proteins in *F13a*$^{+/+}$ versus *F13a*$^{-/-}$ and rFXIII-A$_2$-treated *F13a*$^{-/-}$ versus *F13a*$^{-/-}$ mice. STRING database protein interaction networks for downregulated (F) and upregulated (G) proteins in both *F13a*$^{+/+}$ and rFXIII-A$_2$-treated *F13a*$^{-/-}$ mice versus *F13a*$^{-/-}$ mice. In (G), RNA-binding proteins are indicated in red.

We next examined proteins specifically altered in both *F13a*$^{+/+}$ and rFXIII-A_2-treated *F13a*$^{-/-}$ mice relative to *F13a*$^{-/-}$ mice (Figure 4.4D-E). STRING analysis did not identify any significant enrichment in the 6 proteins downregulated in both *F13a*$^{+/+}$ and rFXIII-A_2-treated *F13a*$^{-/-}$ mice (Figure 4.4F). Conversely, many of the RBPs that were upregulated in *F13a*$^{+/+}$ mice relative to *F13a*$^{-/-}$ mice were also upregulated in the rFXIII-A_2-treated *F13a*$^{-/-}$ mice. In total, 11 of the 19 upregulated proteins (58%) are RBPs (P=5.37e-06) implicated in mRNA processing (P=1.74e-06, Figure 4.4G). Collectively, these findings suggest FXIII-A specifically regulates the liver proteome and may promote liver FXIII-B production via RBP-mediated post-transcriptional regulation.

FXIII-A infusion causes kidney ribosome reduction. Given that *F13b* mRNA is also detected in the kidney[4], we evaluated mouse kidney lysates for proteomic changes similar to those observed in the liver. Label-free quantitative proteomic profiling of *F13a*$^{+/+}$, *F13a*$^{-/-}$, and rFXIII-A_2-treated *F13a*$^{-/-}$ kidney lysates (Figure 4.5) revealed 402 differentially regulated proteins, over 4-fold more than in the liver. Similar to the liver proteomics, both principal component analysis (Figure 4.5B) and hierarchical clustering (Figure 4.5C) separated the three groups of mice. Although overall more differentially regulated proteins were detected in the kidney than the liver, fewer proteins were altered in both *F13a*$^{+/+}$ and rFXIII-A_2-treated *F13a*$^{-/-}$ mice relative to *F13a*$^{-/-}$ mice (Figure 4.5D-E). In contrast to the liver, STRING analysis did not reveal a significant number of interactions among proteins altered in both *F13a*$^{+/+}$ and rFXIII-A_2-treated *F13a*$^{-/-}$ mice relative to *F13a*$^{-/-}$ mice (Figure 4.5F-G). Interestingly, a large number of proteins (98) were downregulated in the rFXIII-A_2-treated *F13a*$^{-/-}$ kidneys relative to both *F13a*$^{-/-}$ and *F13a*$^{+/+}$ kidneys (Figure 4.6A). STRING functional analysis showed multiple enrichments,

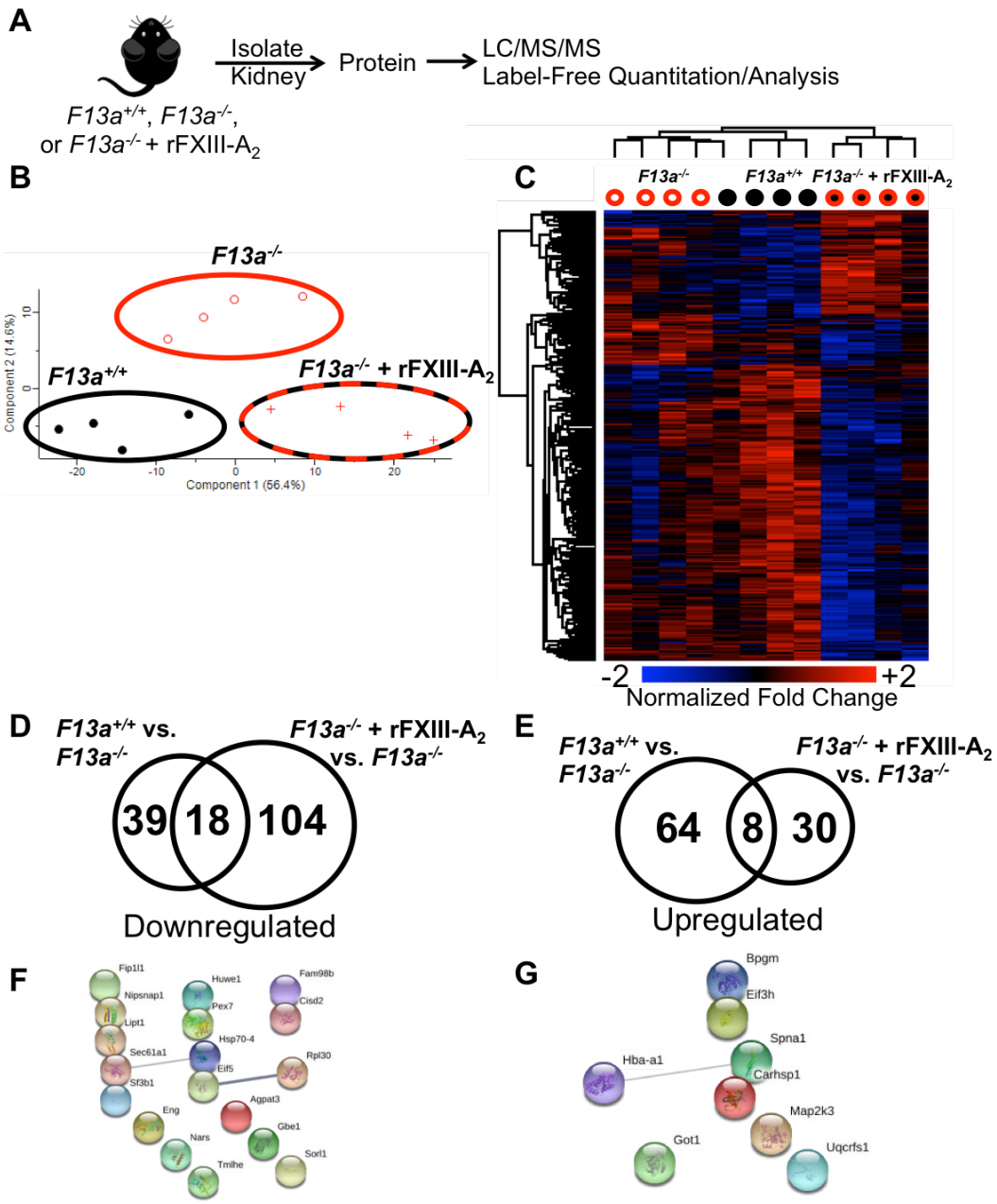

Figure 4.5. FXIII-A and the mouse kidney proteome. (A) Schematic of label-free, quantitative proteomic profiling of *F13a*$^{+/+}$, *F13a*$^{-/-}$, and rFXIII-A$_2$-treated *F13a*$^{-/-}$ kidney lysates (N=4 mice per group). Data was analyzed using MaxQuant and Perseus. (B) Principal component analysis separates the three groups of mice, each dot representing one mouse. (C) Heat map of significantly altered (ANOVA P<0.05) proteins between mouse groups following hierarchical clustering (blue=downregulated, red=upregulated). Venn diagrams show the number of downregulated (D) and upregulated (E) proteins in *F13a*$^{+/+}$ versus *F13a*$^{-/-}$ and rFXIII-A$_2$-treated *F13a*$^{-/-}$ versus *F13a*$^{-/-}$ mice. STRING database protein interaction networks for downregulated (F) and upregulated (G) proteins in both *F13a*$^{+/+}$ and rFXIII-A$_2$-treated *F13a*$^{-/-}$ versus *F13a*$^{-/-}$ mice.

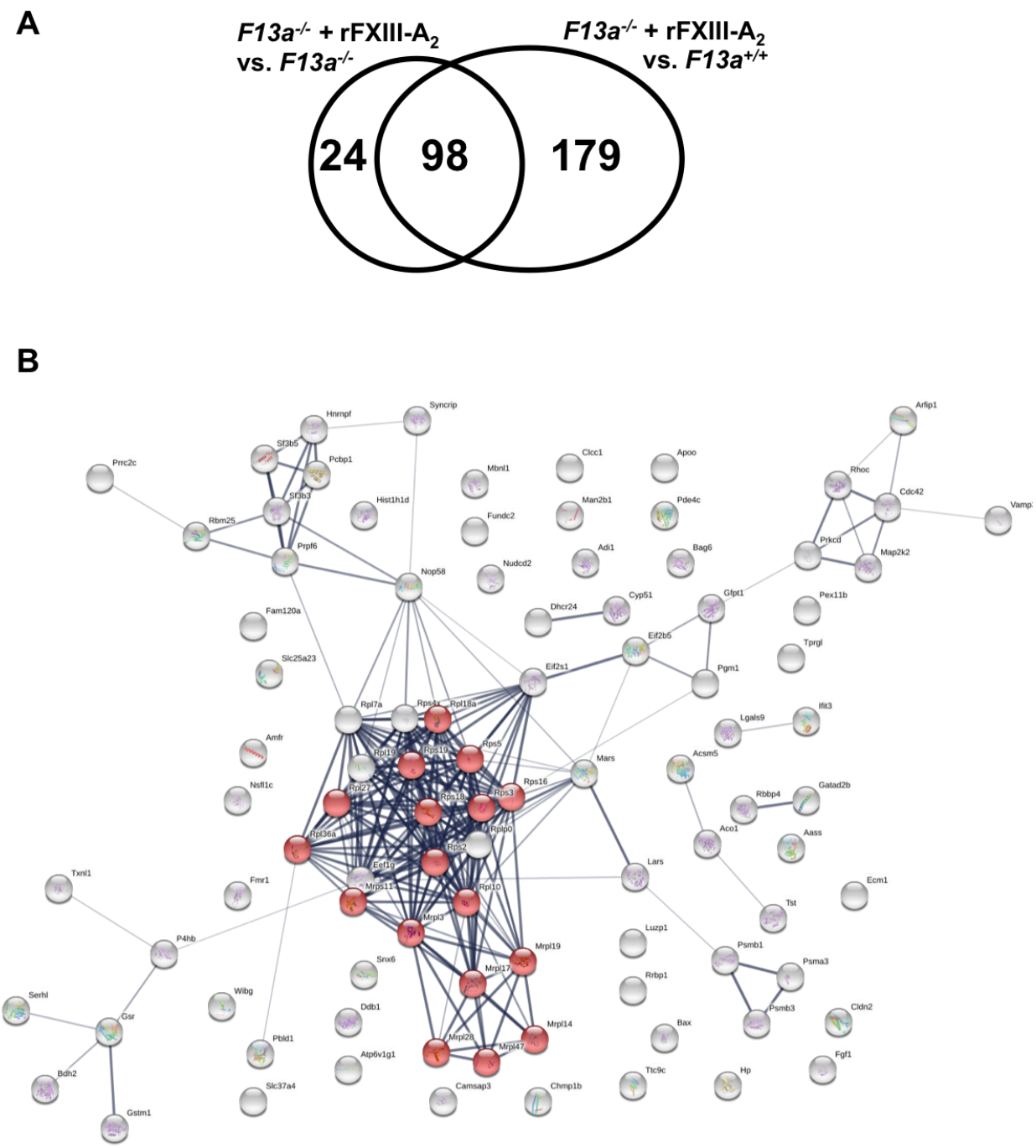

Figure 4.6. Ribosomal structural proteins are downregulated in rFXIII-A_2-infused mouse kidneys. (A) Venn diagram showing the number of downregulated proteins in rFXIII-A_2-treated *F13a*$^{-/-}$ versus *F13a*$^{+/+}$ and *F13a*$^{-/-}$ mice. (B) STRING database protein interaction network for proteins in (A). Ribosomal structural proteins are indicated in red.

notably of ribosomal structural proteins (*P*=1.02e-16) and proteins involved in peptide metabolism (*P*=4.95e-18, Figure 4.6B). These markedly divergent trends in *F13a*$^{+/+}$ and rFXIII-A_2-treated mice suggest that FXIII-A does not exhibit the same regulatory specificity for the kidney as observed in the liver and that the kidney is not the site of FXIII-A-mediated FXIII-B production.

FXIII-A increases FXIII-B production in hepatocellular carcinoma cells. To determine the cellular mechanism of FXIII-A-mediated FXIII-B induction, we developed an in vitro model of FXIII-B production. Tie2^{+}-Cre RiboTag pulldown/RNAseq experiments revealed *F13b* mRNA was enriched in liver parenchymal cells, not endothelium (Audrey C. A. Cleuren, personal communication). Therefore, we measured FXIII-B production in hepatocellular carcinoma (Huh7) cells. We detected low-level basal FXIII-B production in Huh7 culture supernatants and confirmed this finding using siRNA knockdown (Figure 4.7A). IL-6 or LPS treatment did not change FXIII-B production (data not shown). Conversely, rFXIII-A_2 treatment increased Huh7 FXIII-B production nearly 2-fold relative to vehicle (1.98-/+0.86-fold, N=5). rFXIII-A_2 did not increase Huh7 proliferation (Figure 4.7B). We also observed similar FXIII-B production trends in a second hepatocellular carcinoma line (HepG2, data not shown). Collectively, these data confirm our proteomics data suggesting a liver-specific effect of FXIII-A. These data also suggest FXIII-A acts directly on hepatocytes to induce FXIII-B production.

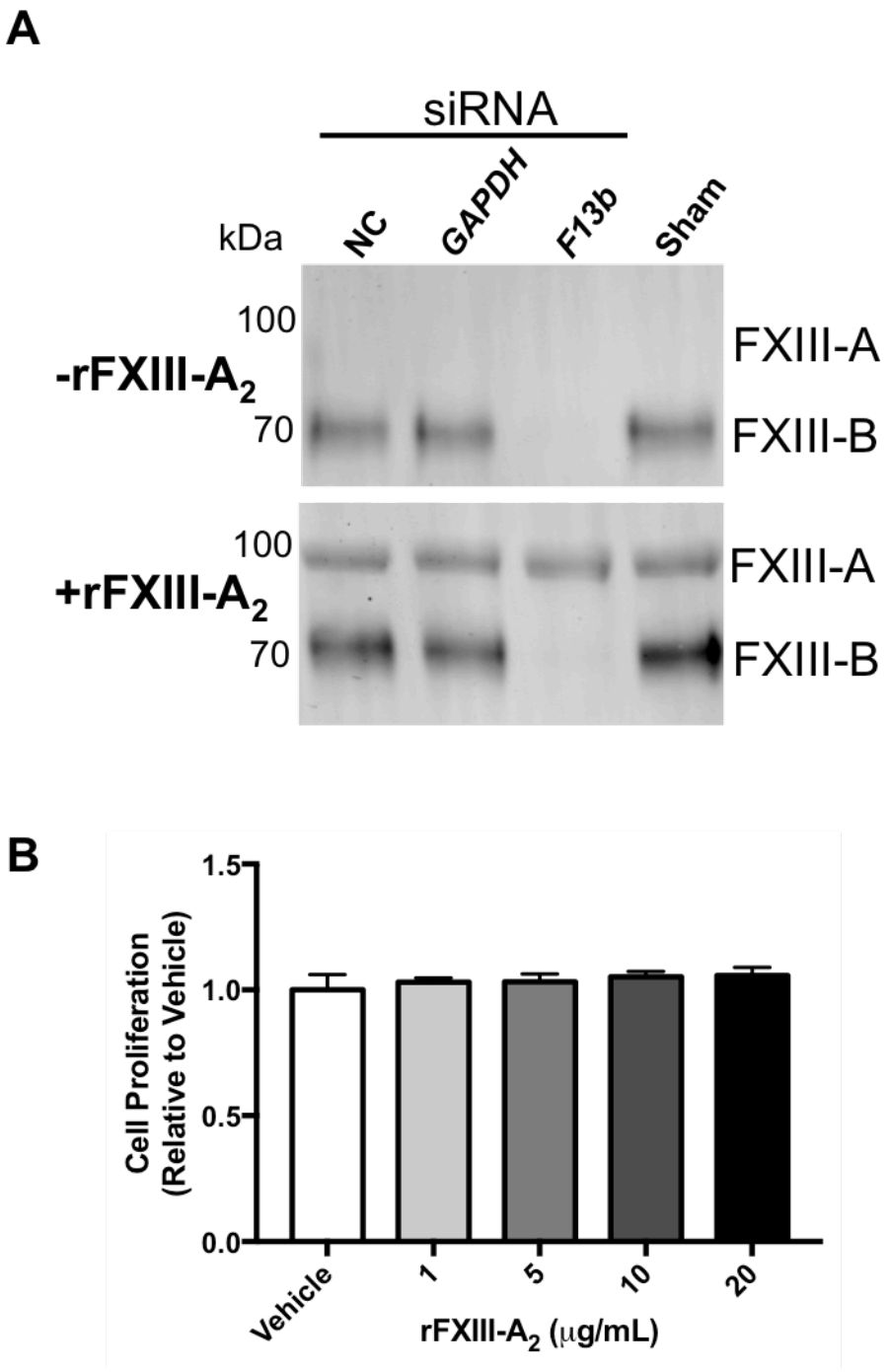

Figure 4.7. rFXIII-A_2 increases FXIII-B production in Huh7 cells. (A) Huh7 culture supernatants from cells grown in the absence or presence of rFXIII-A_2 (20 μg/mL) were collected 3 days following transfection with the indicated siRNAs (NC=Negative Control, Sham did not receive siRNA). Culture supernatant was concentrated 10-fold and FXIII-B visualized using Western blot with anti-FXIII-B antibody. Representative of N=5 Huh7 culture experiments. (B) Huh7 proliferation following treatment with the indicated concentration of rFXIII-A_2 was determined with MTT assay and compared to vehicle.

4.5 Discussion

The role of FXIII-B in stabilizing FXIII-A is well established[4,7,14,18]; however, clinical observations suggesting FXIII-A regulates FXIII-B[7,8] have not been investigated. Our study combines mouse models, proteomics, and cell biology to provide insight into this unique reciprocal regulatory mechanism. First, our data indicate FXIII-A induces FXIII-B production. This finding is important as it exposes unique, inter-tissue crosstalk critical for normal hemostasis and identifies in vivo and in vitro model systems for mechanistic studies. Second, our proteomics studies show that FXIII-A specifically increases RBPs in the mouse liver, but not in the kidney. This exciting finding identifies a tissue-specific effect of FXIII-A. Third, our analysis reveals several upregulated RBPs are post-transcriptional regulators. Together with our observation that FXIII-A does not alter *F13b* transcript levels, these findings suggest FXIII-A may regulate FXIII-B, and possibly other proteins, via a post-transcriptional regulatory mechanism. Further investigation of these findings will extend our understanding of FXIII-A-mediated liver regulation and the role of RBPs in the liver.

Our in vivo studies indicate FXIII-A regulates FXIII-B via two mechanisms. First, we found that FXIII-A slightly reduces FXIII-B clearance (Figure 4.2). This stabilizing effect is consistent with indirect estimates from pharmacokinetic modeling of rFXIII-A_2 infusion in monkeys (uncomplexed FXIII-B $t_{1/2}$ ~4 hours)[18]. This short estimated half-life is surprising given that FXIII-B both profoundly stabilizes FXIII-A and circulates bound to fibrinogen[9] (fibrinogen $t_{1/2}$ ~4 days)[19]. We observed that fibrinogen does not mediate FXIII-B levels (Figure 4.1C), suggesting a unique, uncharacterized FXIII-B clearance mechanism. We[9] and others[20] have observed spontaneous precipitation of isolated FXIII-B in certain experiments. Perhaps uncomplexed FXIII-B is less soluble than FXIII-A-bound FXIII-B, and this reduced solubility

promotes FXIII-B clearance. Alternatively, FXIII-B may possess a clearance-promoting epitope that is obscured in FXIII-A_2B_2. Further study is required to determine how FXIII-B is cleared from plasma.

Second, our data show FXIII-A also induces de novo FXIII-B production. In our model, FXIII-A enters a phase of slow decay 6-9 hours post-infusion, indicating all infused FXIII-A bound circulating FXIII-B. However, FXIII-B levels rise until 30 hours post-infusion (Figure 4.1E-F). This trend in FXIII-B levels is consistent with observations in both monkeys[10] and humans[7]; FXIII-B continues to rise even in the absence of uncomplexed FXIII-A. These findings indicate that the body increases plasma FXIII-B to maintain a pool of excess FXIII-B. Whether this response is to protect newly-synthesized FXIII-A or is related to uncharacterized FXIII-B functions is unknown. Together with the observation that FXIII-A stabilizes FXIII-B, these data reveal a unique pharmacokinetic relationship between FXIII subunits. We are currently developing a novel, indirect pharmacokinetic model of this relationship that incorporates both FXIII-A-mediated FXIII-B stabilization and induction.

Our discovery that FXIII-A upregulates liver RBPs begins to uncover the mechanism of FXIII-A-mediated FXIII-B induction. Although there are more than 1500 RBPs, the function and specificity of these proteins remain largely unknown.[21] RBPs identified in our study are implicated in a wide array of post-transcriptional processes ranging from splicing[22,23] to mRNA transport[24] and translation[25,26], suggesting FXIII-A may modulate post-transcriptional regulation of existing *F13b* RNA in mice. This premise is consistent with our observations that FXIII-A does not increase *F13b* transcription (Figure 4.3) or promote release of FXIII-B protein from intracellular stores (data not shown). However, how the identified RBPs may post-transcriptionally regulate *F13b* RNA is still unclear. Analysis of the human *F13B* mRNA

transcript with RBPmap, a database of RBP binding motifs[27], identifies many RBP binding sites in the *F13B* transcript sequence, including sites for two upregulated liver RBPs (heterogeneous nuclear ribonucleoprotein C and TAR DNA-binding protein 43). Both these proteins are implicated in translational regulation.[25,26] Interestingly, the large number of RBP binding sites on *F13B* mRNA is consistent with RBP-knockdown experiments in HepG2 cells that found dozens of RBPs regulate *F13B* transcript levels (Daniel Dominguez, personal communication). However, this study only assessed *F13B* transcript levels and not *F13B* translation. Moreover, RNA structure, and not sequence alone, is a critical mediator of RBP-RNA interactions.[28] Future study will validate the FXIII-A-upregulated RBPs and identify which regulate *F13B* transcripts.

Beyond regulation of FXIII-B, our findings reveal a newly-recognized role of FXIII-A in modulating liver function. Yet, how FXIII-A-mediated RBP changes regulate the liver is unclear. RBPs have diverse regulatory functions. Genetic deletion of certain FXIII-A-regulated RBPs is embryonic lethal in mice.[29-31] Furthermore, dysregulation of RBPs is implicated in human disease, including neurodegeneration[32], cardiovascular complications[33], and cancer[34]. Interestingly, two of the upregulated liver RBPs identified in our study (polyadenylate binding protein 1 and U6 snRNA-associated Sm-like protein) are also upregulated in human hepatocellular carcinoma.[35] We are currently performing parallel RNAseq and ribosome profiling of $F13a^{+/+}$ and $F13a^{-/-}$ liver RNA to determine the transcriptional and translational consequences of the differential RBP profiles. Future studies will also examine the signaling mechanism connecting FXIII-A and increased liver RBPs. Collectively, these experiments will provide insight into how FXIII-A and RBPs contribute to liver function in health and disease.

FXIII-A is found in both plasma and cellular compartments[16], and the relative contributions of each to liver function is unknown. Interestingly, both principle component analysis and

hierarchical clustering of differentially regulated liver proteins clearly separated the three groups of mice (Figure 4.4B-C). As only plasma FXIII-A was reconstituted in the rFXIII-A_2-infused mice, analysis of differentially-expressed proteins in these mice versus *F13a*$^{+/+}$ mice may illuminate the relative effects of plasma and cellular FXIII-A. For example, we showed that proteins involved in gluconeogenesis/glycolysis and metabolism were upregulated in *F13a*$^{+/+}$ mice and not rFXIII-A_2-treated *F13a*$^{-/-}$ mice. Whether cellular FXIII-A in the liver drives this difference is unclear.

Although *F13b* mRNA is detected in the mouse kidney (Figure 4.3 and [4]), our data suggest this tissue is not the site of FXIII-A-mediated FXIII-B production. Whereas liver proteomics revealed significant upregulation of RBPs in both *F13a*$^{+/+}$ and rFXIII-A_2-treated *F13a*$^{-/-}$ mice, we did not detect similar enrichment in the kidney. Interestingly, a large number of cytoplasmic and mitochondrial ribosome structural proteins were downregulated in rFXIII-A_2-infused mice relative to uninfused (*F13a*$^{+/+}$ and *F13a*$^{-/-}$) mice (Figure 4.5 and 4.6). Ribosome reduction was recently identified as a proteomic biomarker of arsenic-induced cell toxicity[36], and mitochondrial dysfunction is observed during acute kidney injury[37]. Although our rFXIII-A_2 dose (4 mg/kg) was ~10-fold higher than a human therapeutic dose, this dosage was well-tolerated in previous mouse studies.[14] Our dosage is also well below the 22.5 mg/kg required for overt toxicity in monkeys.[10] At this high dose, excess infused rFXIII-A_2 becomes activated, crosslinks circulating fibrinogen, and causes systemic coagulopathy.[10] This observation is consistent with experiments where we infused *F13a*$^{-/-}$ mice with pre-activated rFXIII-A_2 (data not shown). However, at the 4 mg/kg dose of unactivated rFXIII-A_2 we did not detect any intravascular fibrinogen crosslinking (data not shown). Indications of kidney cell stress in rFXIII-A_2-infused mice may signify that

rFXIII-A_2 accumulates in the kidneys, but further investigation is required to support this premise.

Our study has potential limitations. First, we used human, not mouse, rFXIII-A_2 in this study. However, we (Figures 4.1) and others[14] showed mouse FXIII-B enhances circulation of human rFXIII-A_2, indicating rFXIII-A_2 binds mouse FXIII-B. Moreover, we observed similar rFXIII-A_2-mediated FXIII-B induction in both our murine and human model systems. Second, *F13a*$^{-/-}$ mice are only ~90% FXIII-B-deficient. Therefore, we did not perform our FXIII-B subunit clearance studies (Figure 4.2) in a completely FXIII-B-free system. However, we corrected for background FXIII-B when determining the FXIII-B present at each time-point. Lastly, the proteomic changes in both liver and kidney tissue require further validation. However, the strength of liver RBP enrichment, and the use of both genetic (*F13a*$^{+/+}$) and pharmacologic (rFXIII-A_2-infused *F13a*$^{-/-}$) models of FXIII-A sufficiency strongly suggest FXIII-A upregulates these proteins. Planned ribosome profiling and Western blotting experiments will further validate these proteins and identify additional proteins of interest.

In summary, our data expose the unique, inter-tissue, reciprocal regulation of FXIII-A and -B subunits. Although other examples of reciprocal regulation exist in biology, these are largely at the intracellular[11,38] or whole-cell[12,39] levels. To our knowledge, we are the first to describe an inter-tissue, systemic, regulatory relationship between protein subunits. FXIII-B stabilizes FXIII-A, and FXIII-A promotes FXIII-B stability and production. Our observation that FXIII-A upregulates liver RBPs provides insight into potential post-transcriptional regulation of FXIII-B and uncovers a newly-recognized liver-regulatory function of FXIII-A. Continued study will refine the mechanism of FXIII-A-mediated FXIII-B production and expand our understanding of how FXIII-A and RBPs regulate liver function in health and disease.

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CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

5.1 Summary and future directions

Current anticoagulant therapies target coagulation enzymes upstream of fibrin formation and are associated with a risk of bleeding.[1] Basic studies examining the fundamental mechanisms underlying coagulation are therefore required to identify new therapeutic strategies that will reduce thrombosis yet preserve physiologic hemostasis. The studies presented in this dissertation investigate the interactions between factor XIII (FXIII), fibrin(ogen), and red blood cells (RBCs) at the terminal end of the coagulation cascade. Our findings greatly expand our understanding of how this axis functions in coagulation and the regulation of its components. Importantly, these studies emphasize the importance of basic biological studies to support translational research efforts.

In Chapter 2, we determined the mechanism of FXIIIa-mediated RBC retention in clots.[2] This study was an important follow-up to our discovery that FXIIIa promotes RBC retention in clots.[3] We used several assays to first test the hypothesis that FXIIIa directly crosslinked RBCs to the fibrin network. We did not find any evidence of direct RBC-crosslinking to clots. Instead, we found that FXIIIa promotes RBC retention through its fibrin crosslinking function. Interestingly, our experiments using recombinant fibrinogen variants and selective FXIIIa inhibitor dosing revealed that crosslinking of the fibrin α-chains, but not γ-chains, was crucial for normal RBC retention in clots. This finding suggests that α-chain-dependent stiffening[4-7] of the fibrin network is necessary to trap RBCs within a contracting clot. If the fibrin is insufficiently

crosslinked, the RBCs are able to escape the clot, resulting in reduced RBC retention and reduced clot size.

The implications of this study are multifaceted. First, we identified a pathophysiologic function of α-chain crosslinking in mediating RBC retention. The role of α-chain crosslinking in modifying clot biophysical properties has been a recent focus of investigation[5-7], and our findings provide an important connection between those studies and the biological function of this crosslinking in vivo. Second, these findings reveal the mechanism of FXIIIa-mediated RBC retention in clots, an important advancement in the development of therapies targeting FXIIIa to reduce venous thrombosis.[3] Interestingly, our observation that γ-γ dimer and α-chain crosslinking are inhibited at different levels of FXIIIa inhibitor suggest that complete inhibition of FXIIIa may not be necessary to reduce venous thrombus size. The dose of FXIIIa inhibitor could be titrated to inhibit primarily α-chain crosslinking and leave γ-γ dimer formation intact. Although this titration would be clinically difficult and the contribution of γ-γ dimer formation to clot mechanical strength is limited[5-7], preservation of some FXIIIa activity while still reducing thrombus size may reduce the risk of treatment-associated bleeding.

In Chapter 3, we characterized the binding interaction between zymogen FXIII-A_2B_2 and fibrinogen.[8] This study extended our prior observation that this interaction is a crucial mediator of RBC retention in murine clots[3] into a human system. Our use of several recombinant fibrinogen variants and multiple assays examining FXIII-fibrinogen binding (e.g. glycine precipitation, surface plasmon resonance, FXIII activation/fibrin crosslinking) not only revealed that the same fibrinogen residues (γ390-396) implicated in mouse FXIII-fibrinogen binding interactions[3] mediate this interaction in humans, but also clarified a controversial topic in the literature. The FXIII-A_2B_2 binding site on human fibrinogen has been unclear since the

interaction was first discovered over 30 years ago.[9] Prior studies implicated the alternatively spliced γ′-chain[10,11] and residues in the αC region as FXIII-A_2B_2 binding sites.[12] Our study used recombinant fibrinogen variants to investigate these prior observations and clarify the fibrinogen residues involved in this interaction. We showed that the γ′-chain is not necessary for FXIII-A_2B_2 binding and exposed fibrinogen γ-chain residues 390-396 as crucial mediators of this interaction. Importantly, we also demonstrated that the FXIII-B subunits bind fibrinogen and that the pool of excess FXIII-B in plasma circulates bound to fibrinogen.

These findings are important for several reasons. First, we clarified a controversial literature and were able to develop a model of FXIII-fibrinogen binding and activation that incorporates findings from decades of studies. Second, we identified the importance of fibrinogen γ-chain residues 390-396 in binding FXIII-A_2B_2. As we previously identified this interaction as a key regulator of venous thrombus size[3], our findings expose another avenue to target FXIII to reduce venous thrombosis. Instead of inhibiting FXIIIa activity, a therapy could instead target the FXIII-fibrinogen interaction. This would delay FXIII activation and reduce thrombus size, while still maintaining a patient's ability to eventually crosslink a clot. Given that mice with disrupted FXIII-fibrinogen binding exhibit reduced thrombus size in venous thrombosis models[3], yet do not have a bleeding phenotype in hemostasis models, suggests this may be a viable anti-thrombotic strategy. However, therapeutically targeting protein-protein interactions is difficult[13], and further study is required to identify the precise FXIII-B and fibrinogen involved in binding. Chemical crosslinking with mass spectrometry, hydrogen-deuterium exchange, site-directed mutagenesis, and molecular modeling experiments could further clarify the respective FXIII and fibrinogen binding regions and implicate specific residues involved in binding. Such studies would inform efforts to therapeutically target FXIII, and therefore warrant future investigation.

In Chapter 4, we investigated clinical observations that suggest FXIII-A regulates FXIII-B.[14,15] Using a diverse approach incorporating mouse models, proteomics, and cell culture, we found that FXIII-A both stabilizes FXIII-B in circulation and induces de novo liver FXIII-B production. FXIII-A did not change liver *F13b* transcript levels, but increased liver RNA-binding proteins (RBPs). This finding suggests FXIII-A may signal the liver to alter post-transcriptional regulation of *F13b* transcripts. We did not observe a similar RBP increase in the kidney, suggesting the effect of FXIII-A is liver-specific. Consistent with this premise, cultured human hepatocellular carcinoma cells produced FXIII-B and exhibited increased FXIII-B production when treated with FXIII-A.

This study is exciting as it identifies a unique, inter-tissue reciprocal regulatory relationship between two protein subunits. These findings also suggest a liver-regulatory role of FXIII-A. Still, many mechanistic questions remain. Most importantly, the signaling pathway connecting FXIII-A with increased liver RBP and plasma FXIII-B levels is unclear. Although $Tie2^{+}$-Cre RiboTag pulldown/RNAseq experiments showed *F13b* mRNA was enriched in liver parenchymal cells, it is possible that FXIII-A interacts with the liver endothelium, which in turn signals the parenchyma. However, our in vitro data suggest FXIII-A signals directly to hepatocytes. The hepatocyte receptor for FXIII-A remains unknown, but preliminary in vitro experiments suggest FXIII-A crosslinking activity is not required to induce FXIII-B. Another possibility is that FXIII-A does not directly signal the liver, and instead the liver senses the decrease in uncomplexed plasma FXIII-B following rFXIII-A_2 treatment. Ongoing experiments will blend our proteomics data with transcriptomics, ribosome profiling[16], 3D hepatocyte spheroid culture[17], and in vitro and in vivo siRNA knockdowns to further define the FXIII-A signaling mechanism. Furthermore, data from the $Tie2^{+}$-Cre RiboTag pulldown/RNAseq

experiments will also help identify any endothelial- versus hepatocyte-specific effectors. Collectively, these studies will define the liver-specific effect of FXIII-A and provide insight into how FXIII-A and RBPs affect liver function.

In summary, this dissertation clarifies the interactions between FXIII, fibrin(ogen), and RBCs in coagulation. The studies presented here inform continued efforts to therapeutically target FXIII and begin to characterize a newly-recognized regulatory function of FXIII-A.

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