SUPPLEMENTAL METHODS

Vector constructs

The control vector plasmid was designated as *pAAV-AAT-co-hFIX-R338L-Padua* and contained a codon-optimized (co) human (h) FIX-R338L-Padua cDNA (abbreviated as R338L-Padua) cloned downstream of a liver-specific promoter (α1-anti-trypsin promoter, AAT). The R338L-Padua in this study was identical to the transgene previously used in our initial validation studies that demonstrated its superiority in gene therapy over the wild-type codon-optimized *FIX* transgene[1,2]. All vectors are identical in design and were based on the self-complementary (sc) configuration[3]. The *AAT-co-hFIX-R338L-Padua* expression cassette was cloned into a self-complementary (sc) AAV backbone (*scAAV*)[3] that also contained a mini-intron from minute virus of mice (*MVM*) upstream of the *co-hFIX-R338L-Padua* transgene and a bovine growth hormone polyadenylation signal (*bGHpA*). The test vector plasmid *pAAV-AAT-co-hFIX-CB2679d-GT* was identical in design as the control vector *pAAV-AAT-co-hFIX-R338L-Padua* and expressed a novel codon-optimized human *FIX* cDNA (designated as CB 2679d-GT) from the same liver-specific AAT promoter in a scAAV backbone. The CB 2679d-GT transgene encodes a novel FIX with triplet substitutions (i.e. R318Y, R338E, T343R) known to increase catalytic activity, provide resistance to antithrombin inhibition and improve the affinity for activated FVIII[4,5]. Both R338L-Padua and CB 2679d-GT constructs contain the T148A residue; a known polymorphism in FIX[6]. Therefore, the only difference between the *pAAV-AAT-co-hFIX-R338L-Padua* and *pAAV-AAT-co-hFIX-CB2679d-GT* vector plasmids are the R318Y,

R338E, T343R triplet mutations in CB 2679d-GT. To generate the test vector plasmid *pAAV-AAT-co-hFIX-CB2679d-GT,* we first synthesized a gene fragment corresponding to the CB 2679d-GT transgene and cloned it via the restriction sites *NheI* at the 5' end and *BglII* at the 3' end into the *pAAV-AAT-co-hFIX-R338L-Padua* control plasmid to replace the *co-hFIX-R338L-Padua* transgene. Both plasmids were verified by restriction digestion and Sanger sequencing using 13 different primers spanning the entire expression cassette and the *scAAV* backbone.

Vector production and purification

To produce the AAV vectors, AAV-293 human embryonic kidney (HEK) cells were co-transfected by calcium phosphate transfection (Invitrogen Corp, Carlsbad, CA, USA) with the AAV plasmids of interest *pAAV-AAT-co-hFIX-R338L-Padua* or *pAAV-AAT-co-hFIX-CB 2679d-GT*, a chimeric AAV packaging construct encoding AAV/DJ8 (Bio-connect, The Netherlands)[7] and an adenoviral helper plasmid, as described[8,9]. The AAV/DJ8 was chosen since it was derived by DNA family shuffling technology and selection for increased hepatotropic properties and increased resistance to neutralization by pooled human antisera (intravenous immunoglobulin or IVIG)[7]. The codon-optimized sequences and the corresponding AAV vector plasmid maps of the R338L-Padua and CB 2679d-GT vectors are provided as Supplementary Figure 1. Codon-optimization was carried out using a proprietary algorithm (BaseClear). All plasmids required for the AAV production were extracted using an endotoxin free maxiprep protocol (Thermo Fischer Scientific, Belgium). Two days post transfection; cells were harvested and stored in resuspension buffer for subsequent purification by isopycnic ultracentrifugation. Harvested cells were lysed by successive freeze/thaw

cycles and sonication, treated with benzonase (Novagen, Madison, WI, USA), deoxycholic acid (Sigma- Aldrich, St Louis, MO, USA) and subsequently subjected to three successive rounds of cesium chloride (Invitrogen Corp, Carlsbad, CA, USA) density gradient ultracentrifugation. Fractions containing the AAV vector were collected, concentrated and dialyzed into 1 mM $MgCl_2$ in Dulbecco's phosphate buffered saline (PBS) (Gibco, BRL). The corresponding purified AAV/DJ8 vector batches were designated as R338L-Padua and CB 2679d-GT. The vector titers (in viral genomes (vg) per ml) were determined by quantitative real-time polymerase chain reaction (qPCR) using vector-specific primer pairs. For all vectors, primers specific for the *bGHpA* sequence were used. The forward and reverse primers used were *5'- GCCTTCTAGTTGCCAGCCAT-3'* and *5'-GGCACCTTCCAGGGTCAAG-3'*, respectively. Reactions were performed with SYBR® Green PCR Master Mix, according to the manufacturer's instructions on an ABI 7500 Real-Time PCR System. Known copy numbers (10^2–10^8) of the respective vector plasmids were used to generate the standard curves.

Vector quality and purity control

The quality of the research-grade vector preparations was controlled on the basis of sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE). Different vector titers (i.e., $1.25x10^{11}$ vg and $6.25 x10^{10}$ vg) of the R338L-Padua and CB2679d-GT vector preparations were added to 2x Laemmli Sample Buffer (BioRad) in a 1:1 ratio before being denatured at 95°C for 5 min. 10 µl of the sample preparations and 5 µl of PageRuler Prestained Protein Ladder (Thermoscientific) were added into a Mini-PROTEAN TGX gel (4-15%) (BioRad) which was submerged in 1x

Tris/Glycine/SDS running buffer (BioRad). The gel was run at 50 V until the samples and protein ladder entered the resolving gel and subsequently at 100 V until the sample buffer reached the lower limit of the gel. After careful removal, the gels were stained with either Sypro™ Ruby Protein Gel Stain (Thermofisher) or the ProteoSilver™ Silver Stain Kit (Sigma Aldrich). The AAV capsid proteins VP1 (82 kDa), VP2 (67 kDa), and VP3 (60 kDa) are indicated with arrows (Suppl. Fig. 2). SDS-PAGE (Suppl. Fig. 2) revealed that there was no difference in quality and purity between the research-grade R338L-Padua and CB2679d-GT vector preparations at the different vector doses tested, consistent with the comparable vector titers, transduction efficiencies (Fig. 2E-H) and safety profiles (Suppl. Fig. 5: AST-ALT/ H&E staining). In particular, there were no significant differences in residual protein impurities present in both of the research-grade R338L-Padua and CB2679d-GT vector preparations.

FIX expression analysis and phenotypic correction

The institutional Animal Ethics Committee prior to initiating the experiments approved all animal experiments. A colony of hemophilia B mice ($FIX^{-/-}$)[10] on a C57BL/6 background was established in house. The *in vivo* performance of the R338L-Padua and CB 2679d-GT vectors were assessed in hemophilia B mice ($FIX^{-/-}$) by intravenous injection of three different vector doses ($1x10^9$, $5x10^9$, and $1x10^{10}$ vg/mouse) in experiment. The experiment was also repeated (at the doses of $5x10^9$, $1x10^{10}$ vg/mouse) and the representative experimental data were represented in the figures. Whole blood was collected into buffered citrate by phlebotomy of the retro-orbital plexus at different times points post vector administration. Blood was collected in 1.5

ml eppendorf tubes containing 20% sodium citrate buffer using non-heparinized capillaries. To obtain the plasma, the blood was centrifuged for 3 min at 13000 rpm. The plasma was aliquoted, placed on dry ice and stored at -80°C. Plasma from non-injected or vehicle-injected mice, and C57BL/6 mice were used as negative and positive controls, respectively. The FIX protein levels in the plasma were determined using an enzyme-linked immunosorbent assay (ELISA) specific for hFIX antigen (ASSERACHROM IX: Ag Enzyme Immunoassay for Factor FIX; Diagnostica Stago, France) according to the manufacturer's instructions. To measure CB 2679d-GT and R338L-Padua antigen levels by ELISA and exclude any potential bias, FIX standard curves were constructed with known amounts of the purified respective recombinant CB 2679d-GT or R338L-Padua proteins (Cambridge Protein Works). In both cases, the purified recombinant proteins in the current study contained the FIX T148A amino acid substitution. The analytical sensitivity (AS) is equal -by definition- to the slope of the calibration curve and is very similar for the R338L-Padua and the CB2679d-GT calibration curves (R338L-Padua AS = 0.98-1.14; CB2679d-GT AS = 0.99-1.03). The detection limit (DL) of the FIX antigen assays is <1.95 ng/ml for both FIX-Padua and FIX- CB 2679d-GT. Considering that the physiologic concentration of wild-type FIX is equal to 5 µg/ml (100%), this would correspond to 0.04% which is below the therapeutic threshold of 1%. The FIX activity was measured using an activated partial thromboplastin time (aPTT) assay as per the manufacturer's instructions (C.K. PREST kit and Start Max; Stago). FIX activity levels were also determined in mouse plasma samples using a single-stage, aPTT-based Factor IX clotting assay. The assay was performed on an ACL-TOP instrument using the manufacturer's recommended reagents (HemosIL reagent set designed for Instrumentation Laboratories). Calibration was performed against the respective reference plasma from the

manufacturer, which is traceable to the WHO standard (09/172). Each sample was run in true duplicate, using required number of dilutions to obtain valid results. Sample dilutions ranged from 1:10 to 1:48.5. The specific activity was calculated based on these FIX activity levels determined with the WHO FIX standard per mg FIX protein as determined by ELISA.

The tail-clip model was performed to assess the phenotypic correction using a modified version of a protocol described earlier[11]. Briefly mice were anesthetized, and the tails were placed in a pre-warmed saline solution for two minutes and subsequently cut at a 2.5 mm diameter. The tails were then immediately returned to the 37°C saline solution and monitored for bleeding or clotting for 30 minutes. For humane reasons, vehicle control mice were euthanized after 120 minutes, although they were still bleeding at that time. Blood-containing saline was subsequently centrifuged at 520 g for 15 min at 4°C to collect erythrocytes, which were then resuspended in 18 ml of lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA). Lysis proceeded for 10 minutes at room temperature and samples were again centrifuged at 520 g for 10 min at 4°C. The absorbance of the supernatants was measured at 570 nm spectroscopically to determine the amount of hemoglobin as an indication of blood loss. Likewise, the clotting time for these mice was recorded.

Immune responses

Anti-FIX antibody titers were analyzed using a modified ELISA protocol. Briefly, 96-well microtiter plates were coated with either purified recombinant R338L-Padua or CB 2679d-GT (1 µg/ml). Serially diluted reference standards were prepared with

purified mouse IgG (Invitrogen, Europe). The plasma samples from mice injected with the R338L-Padua vector were analyzed using the plates that were coated with the purified R338L-Padua protein, whereas the CB 2679d-GT coated plates were used to assay the plasma samples from mice injected with the CB 2679d-GT vector. As a positive control, mice were injected with a FIX vector that expressed wild-type FIX from a ubiquitously expressed cytomegalovirus (CMV) promoter that invariably results in high-titer anti-FIX antibodies. The plates were incubated overnight at 4°C. On day two, the samples of mouse plasma were diluted in dilution buffer, loaded on the pre-coated plates and incubated for two hours at room temperature. Experimental plasma samples were obtained from mice injected with different dose levels of the AAV vectors. The plates were then incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Invitrogen, Europe) as a secondary antibody. Anti-hFIX antibody levels were subsequently measured following incubation with the detection buffer constituting 12 ml 0.01M sodium citrate, 12 mg *o*-phenylenediamine and 2.5 µl hydrogen peroxide (Invitrogen, Europe). The colorimetric reaction was monitored by determining the absorbance at 450 nm.

To analyze the immune risk of gene therapy with CB 2679d-GT versus R338L-Padua in an *in vivo* hemophilia B mouse model, hemophilic ($FIX^{-/-}$) mice were injected with either $5x10^9$ vg/mouse R338L-Padua (n=5), $5x10^9$ vg/mouse CB2679d-GT (n=5) or PBS (n=4). Plasma was collected from these mice at week 2, 4, and 6 post-injection to analyze anti-FIX antibody levels. At week 8 post-injection, all cohorts were immunized by injecting the corresponding FIX protein (purified R338L-Padua or CB 2679d-GT) (5 µl) together with Incomplete Freud's Adjuvant (IFA) (200 µl, Sigma-Aldrich). The vehicle control cohort (PBS) was immunized with the CB2679d-GT

protein. Plasma was collected at weeks 2 and 3 post-immunization to analyze the anti-FIX antibody levels (Suppl. Fig. 4A).

Liver toxicity

To assess for liver toxicity, aspartate aminotransferase (AST) and alanine transaminase (ALT) activity was determined in plasma using AST (MAK055-1KT, Sigma Aldrich, MO, USA) and ALT activity assay kits (MAK052-1KT, Sigma Aldrich, MO, USA) according to the manufacturer's instructions. Besides AST and ALT, hematoxylin (Sigma) and eosin (Sigma) staining was conducted 36 weeks post-vector injection on liver sections from hemophilic FIX knockout mice injected with either the R338L-Padua (n=5) or CB 2679d-GT vectors (n=5) (vector dose: $1x10^9$ vg/mouse) (Suppl. Fig. 5E-H). Wild-type C57BL/6 or PBS-injected hemophilia B ($FIX^{-/-}$) mice were used as controls.

Pathological activation of coagulation

To assess pathological activation of coagulation, D-dimer levels were determined in plasma using a D-dimer specific ELISA (Hyphen Biomed) according to the manufacturer's instructions.

Transduction efficiency and biodistribution

Mice were euthanized and a panel of organs was collected for further DNA and RNA analyses. Genomic DNA and RNA was extracted from different tissues using the AllPrep DNA/RNA Mini Kit (Qiagen, Chatsworth, CA, USA) based on the manufacturer's instructions. Typically, 150 ng genomic DNA was analyzed using

qPCR on an ABI Prism 7900HT (Applied Biosystems, Foster City/CA, USA) and GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) with bghpA specific forward (*5'-GCCTTCTAGTTGCCAGCCAT-3')* and reverse *(5'-GGCACCTTCCAGGGTCAAG-3'*) primers. To generate standard curves, known copy numbers of the corresponding vector plasmid were used. The isolated RNA was used for cDNA synthesis. 100 ng of RNA was reverse transcribed into cDNA by the GoScriptTM Reverse Transcription system (Promega, Madison, WI, USA) and analyzed by qPCR (ABI Prism 7900HT, Applied Biosystems, Foster City/CA, USA) and GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) with bghpA specific forward (*5'-GCCTTCTAGTTGCCAGCCAT-3')* and reverse *(5'-GGCACCTTCCAGGGTCAAG-3'*) primers. The expression levels were normalized to murine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression, obtained by using the forward primer *5'-TGTGTCCGTCGTGGATCTGA-3'* and reverse primer *5'-GCCTGCTTCACCACCTTCTTGA-3'.* Expression levels were determined based on the $2^{-\Delta cT}$ method[12].

Statistics

Data were analyzed using the Microsoft Excel Statistics package. Values are presented as the mean ± SEM. A comprehensive global two-way repeated measures analysis of variance for the data sets was used to evaluate the FIX activity levels (U/mL). Phenotypic analyses were evaluated by unpaired Student's t-test.

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: AAV plasmid maps and corresponding codon-optimized sequences. (A-B): AAV plasmid map of pAAV-AAT-co-hFIX-R338L-Padua and corresponding codon-optimized plasmid sequence (A). AAV plasmid map of pAAV-AAT-co-hFIX-CB2679d-GT and corresponding codon-optimized plasmid sequence (B).

Supplementary Figure 2: Vector quality and purity control. SDS-PAGE results with Sypro- (left) and Silver staining (right): $1.25x10^{11}$ vg of R338L-Padua vector (lane 1), $1.25x10^{11}$ vg of CB 2679d-GT vector (lane 2), $6.25x10^{10}$ vg of R338L-Padua vector (lane 3), $6.25x10^{10}$ vg of CB 2679d-GT vector (lane 4), and Prestained Protein Ladder (lane 5) were added. AAV capsid proteins VP1 (82 kDa), VP2 (67 kDa), and VP3 (60 kDa) are indicated with a black arrow.

Supplementary Figure 3: Comparative genomic DNA and RNA analysis of the Liver samples. (A-B): AAV copy number in liver samples for the mice injected with R338L-Padua or CB 2679d-GT at the doses of $5x10^{9}$ and $1x10^{10}$ vg/mouse were analyzed using quantitative real time PCR to evaluate the transduction efficiencies of both the vector groups (A). The same set of mice were analyzed for RNA expression in liver samples by qRT-PCR to confirm that there is no significant difference in RNA expression profiles for the different vector groups (B). RNA expression of the transgenes was normalized to mouse GAPDH expression. Results are presented as

mean ± SEM. *P<0.05; **P<0.01; ***P<0.001 (Student t test); N.S., not significant (P>0.1).

Supplementary Figure 4: Assessing immune responses against CB 2679d-GT and R338L-Padua. (A-C): Schematic representation of the immune challenge (A). FIX specific antibodies were determined by ELISA in immunized hemophilia B mice ($FIX^{-/-}$) injected with either R338L-Padua ($5x10^9$ vg/mouse, n=5), CB 2679d-GT ($5x10^9$ vg/mouse, n=5), or PBS (n=4). Immunization was performed by injecting the corresponding FIX protein (purified R338L-Padua or CB 2679d-GT) (5 µl) together with Incomplete Freud's Adjuvant (IFA) (200 µl, Sigma-Aldrich). Vehicle control mice (PBS) were immunized with CB 2679d-GT protein (B). Individual FIX specific antibody concentration in hemophilic ($FIX^{-/-}$) mice injected with CB 2679d-GT ($1x10^9$ vg/mouse, n=6) (C). Results are presented as mean ± standard error of the mean.

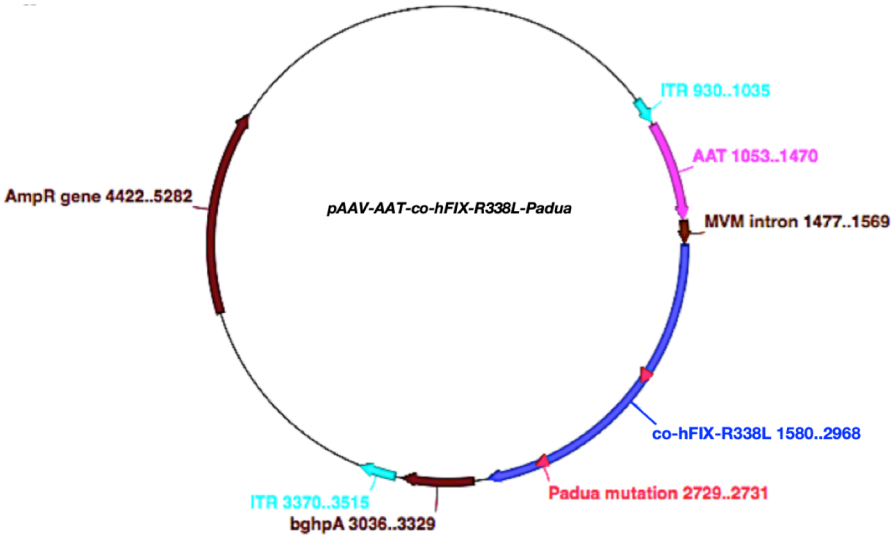
Supplementary Figure 5: Evaluating liver toxicity. (A-B): AST activity levels in mice injected with R338L-Padua or CB 2679d-GT at the three doses of $1x10^9$, $5x10^9$, and $1x10^{10}$ vg/mouse compared to the control vehicle group at two timepoints of one week (A) and three weeks (B) post-injection. **(C-D):** ALT activity levels in mice injected with R338L-Padua or CB 2679d-GT at the three doses of $1x10^9$, $5x10^9$, and $1x10^{10}$ vg/mouse compared to the control vehicle group one week (C) and three weeks (D) post-injection. In normal healthy mice, the expected levels of ALT are up to 60 mU/ml and AST falls in the range of 50-100 mU/ml. **(E-H):** Hematoxylin/eosin staining of week 36 post-injection liver sections from hemophilia B ($FIX^{-/-}$) mice injected with either

R338L-Padua ($1x10^9$ vg/mouse, n=5) (E) or CB 2679d-GT ($1x10^9$ vg/mouse, n=5) (F), C57BL/6 positive control mice (G), and vehicle injected hemophilia B ($FIX^{-/-}$) mice (PBS) (H).

Supplementary Figure 6: Evaluating pathological activation of coagulation. (A-B): D-dimer levels of week 5 post-injection plasma of hemophilia B ($FIX^{-/-}$) mice injected with CB 2679d-GT ($5x10^9$ vg/mouse, n=7 (A); $1x10^{10}$ vg/mouse, n=5 (B)), R338L-Padua ($5x10^9$ vg/mouse, n=8 (A); $1x10^{10}$ vg/mouse, n=8 (B)), or vehicle (PBS) (n=4), and C57BL/6 positive control mice (n=4) were determined by ELISA (Hyphen, BioMed). The D-dimer positive control is shown (dotted line) and represents a known amount of D-dimer (1.5-2 µg/ml). Results are presented as mean ± standard error of the mean. N.S., not significant (P>0.1); (Student t test).

Supplementary Figure 1

A



pAAV-AAT-co-hFIX-R338L-Padua plasmid sequence

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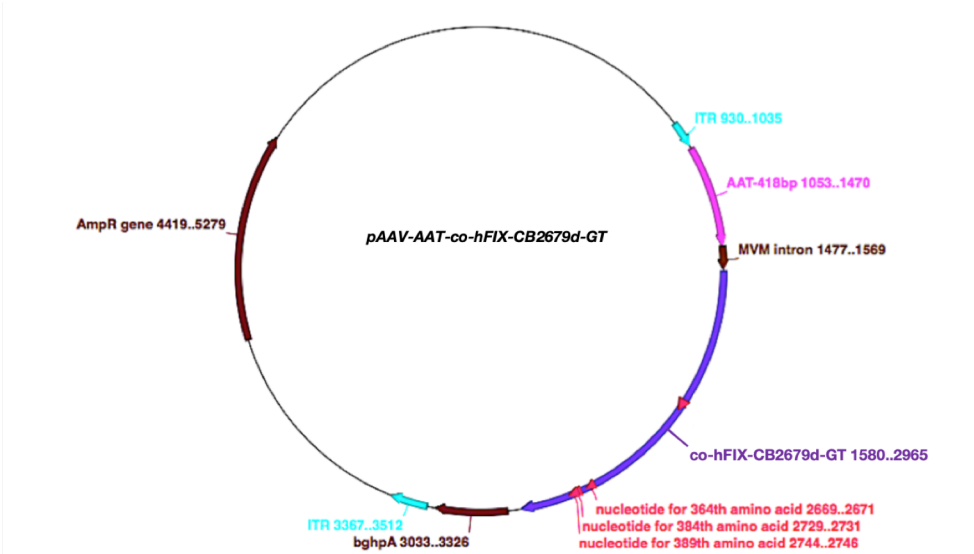
	5' – ITR & 3' – ITR
	AAT
	MVM Intron
	co-hFIX-R338L
	bghpA

Supplementary Figure 1

B



pAAV-AAT-co-hFIX-CB2679d-GT plasmid sequence

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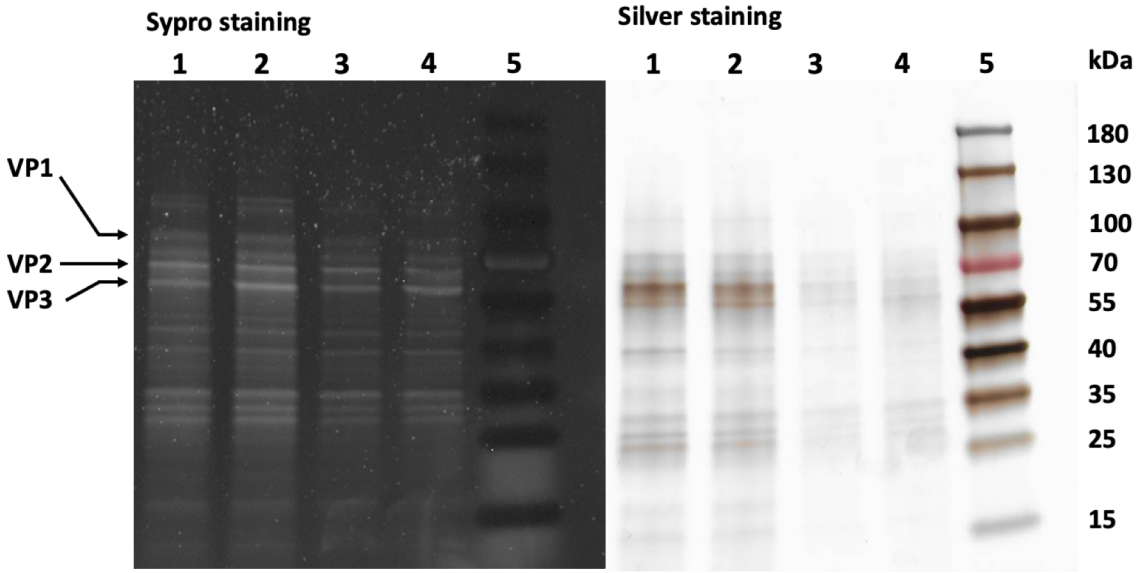
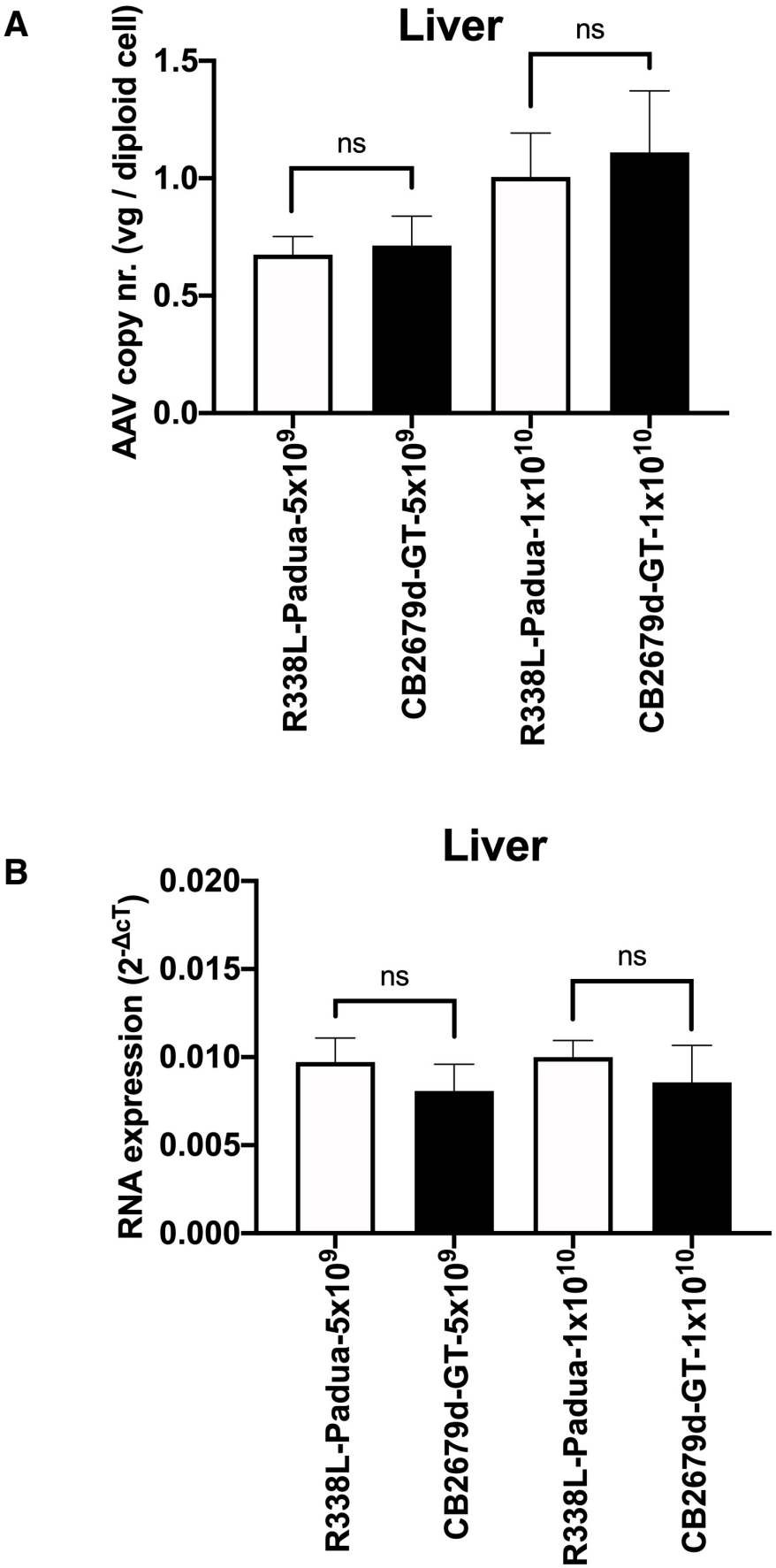
GGGAGTGG 3'

	5' – ITR & 3' – ITR
	AAT
	MVM Intron
	co-hFIX-CB2679d-GT
	bghpA

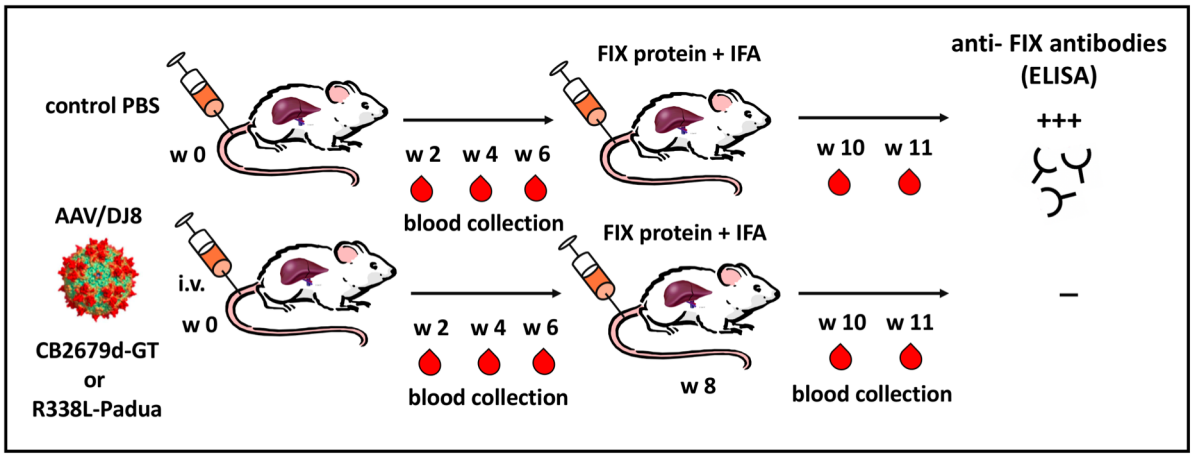
Supplementary Figure 2

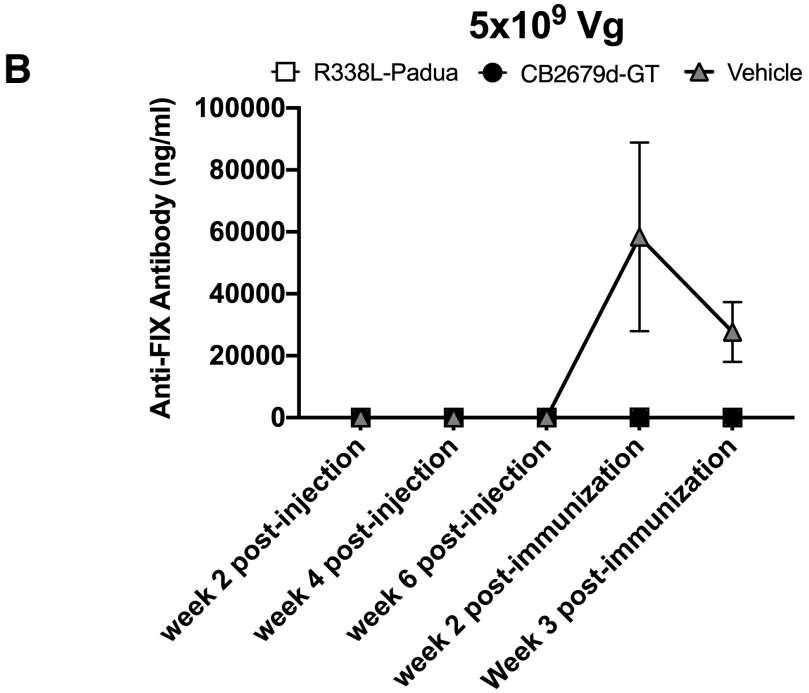


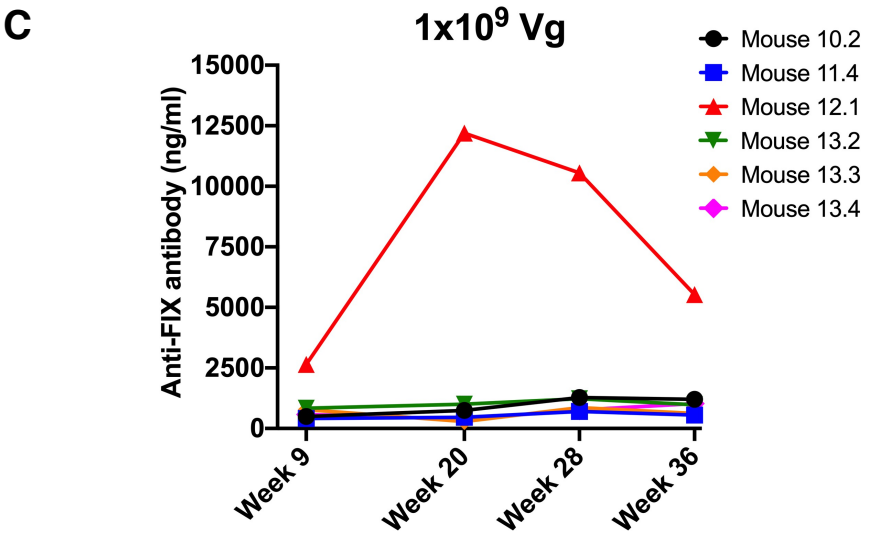
Supplementary Figure 3



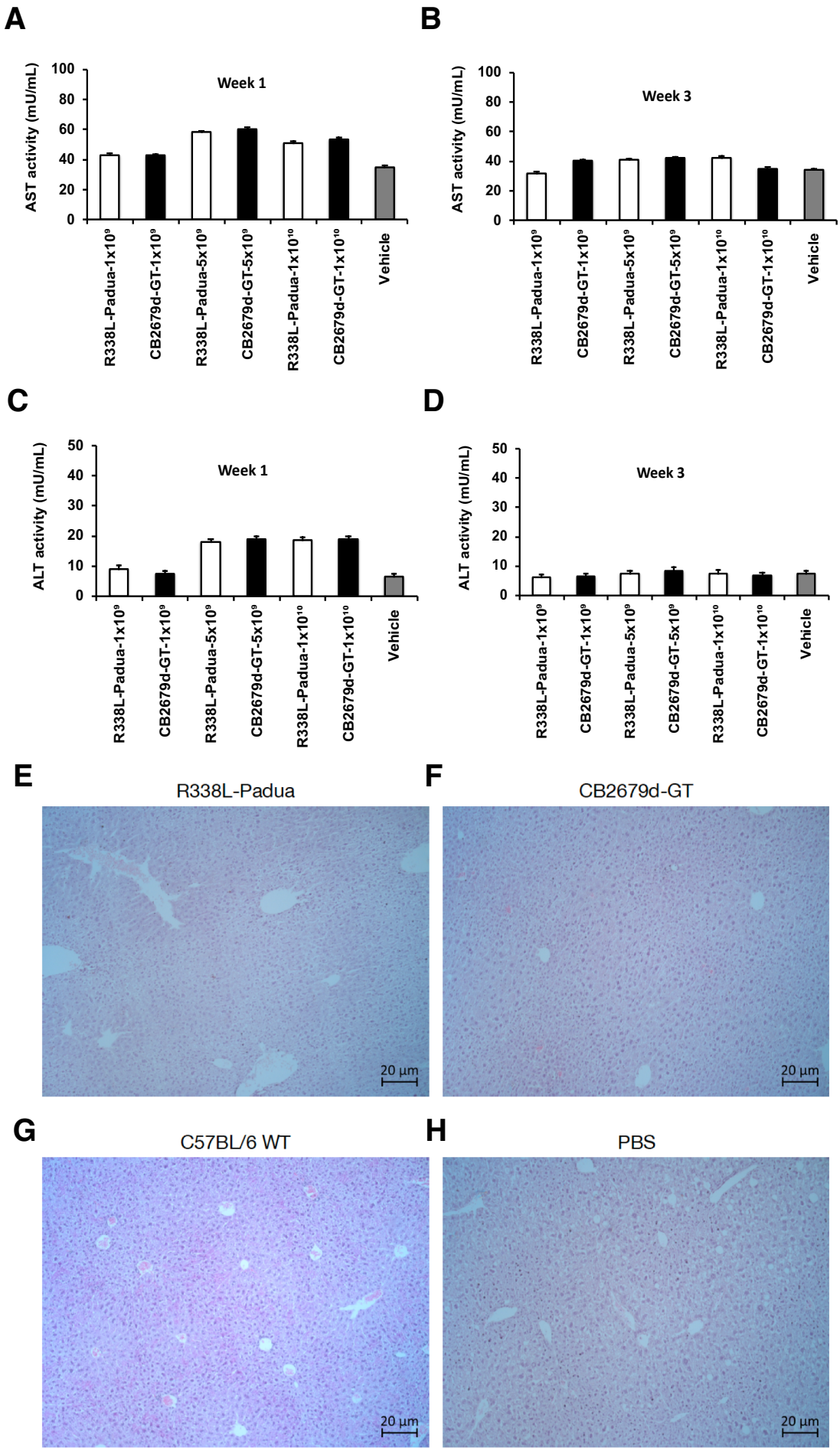
Supplementary Figure 4







Supplementary Figure 5



Supplementary Figure 6