Supplemental Materials and Methods

Viral integration number in pFVIII-iMKs

DNA was extracted from pFVIII-iMKs using DNeasy® Blood and Tissue Kit (Qiagen). Viral integration was determined via Lenti-X™ Provirus Quantitation Kit (Clontech). Total RNA was isolated from $2x10^6$ iMKs using RNeasy Micro Kit (Qiagen). Total RNA (500 ng) from each sample was used to generate cDNA using High Capacity Reverse Transcription Kit (ABI). Message RNA level of target genes was determined by quantitative RT-PCR using Taqman Probes with TaqMan® Fast Advance Master Mix (ABI) via 7900HT Real-Time Cycler. Taqman Probes used were: FVIII (*F8*) Hs00252034_m1, integrin alpha-IIb (*ITGA2B*) Hs01116228_m1, Tata Binding Protein (*TBP*) Hs00427620_m1, and Platelet factor 4 (*PF4*) Hs00427220_g1. Relative transcript levels were quantified using 2^-ΔΔCt and calculated against TBP standard[1]. All kits were used according to manufacturer's instructions

pFVIII antigen level in pFVIII-iMKs

FVIII antigen levels in iMKs were determined using an ELISA for hFVIII as previously described[2,3]. Levels were compared to recombinant full-length hFVIII (Advate, Shire) and recombinant hBDFVIII (Xyntha, Pfizer).

Apoptotic studies of iMKs

Analysis of apoptosis in the iMKs was performed through investigation of TUNEL-labeling using APO-bromodeoxyuridine (BrdU) TUNEL Assay Kit (Invitrogen). During TUNEL protocol, samples were stained and analyzed via flow cytometry using APC-labeled anti-CD42b (BD Biosciences)[4] and Alexa Fluor 488 labeled Anti-BrdU for degree of apoptosis[2]. All samples were compared to non-transduced controls. To further investigate apoptosis, $1x10^6$ iMKs samples were incubated with 5 µl annexin V FITC, 10 µl CD42b APC (BD Biosciences) in 100 µl Tyrode's buffer (Sigma) with 0.1% bovine serum albumin (BSA) at room temperature (RT) for 30 minutes. Post incubation cells were washed twice and then resuspended in Tyrode's buffer with 0.1%. Annexin V and CD42b were analyzed via flow cytometry[5].

Flow cytometry analysis of iMks

Day 6 pFVIII-iMKs, controls, and mouse whole blood were stained and analyzed on FACSCanto (BD Biosciences) as previously described[4,6]. Antibodies used in these studies are listed in Supplemental Table 1.

Supplement Table 1. **Summary of commercial antibodies used in this paper**. All are characterized in this Table except for the two FVIII inhibitors, ESH8 and GMA-802 – including their indicated usage.

Target	Source	Reactivity	Label	Company	Catalog Number	Usage
CD42b	Monoclonal Mouse	Human	APC	BD Biosciences	551061	Flow Cytometry
CD42b	Monoclonal Mouse	Human	PE	BD Biosciences	555473	Flow Cytometry
CD41a	Monoclonal Mouse	Human	APC	BD Biosciences	559777	Flow Cytometry
CD41a	Monoclonal Mouse	Human	PE	BD Biosciences	555467	Flow Cytometry
CD42a	Monoclonal Mouse	Human	PerCP	BD Biosciences	340537	Flow Cytometry
CD42a	Monoclonal Mouse	Human	PE	BD Biosciences	558819	Flow Cytometry
Annexin V	Bacteria	Human	FITC	BD Biosciences	556420	Flow Cytometry
PAC-1	Monoclonal Mouse	Human	FITC	BD Biosciences	340507	Flow Cytometry
BrdU [PBR-1]	Monoclonal Mouse	Human	Alexa Fluor 488	Invitrogen	A23210	Flow Cytometry

PE: Phycoerythrin
APC: Allophycocyanin
FITC: fluorescein isothiocyanate

iMK responsiveness to an agonist

To examine in vitro responsiveness of iMKs, $1x10^6$ iMKs were incubated with 1 unit of thrombin/ml (T8885-1VL, Invitrogen), in Tyrode's buffer with 0.1% BSA in a final volume of 100 µl containing 1:2000 APC-labeled anti-CD42b antibody and FITC-labeled PAC-1 as previously described[7]. The cells were incubated at 37°C for 30 minutes. Post incubation, 500 µl of ice-cold 0.1% Tyrode's buffer was added. PAC-1 and CD42b binding were then analyzed via flow cytometry.

Supplement Table 2. Parametric analysis of ROTEM studies in Figures 2A and 2B. * = p<0.05, ** = p<0.001, and *** = p<0.0001 comparing all groups to Null control using two-way ANOVA. N/A = Values could not be determined due to sample not clotting. Clotting time (CT) is the time from the start of the assay until clotting begins. Clot formation time (CFT) is the time from the start of clotting until a firmness of 20 mm is reached. Maximum clot firmness (MCF) is in mm. α-angle is the angle measuring steepness at the point of initial clot formation.

	WT	h38	1%	5%	20%	Null
CT (min)	3.1 ± 1.4***	9.5 ± 1.3***	23.8 ± 0.9	14.3 ± 2.9*	11.7 ± 0.8**	24.3 ± 6.4
CFT (min)	1.8 ± 0.9	2.8 ± 0.4	13.1 ± 6.0	10.9 ± 7.1	5.4 ± 0.9	N/A
MCF	55.6 ±4.0	52 ± 3.7	19.0 ± 13.8	40.0 ± 9.1	51.7 ± 2.9	N/A
α-angle	70 ± 8.3	58.3 ± 3.8	16.7 ± 11.2	34.33 ± 18.8	40.67 ± 3.8	N/A

Supplement Table 3. Parametric analysis of ROTEM studies in Figures 4A and 4B. * = p<0.05, ** = p<0.001, and *** = p<0.0001comparing all groups to Null using two-way ANOVA. N/A = Values could not be determined due to sample not clotting.

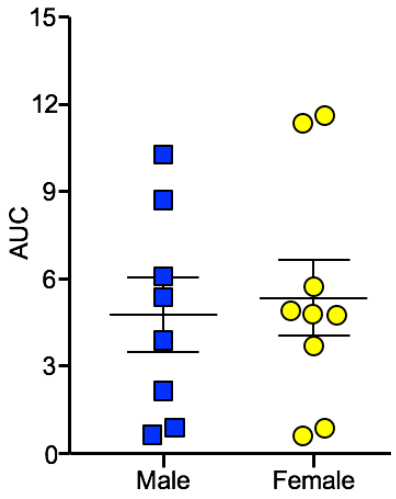
	h38	10 nM Novo7	25 nM Novo7	10nM + P38	Null
CT (min)	9.4 ± 1.4**	13.4 ± 3.8*	7.2 ± 0.4***	7.6 ± 0.3***	23.9 ± 8.2
CFT (min)`	3.1 ± 0.2	7.5 ± 0.1	94.0 ± 0.5	93.1 ± 0.5	N/A
MCF	53.7 ± 4.1	40.5 ± 2.1	56.7 ± 3.5	55.3 ± 3.1	N/A
α-angle	56.7 ± 2.5	34.0 ± 28.2	61 ± 11.5	56.6 ± 4.5	N/A

Supplement Table 4. Parametric analysis of ROTEM studies in Figures 5A and 5B. The (A) and (B) for each column refers to Figures 6A and 6B, respectively. * = p<0.05 and ** = p<0.001 comparing all groups to Null using two-way ANOVA. N/A = Values could not be determined due to sample not clotting.

	BD(A)	RH(A)	NTC(A)	BD(B)	RH(B)	NTC(B)
CT (min)	33.7 ± 16.9	20.8 ± 6.7	37.5 ± 21.9	12.8 ± 1.9*	8.9 ± 1.1	**31.3 ± 11.5
CFT (min)	N/A	14.2 ± 10.1	N/A	7.6 ± 2.5	5.0 ± 2.4	N/A
MCF	23.0 ± 16.2	32.3 ± 15.9	N/A	49.3 ± 9.3	52.5 ± 10.1	17.0 ± 15.4
α-angle	8.3 ± 3.1	24.3 ± 13.7	N/A	32.0 ± 8.0	46.3 ± 13.9	11.5 ± 7.8

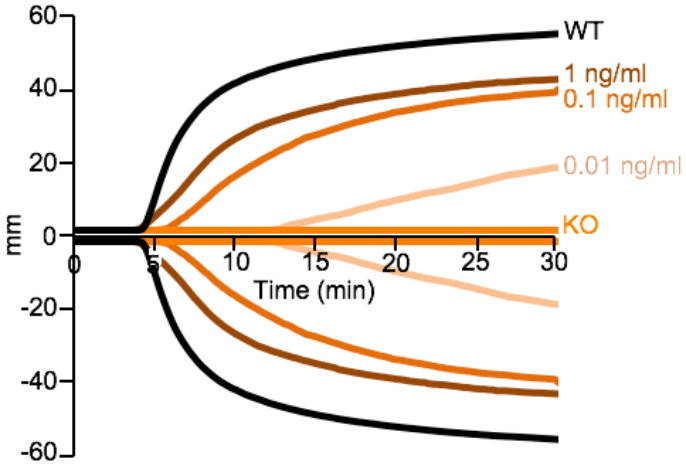
Supplement Table 5. Parametric analysis of ROTEM studies in Figure 5C. * = p<0.05, ** = p<0.001, and *** = p<0.0001 comparing all groups to Null using two-way ANOVA. N/A = Values could not be determined due to sample not clotting.

	BD	RH	BD + 10 nM	RH + 10 nM	Null
CT (min)	16.9 ± 0.5***	14.1 ±4.9***	8.7 ±1.1***	7.5 ± 0.9***	40.1 ±7.5
CFT (min)	17.5 ± 3.0	9.9 ± 2.9	6.8 ± 2.2	6.4 ± 2.1	N/A
MCF	28.0 ± 4.0	40.0 ± 6.0	45.8 ± 7.3	45.3 ± 7.1	N/A
α-angle	18.0 ± 1.0	27.0 ± 5.0	36.5 ± 6.6	37.5 ± 7.4	N/A



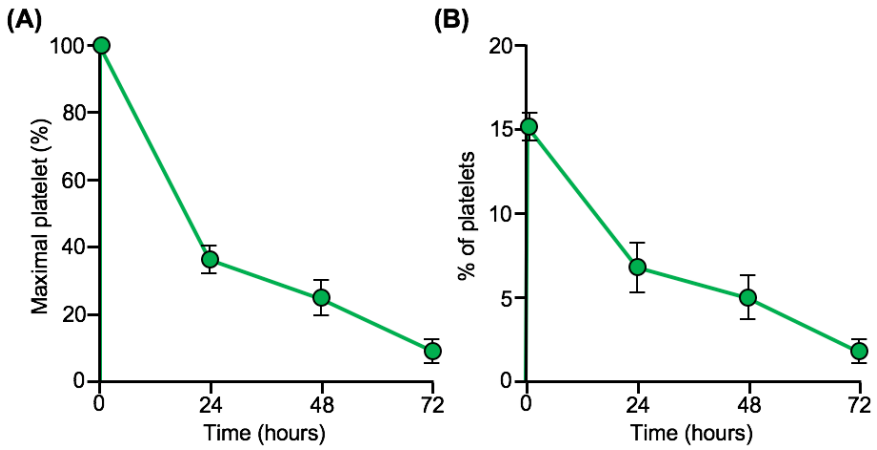
Supplement Figure 1. Sex differences in the $FeCl_3$ carotid artery model.

$FVIII^{null}$ littermate mice of both sexes were studied in the $FeCl_3$ carotid artery injury model at 7-8 weeks of age, each mouse receiving $4X10^6$ p38 platelets intravenously. AUC for total blood flow over the observed 30 minutes following injury are shown as are the mean ± 1 SEM. p=0.74 using a two-tailed Student t test.



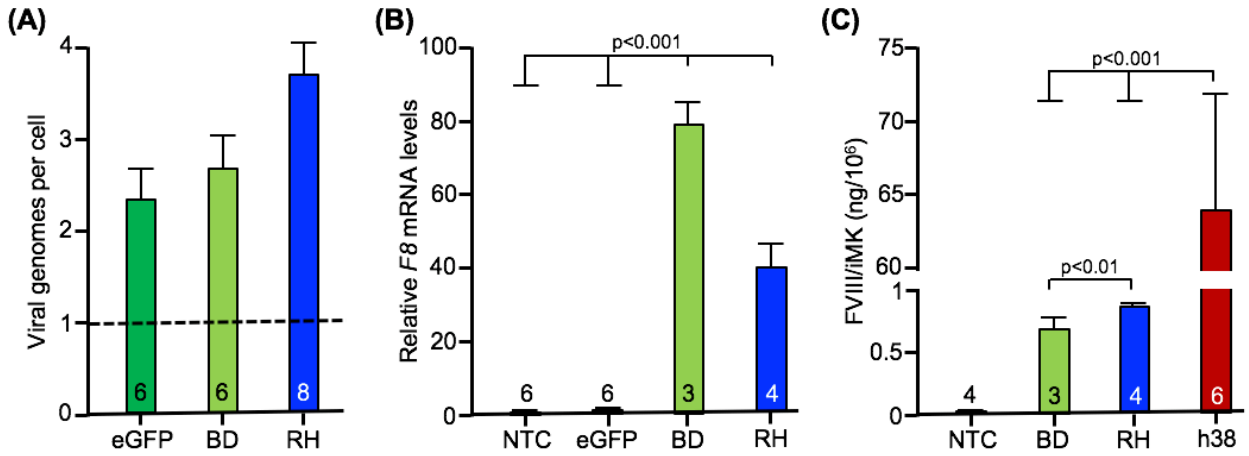
Supplement Figure 2. Addition of soluble rFVIII improves clotting in in vitro ROTEM assay.

Studies similar to Figure 1 except that different concentrations of rFVIII were added to 110 µl of $FVIII^{null}$ (KO) whole blood to achieve the concentrations noted. Each curve represents 4 independent studies.



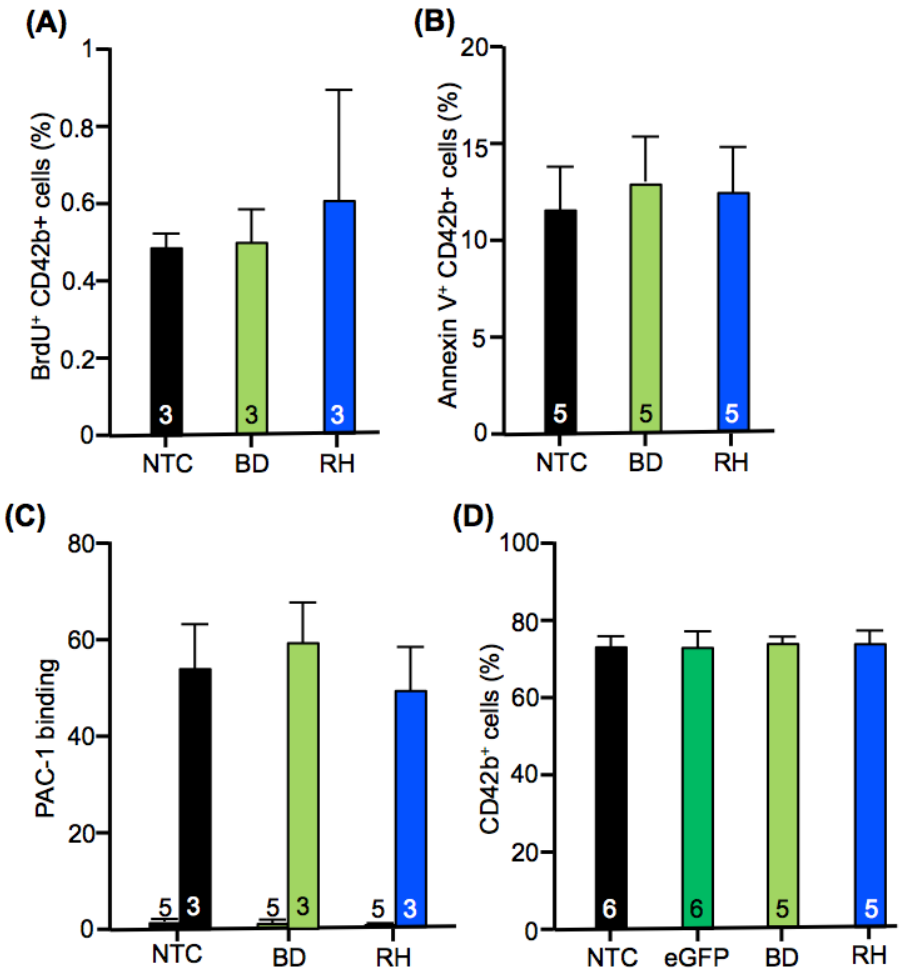
Supplement Figure 3. Half-life of isolated mouse platelets infused into FVIII^{null} mice.

(**A**) Half-life based on infusing 1-4x10^8 human hαIIb-expressing mouse^8 platelets into FVIII^{null} mice. Total hαIIb-expressing platelets were tracked over 72 hours via flow cytometry. Measurement of remaining infused human platelets normalized to level at 5-minutes post-infusion. Mean ± 1 SEM are shown. N = 4 independent studies. (**B**) Calcein-AM 4x10^8 loaded WT platelets infused into FVIII^{null} mice. Percent of circulating platelets that contained calcein are shown. Mean ± 1 SEM are shown. N = 3 independent studies.



Supplement Figure 4. FVIII expression in iMks.

(**A**) DNA from 10^6 iMks was collected and analyzed for lentiviral integration. Viral genomes per cell were relative to non-transduced control (NTC, dashed line). Mean ± 1 SEM are shown with the number of independent experiments indicated in each bar. P values determined by one-way ANOVA. (**B**) 500 ng of total RNA was analyzed for *F8* message. NTC and eGFP-expressing iMks were used as negative controls. Mean ± 1 SEM are shown with number of independent experiments indicated in each bar. P values determined by one-way ANOVA. (**C**) $2x10^6$ iMKs were lysed to extract cellular content. Whole cell lysates were analyzed using human FVIII ELISA. Mean ± 1 SEM are shown with number of independent experiments indicated in each bar. P values determined by one-way ANOVA. BD = pBDFVIII-iMKs, RH = pBDFVIII^{RH}-MKs, and h38 = Line h38 mouse megakaryocytes.



Supplemental Figure 5. pFVIII effects on iMks.

(**A**) TUNEL Assay Kit-stained for BrdU and co-stained for CD42b to examine for pFVIII-iMKs apoptosis. Mean ± 1 SEM are shown with number of independent experiments indicated in each bar. No difference was noted by ANOVA analysis between the examined iMKs. (**B**) Annexin V binding as an indicator of apoptosis of iMKs. Mean ± 1 SEM are shown with number of independent experiments indicated in each bar. No difference was noted by ANOVA analysis between the examined iMKs. (**C**) Agonist responsiveness of iMKs to 1 unit of thrombin/ml and measuring FITC-labeled PAC-1 antibody binding. For each iMK, the left bar is pre-thrombin and the right is post-thrombin activation. Mean ± 1 SEM are shown with number of independent experiments for each iMK condition is shown. $P<0.001$ for pre- versus post-thrombin activation for each iMK condition by one-way ANOVA, but no difference was noted by ANOVA analysis between the examined iMKs. (**D**) Studies of iMK injury as indicated by loss of CD42b signal in the CD41+ iMKs. Percent of CD41+ iMKs that were also $CD42b^+$ are shown. Mean ± 1 SEM are

shown with number of independent experiments indicated in each bar. No difference was noted by ANOVA analysis between the examined iMKs.

References

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