Integration site analysis
The 2bF9 transgene integration site was determined by inverse PCR. Mouse genomic DNA was digested with *TaqI*, *MspI*, or *AciI* followed by ligation into covalent circles using T4 DNA ligase. Two sets of nested-PCR primers were used for the inverse PCR reactions (For 5′ end: external forward, 5′-AACGA TTCGCAGTTAATCCTGG-3′; external reverse, 5′-TTCGCTTTCAAGTCCCTGTT C-3′; internal forward, 5′-ACCATCCCTTCAGACAGGATCA-3′; internal reverse, 5′-TTGAAGCACTCAAGGCAAGCT-3′. For 3′ end: external forward, 5′-AGCCT CAATAAAGCTTGCCTTG-3′; external reverse, 5′-TGATCCCATCTTGTCTTCGT TG-3′; internal forward, 5′-TTCAAGTAGTGTGTGCCCGTCT-3′; internal reverse, 5′-CAGCTGCCTTGTAAGTCATTGG-3′). PCR products were purified for DNA sequencing, and the sequences were matched with the mouse genome by BLAST to identify the insertion site.

APTT assay
APTT assay was performed on a STart 4 Hemostasis Analyzer (Diagnostica Stago, France). 75ul each of 1:10 Diluted platelet lysates, FIX-deficient plasma (Haematologic Technologies) and APTT reagent (Diagnostica Stago) were mixed and incubated at 37°C for 3 minutes. Then 75 µl of 25 mM $CaCl_2$ was added and clotting time was recorded.

Immuno-staining of FIX
Platelets and mononuclear cells from 2bF9 transgenic and FIX^{null} mice (parallel control) were stained with immunoflurescent-or immunogold-labeled anti-hFIX antibodies as previously described.[1] For confocal studies, an affinity purified goat anti-hFIX GAFIX-AP (Affinity Biologicals Inc.) and rabbit anti-VWF (DAKO, Glostrup, Denmark) were used as primary antibodies. AlexaFluor 488-conjugated donkey–anti-goat IgG antibody and AlexaFluor 594-conjugated donkey–anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) were used as secondary antibodies. A Leica TCS SP2 Confocal Laser Imaging System (Leica Microsystems) in the Imaging Core of the Medical College of Wisconsin (Fig. 3A–F) and an Olympus FV1000MPE Multiphoton Laser Scanning Microscope (Olympus, Center Valley, PA) in the Blood Research Institute (Fig. 3G–L) were used to acquire microphotographs from slides mounted in Vectashield (Vector, Burlingame, CA).

EM studies were performed by Clive W. Wells in The Electron Microscopy Facility of the Medical College of Wisconsin. Goat–anti-hFIX GAFIX-AP and mouse–anti-VWF monoclonal antibodies (332.3 and 344.3, produced by our laboratory) were used as primary antibodies, and probed with rabbit–anti-goat IgG (5 nm) and rabbit–anti-mouse IgG (10 nm) colloidal gold probes, respectively. The images were captured by a Hitachi H600 TEM (Hitachi High-Technologies) operating at 75 kV.

For immunohistochemical (IHC) staining, tissues were collected from animals after perfusion and fixed in 10% buffered formalin. Fixed tissues were paraffin-embedded and sectioned. The hematoxylin and eosin (H & E) staining of tissue sections was performed by Barbara Fleming in the Histology Core at Medical College of Wisconsin. Tissue sections were stained using ImmunoHisto Peroxidase Detection Kit (Pierce) and Vectastain ABC (Vector Laborataries, Burlingame, CA) with minor modification of the manufacturer's instructions. Briefly, paraffin-embedded mouse tissue sections were dewaxed, rehydrated, permeabilized with 5% Triton X-100, and blocked in Universal Blocker for 30 minutes. The endogenous peroxidase was

quenched using Peroxidase Suppressor. Goat–anti-hFIX antibody GAFIX-AP was used as a primary antibody at 5 μg/ml for 3 hours. Biotinylated mouse–anti-goat IgG antibody (Pierce) was used as a secondary antibody at 1:200 for 30 minutes. A preformed HRP-labeled Streptavidin/Biotin enzyme Complex (ABC) was used to increase the sensitivity of IHC staining at 1:500 for 30 minutes. The DAB substrate was used for colorimetric detection. Nonspecific isotype control antibodies served as negative control. Images were acquired on a Nikon Eclipse E600 Microscope (Nikon) with a 40x objective/0.75 aperture, equipped with a SPOT Insight camera Model II.2 Color Mosaic and SPOT acquisition software (Diagnostic Instruments, McHenry, IL)

Rotational thromboelastometry (ROTEM) analysis
Blood was collected from the vena cava and mixed with 3.8% sodium citrate at the ratio of 9:1. Three hundred microliters of citrated whole blood was added into a pre-warmed ROTEM cup and the clotting analysis was initiated when 20 μl 0.2 M $CaCl_2$ was added.

Quantitative real-time PCR
Anesthetised mice were subjected to whole body perfusion with sterile phosphate buffered saline to remove as much blood as possible. Organs were harvested and immediately placed in liquid nitrogen. Total RNA was isolated with Trizol (Invitrogen) reagent and treated with TURBO DNase (Ambion, Austin, TX) following the manufacturer's instructions, to eliminate DNA contamination. Then RNA was further purified using RNeasy Mini Kit (Qiagen). 400 ng RNA was used for reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) and random primers (Invitrogen). Real-time PCR was performed using SYBR GreenER (Invitrogen) on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). Primers specific for 2bF9 transgene were 5′-CAGCGCGTGAACATGATCA-3′ and 5′-CCTCTTTGGCCGATTCAGA-3′. Relative expression values were normalized to the housekeeping gene ribosomal protein L19 (*Rpl19*).[3;4]

Figure S1. Quantitative real-time PCR analysis of 2bF9 transgene in mouse organs
2bF9 mRNA was measured in BMT FIX^{null}/2bF9-H mouse organs (heart, brain, lung, liver, kidney, spleen, and platelets). FIX^{null} mouse liver (FIX^{null} liver) and platelets (FIX^{null} plts) were used as negative controls; 2bF9-H platelets (2bF9-H plts) served as a positive control. Housekeeping gene *Rpl19* was used for normalization. The data represent mean ± SD of 3 mice.

Figure S2. IHC staining of representative mouse organs
Fixed FIX^{null}/2bF9-H (A, B, D, E, G, and H), 2bF9-H (J and K), and FIX^{null} (C, F, I, and L) mouse organ sections were immunostained for FIX (A, C, D, F, G, I, J, and L) or with an isotype control (B, E, H, and K). No FIX staining was found in the FIX^{null}/2bF9-H mouse organs (A, D, and G). 2bF9-H BM was used as a positive control. Bars, 50 μm.

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Figure S1

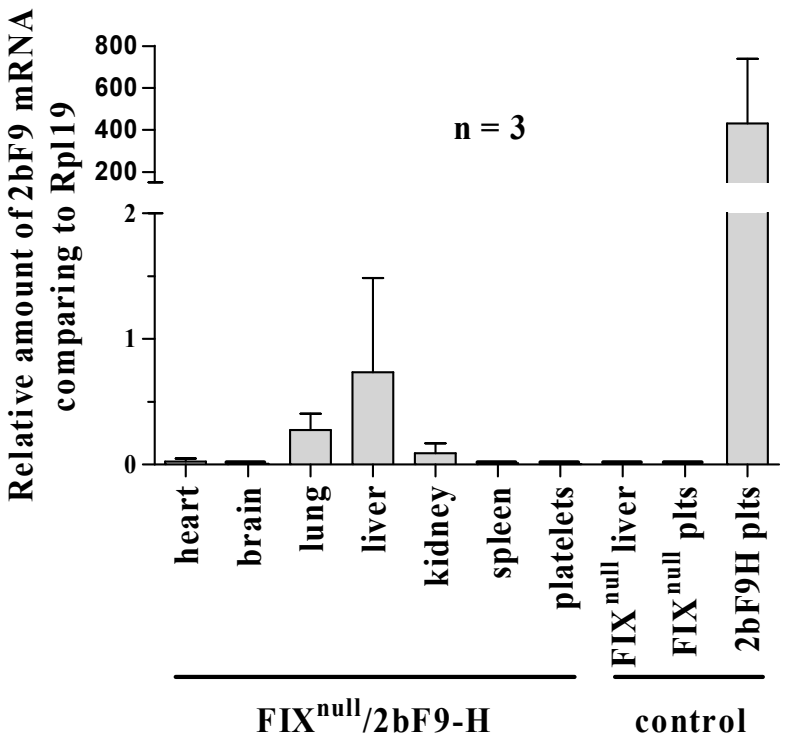
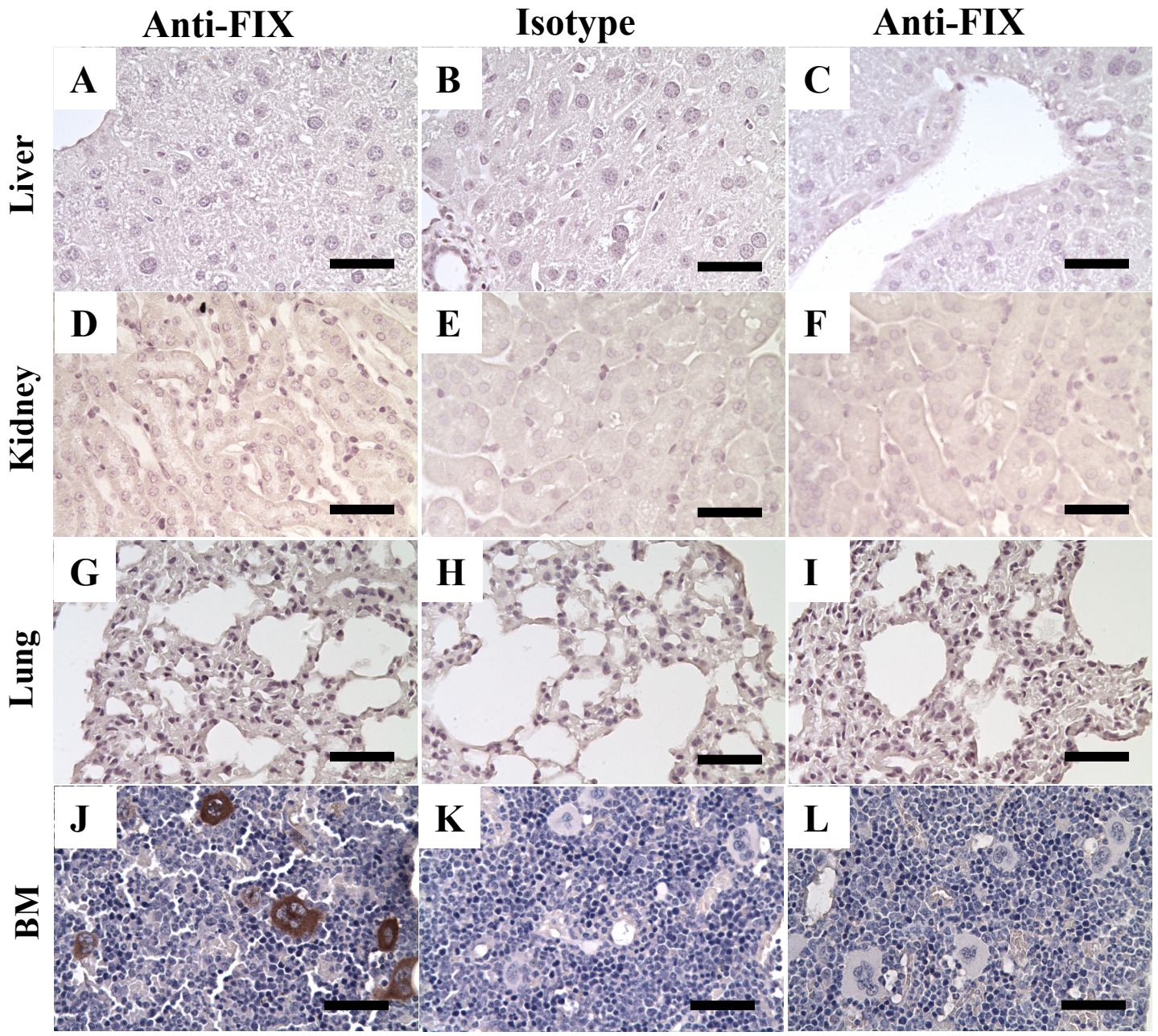

Relative amount of 2bF9 mRNA comparing to Rpl19
800
600
400
200
2
1
0
n = 3
heart
brain
lung
liver
kidney
spleen
platelets
FIX^null liver
FIX^null plts
2bF9H plts
FIX^null/2bF9-H
control

Figure S2


Anti-FIX
Isotype
Anti-FIX
Liver
Kidney
Lung
BM
A
B
C
D
E
F
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H
I
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K
L