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Supplemental Information

CRISPR-Cas9-Mediated *In Vivo* Gene Integration at the Albumin Locus Recovers Hemostasis in Neonatal and Adult Hemophilia B Mice

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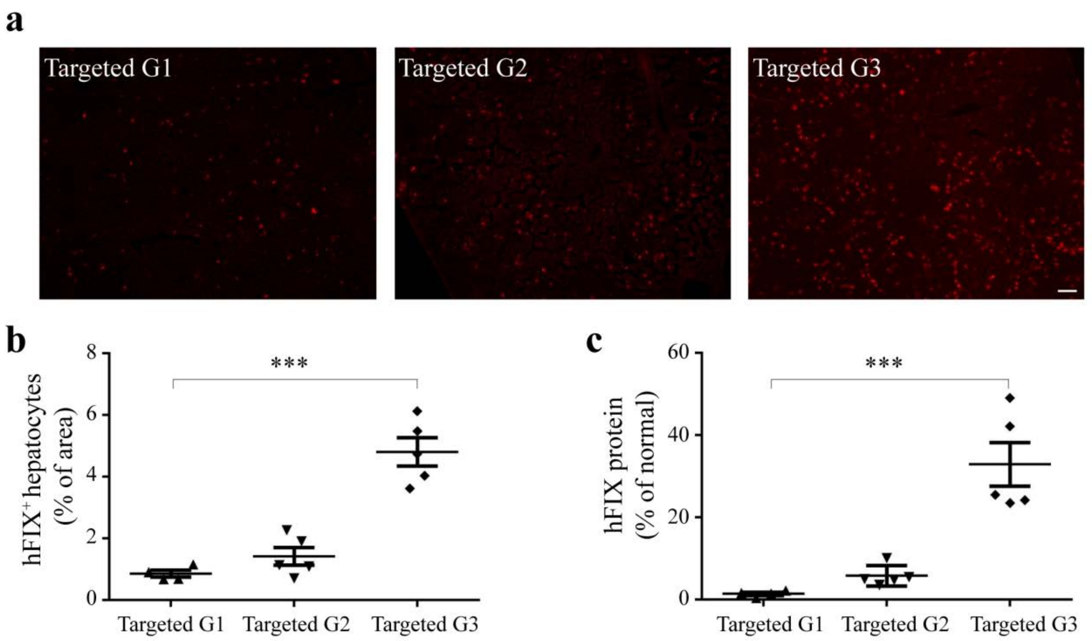
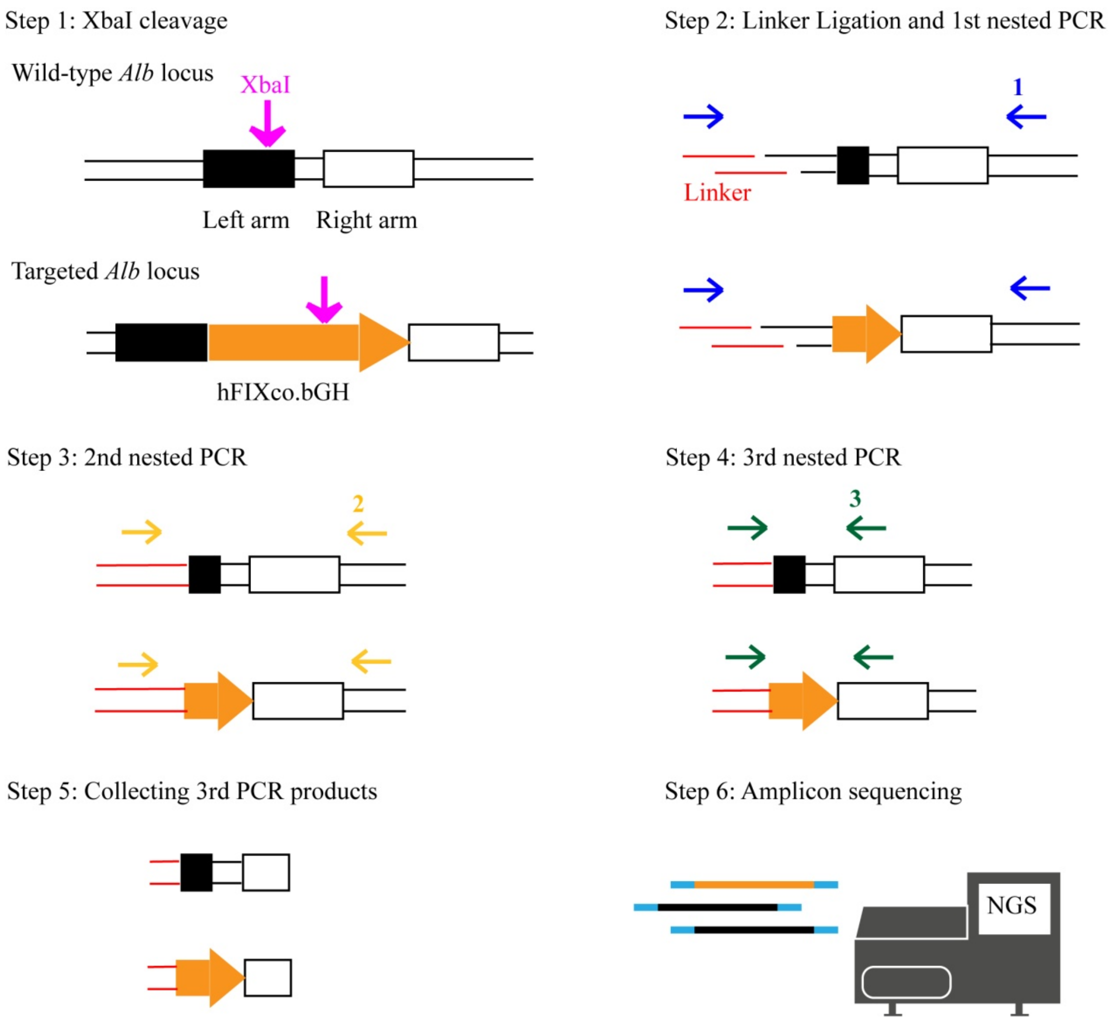
Supplementary Figures



Supplemental Figure 1 Vector dose optimization to improve *in vivo* gene integration.

Hemophilia B mice (8 weeks old) received tail vein injection of 9×10^{10} GC AAV8.SpCas9 and either 9×10^{10} (G1, n=4), 2.7×10^{11} (G2, n=5), or 4.5×10^{11} (G3, n=5) GC of AAV8.sgRNA.donor vector. Liver samples were collected 4 weeks post vector treatment for analysis. (a) Immunofluorescence staining with antibodies against hFIX (red) on liver sections. Stained cells typically showed as single integrated hepatocytes. Scale bar, 100 µm. (b) Quantification of gene correction based on the percentage of area on liver sections expressing hFIX by immunostaining. (c) Plasma hFIX was measured by ELISA at 4 weeks after the dual AAVs treatment. Mean ± SEM are shown. *** $P<0.001$, Dunnett’s test.



Supplemental Figure 2 Scheme of integration rate assessment. Assessment of on-target integration rate begins using XbaI digesting genomic DNA at (step 1). XbaI compatible linker (red) is then ligated to the genomic locus but not to the vector followed by the first round of nested PCR amplification (primer 1 in blue) (step 2). The length of first round of RCR products are similar in both targeted and wild-type alleles, thus allowing for unbiased amplification followed by nest two rounds of nested PCR amplifications (primer 2 in yellow (step 3), and then primer 3 in green (step 4)). The amplicons of the third nested PCR then serve as a template and subject for NGS (step 5 and step 6). The HDR-mediated gene integration rate is determined by the percentage of reads containing the expected hFIXco sequence among the total mapped reads.

Supplemental Tables

Supplemental Table 1. Off-target analysis. Potential off-target sequences for Albumin sgRNA4 identified and scored by Benchling's off-target analysis.

ID	Sequence	PAM	Score	Chromosome	Strand	Position	Mismatches	On-target
sgRNA4	GTAAATATCTACTAAGACAA	TGG	100.000	chr5	-1	90461429	0	
OT1	TTAAATATCAACTAAGACAA	TAG	7.415	chr2	1	14017575	2	FALSE
OT2	CTAAATATATACTAAGACAA	GGG	4.607	chr8	1	105884816	2	FALSE
OT3	TTAAATACCAACTAAGACAA	CAG	2.738	chrX	1	37895074	3	FALSE
OT4	TTAAATACCAACTAAGACAA	CAG	2.738	chrX	-1	37833846	3	FALSE
OT5	ATAAGTATCTGCTAAGACAA	CAG	1.715	chr14	-1	27934452	3	FALSE
OT6	TGAAATATCTTCTAAGACAA	AGG	1.715	chrX	-1	52027245	3	FALSE
OT7	CTAAGTATCTAATAAGACAA	AGG	1.582	chr1	-1	47526830	3	FALSE
OT8	CCAAATATCTAGTAAGACAA	AAG	1.582	chr15	-1	69401691	3	FALSE
OT9	GTATGTATATACTAAGACAA	TGG	1.579	chr5	1	58977566	3	FALSE
OT10	GAAATTATCTACTAAGTCAA	TGG	1.478	chr14	-1	100457044	3	FALSE

Supplemental Table 2. Primers and sequences for construction of plasmids and analysis of gene target and expression.

Name	**Sequence**	**Note**
Albumin sgRNA1_Fwd	CACCGCATCTTCAGGGAGTAGCTT	Albumin target sequence 1
Albumin sgRNA1_Rev	AAACAAGCTACTCCCTGAAGATGC	
Albumin sgRNA2_Fwd	CACCGCACAGATATAAACACTTAA	Albumin target sequence 2
Albumin sgRNA2_Rev	AAACTTAAGTGTTTATATCTGTGC	
Albumin sgRNA3_Fwd	CACCGGAAAATCTGAAGGTGGCAA	Albumin target sequence 3
Albumin sgRNA3_Rev	AAACTTGCCACCTTCAGATTTTCC	
Albumin sgRNA4_Fwd	CACCGTAAATATCTACTAAGACAA	Albumin target sequence 4
Albumin sgRNA4_Rev	AAACTTGTCTTAGTAGATATTTAC	
mAlb_PointMF	CCTGCTCGACCATGCTATACT	Albumin PCR for Surveyor and NGS assay
mAlb_PointMR	CAGGCCTTTGAAATGTTGTT	
Albumin_qPCR_Fwd	TGGCAAAATGAAGTGGGTAA	Wild type mAlb qPCR primer
Albumin_qPCR_Rev	GGGCGATCTCACTCTTGTGT	
F9_qPCR_Fwd	TGGCAAAATGAAGTGGGTAA	Chimeric m-hFIX qPCR primer
F9_qPCR_Rev	TCTCGTGGTCCAGAAACACT	
GAPDH_qPCR_Fwd	AAGGTCATCCCAGAGCTGAA	GAPDH qPCR primer
GAPDH_qPCR_Rev	CTGCTTCACCACCTTCTTGA	
Cas9_qPCR_Fwd	GGACTCCCGGATGAACACTA	SpCas9 qPCR and copy number assay primer
Cas9_qPCR_Rev	TCGCTTTCCAGCTTAGGGTA	
Linker_Fwd	GACCCGGGAGATCTGAATTCAGTGGCACAGCAGTTAGG	XbaI compatible Linker
Linker_Rev	CTAGCCTAACTGCTGTGCCACTGAATTCAGATC	
LM_P1	GACCCGGGAGATCTGAATTC	LM nest PCR1
LM_P2	CAAAACTATAGTTCATAGACCTTTCCAG	
LM_P3	GATCTGAATTCAGTGGCACAG	LM nest PCR2
LM_P4	ATAAGATTCCAGACTTAACAAGTTAGA	
LM_P5	GGCACAGCAGTTAGGCTAGA	LM nest PCR3
LM_P6	TCTGTGCATTCAAACTCAAGAT	

Supplemental Table 3. PCR primer sequences for detecting potential off-target effects by NGS assay.

Primer Name	Sequence	Note
OT1_P1Fwd	GCAAGGGCATAACCAGGACT	Primers for OT1
OT1_P1Rev	TGCTGCTAAACTGCACTCCA	
OT1_P2Fwd	TACATGCATATGTGTTCATGGACAC	
OT1_P2Rev	GCCTTCTATACTCTTGCAACCATGT	
OT2_P1Fwd	GCAACCTTTTCACTGCGTCC	Primers for OT2
OT2_P1Rev	CCTGGCCTGGGAATGGATAC	
OT2_P2Fwd	AGGTTCCAATGCCGAAAGCC	
OT2_P2Rev	TGCTCACCTCCCTCCTGACA	
OT3_P1Fwd	AGAATCTTAACTCCTCTGATACCG	Primers for OT3
OT3_P1Rev	CCCTTTGCAAGCAAACAACG	
OT3_P2Fwd	GTCCCTTTCCTGACTGTCAGTGT	
OT3_P2Rev	GGGCAGAAGTATATGATGAATGTG	
OT4_P1Fwd	TTCATGGCCCTGTTCCATCC	Primers for OT4
OT4_P1Rev	AGTCCTCTGATGCAGTGTCA	
OT4_P2Fwd	GCTCCTCTTCCTGCACAGCT	
OT4_P2Rev	CAGCCCCAGACTTTCAGTCTT	
OT5_P1Fwd	AGCACTTGGCATGCTTGAAC	Primers for OT5
OT5_P1Rev	TCCCAAGATGCATACCGTGC	
OT5_P2Fwd	GCCTCACTGTCTCTATGAGCCA	
OT5_P2Rev	ACATCTACCTGAATTCCCTGTGAC	
OT6_P1Fwd	CTTCGGAATGCATCGAGCTG	Primers for OT6
OT6_P1Rev	CCAGCAGAGCTATGTAGCCA	
OT6_P2Fwd	GCAGATCTCATCTCAAGTTATAGCT	
OT6_P2Rev	CCTTTGGTTTAGATAAGCTGATTC	
OT7_P1Fwd	CCAGGCTCAAGAGGACTCAG	Primers for OT7
OT7_P1Rev	TGGGCTCTTAATGGTGGCTT	
OT7_P2Fwd	TTAGAATTTCCTTTGGAGTGTTCAG	
OT7_P2Rev	CAACTACACTATAGACCTGGGTTGA	
OT8_P1Fwd	CAGTTGGGTCTGGCATAGGG	Primers for OT8
OT8_P1Rev	CACGGACTGTAGGTGGGAAA	
OT8_P2Fwd	TCTGTCAGTCACAAGACCTCTGC	
OT8_P2Rev	GCAATCTATAATCTGGGAGATTGG	
OT9_P1Fwd	TGTGGCAAATGAGAGCGAAGT	Primers for OT9
OT9_P1Rev	TGCCTGTGATTGATGAGCAAG	
OT9_P2Fwd	GTATTTTGATATACATCAGCCTCGA	
OT9_P2Rev	CTAACTAGCATCACATCAAACAAGA	
OT10_P1Fwd	ATGGGGCTCACGGTTTTTCT	Primers for OT10
OT10_P1Rev	CGGGGTTAAGATCGCACTCA	
OT10_P2Fwd	ATTCTGCCCTAATTGCATAACTG	
OT10_P2Rev	GGAAATGAGAGCCTCTGGACA	