Supplemental material to:

**Title:** Defenestrated endothelium delays liver directed gene transfer in Hemophilia A mice

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Table S1: Sequences of primers used in this study

| Primer           | Forward                        | Reverse                        |
|------------------|--------------------------------|--------------------------------|
| eNOS             | CCAGCACCAGGAGGCTAGC            | AGGGTGTCGTAGGTGATG             |
| Ehd3             | CGCCGTGCTTGAAAGTATCAG          | ATAAATCGGTCCACCCGCTC           |
| Endosialin/Cd248 | TTCAACCTGTTTCCAGCGA            | CTGAAGGCTGTTCACGCAC            |
| Gata4            | ACCCTGGAAGACACCCCCAAT          | CCACAGGCATTGACAGGTGA           |
| ICAM1            | GTT TAA AAA CCA GAC CCT GGA ACT| CGT CTG CAG GTC ATC TTA GGA G  |
| Id1              | CGCTCAGCACCCTGAACGCGC          | TCCGCTGGCTGCGGTAGTG            |
| Maf              | AGGATGCTCTCAAGAAGTGGC          | GGTCTCCACCGGTTTCTTTT           |
| Stabilin2        | CACTATGCTGGGGATGGACG           | GGGAGCGTAGGTGGAATACG           |
| Factor VIII      | CTTCACTCCAGGGAGGACTA           | TCCACTTGCAACCATTGTTTGT         |
| 18S              | CGG CTA CCA CAT CCA AGG AA     | GCT GGA ATT ACC GCG GCT        |

Table S2: Primary antibodies used in IHC/IF assays in this study

| Antibody         | Company                                | Concentration |
|------------------|----------------------------------------|---------------|
| GFP (anti rabbit)| Abcam, Cat # ab290,                    | 1:500         |
| CD31 (anti Rabbit)| Novus biologicals, Cat # NB100-2284,   | 1:100         |
| EpCAM (anti mouse)| Biolegend, Cat # 102414,               | 1:150         |
| Lyve1 (anti rabbit)| ReliaTech GmbH (Germany) Cat # 103_PA50| 1:150         |
| Caspase 3 (anti rabbit) | Abcam, Cat # ab13847 | 1:100         |

Table S3: Liver enzyme analysis of control and F8TKO mice 1 month post AAV8-GFP treatment.
Liver enzymes | Control (N=4) | F8TKO (N=4)
--- | --- | ---
ALT | 64 | 69
AST | 55 | 48
Total Bilirubin | 0 | 0
Direct Bilirubin | 0.1 | 0.15

Supplemental Method:

Real time intravital imaging:

Mice were anesthetized with an intraperitoneal (i.p.) injection of 100 mg kg⁻¹ of body weight ketamine HCl (100 mg ml⁻¹; Henry Shein Animal Health; Dublin, OH) and 20 mg kg⁻¹ of body weight xylazine (20 mg ml⁻¹; LLOYD Laboratories; Shenandoah, IA) and repositioned supine. The overlying skin and fat were removed, exposing the right lobe of the liver. Describes the surgical procedure in detail. Intravascular fluorescent dyes used included 200 g of TXR dextran and 100 g of AF488-anti CD31 Ab. TXR dextran (MW 70,000) was used to visualize blood flow through the liver sinusoids, while CD31 was used to visualize liver sinusoidal endothelial cells. The Nikon MPE multi-photon excitation microscope was used for the microscopy.

Quantification of SEM Images:

Measurement of total number of fenestrae/FOV: To determine the total number of endothelial cell fenestre, the total number of fenestrae (small pore like structures) in each FOV was counted and repeated with at least 10 FOV in each genotype studied (N=3).
Measurement of pore grouping /FOV: To measure the pore grouping of liver sinusoidal endothelial cells, we manually counted groups of pores of LSCES /FOV. Each data point represents average of 10 FOVs.

Immunohistochemistry (IHC) and immunofluorescence (IF): Tissue sections (4-6μm) were stained with Prussian blue as described elsewhere2. We used primary antibodies as shown in table-2. Nikon A1 Spectral Confocal microscopes were used to capture images at CBI Pitt.

Western Blot: Immunoblotting was performed as described elsewhere2. The following primary antibodies were used: GFP (1:500, ABCAM), CD31 (1:500 Novus biologicals), VEGF (1:500, ABCAM) and GAPDH (1:1500, ABCAM).

mRNA isolation and real time polymerase chain reaction: mRNA was isolated and purified from livers of SS and SS-Selp−/− mice (n=4/group). mRNA was isolated using Trizol (Invitrogen). RT-PCR was performed as described elsewhere2. 18S and GAPDH were used to normalize the mRNA expression data. Sequences of primers used are added in table-1.

Biochemical analysis: Enzyme-linked immunosorbent assay (ELISA) was used to measure levels of GFP (ABCAM; cat #ab171581), and Immunoglobulin G (IgG) (ABCAM; cat #ab151276).

Supplemental Figures and Legends
Figure S1: Defenestrated endothelium delays liver directed gene transfer in hemophilia-A mice.

(A) Quantification of GFP positive cells in control and F8TKO liver 15- and 30-days post AAV8-GFP administration. (B) qRT-PCR showing the absence of FVIII in F8TKO liver. (C) ELISA assay
of total liver GFP amount in control and B6; 129S-F8tm1Kaz/J mice at 30 days post AAV8-GFP injection (IV). (D) Detection of IgG antibody in serum samples of control (littermate heterozygous female) and F8TKO mouse at baseline and after 10-30 days post AAV8-GFP injection. (E) Immunofluorescence of GFP staining in control and B6; 129S-F8tm1Kaz/J mice 30 days post AAV8-GFP injection (IV). (F) Western blot analysis of GFP in control and B6; 129S-F8tm1Kaz/J mice after 30-days post AAV8-GFP administration (IV). Bar chart shows quantification of GFP levels compared to GAPDH. (G) Western blot analysis of GFP in control and F8TKO mice after 60-days post AAV8-GFP administration (IV). Bar chart shows quantification of GFP levels compared to GAPDH (H) qRT-PCR analysis exhibits increased mRNA expression of markers of liver sinusoidal endothelial cells in F8TKO liver as compared to control liver. (I) qRT-PCR analysis exhibits increased mRNA expression of markers of extra cellular matrix in F8TKO liver as compared to control liver. Scale bar 10 um.

Supplemental Movie Legends

Movie S1. Visualization of blood flow and sinusoidal endothelial cells in a control mouse at baseline after administration of TXR-dextran and CD31-488 prior to imaging. The sinusoids in a control mouse liver visualized by carotid artery injection of TXR-dextran (red). AF488-CD31 (green) was used as a marker for sinusoidal endothelial cells to observe baseline expression of LSECs in control liver. Original acquisition rate. Scale bar 20 uM.

Movie S2. Visualization of blood flow and sinusoidal endothelial cells in a control mouse 15 days after AAV8-GFP injection with administration of TXR-dextran and CD31-488 prior to imaging. The sinusoids in a control mouse liver visualized by carotid artery injection of TXR-
dextran (red) and AF488-CD31 (green; as a marker for sinusoidal endothelial cells). No change in total number of CD31 positive sinusoidal endothelial cells were seen after 15 days of AAV8-GFP injection as compared to control (baseline). Original acquisition rate. Scale bar 20 uM.

**Movie S3. Visualization of blood flow and sinusoidal endothelial cells in a FVIII null mouse at baseline after administration of TXR- dextran and CD31-488 prior to imaging.** The sinusoids in a F8TKO mouse liver visualized by carotid artery injection of TXR-dextran (red). AF488-CD31 (green) was used as a marker for sinusoidal endothelial cells to observe baseline expression of LSECs in a F8TKO mice at baseline. Endothelial cells appeared comparable to control at baseline. Original acquisition rate. Scale bar 20 uM.

**Movie S4. Visualization of blood flow and sinusoidal endothelial cells in a FVIII null mouse 15 days after AAV8-GFP injection with administration of TXR- dextran and CD31-488 prior to imaging.** The sinusoids in a F8TKO mouse liver visualized by carotid artery injection of TXR-dextran (red). AF488-CD31 (green) was used as a marker for sinusoidal endothelial cells to observe baseline expression of LSECs in a F8TKO mice after AAV8-GFP treatment. Significant loss in CD31 positive sinusoidal endothelial cells were seen after 15 days of AAV8-GFP injection as compared to control. Original acquisition rate. Scale bar 20 uM.
References:

1. Vats, R. *et al.* Impaired bile secretion promotes hepatobiliary injury in Sickle Cell Disease. *Hepatology* (2020). doi:10.1002/hep.31239

2. Pradhan-Sundd, T. *et al.* Dual catenin loss in murine liver causes tight junctional deregulation and progressive intrahepatic cholestasis. *Hepatology* (2017). doi:10.1002/hep.29585