Deciphering the Ets-1/2-mediated transcriptional regulation of *F8* gene identifies a minimal *F8* promoter for hemophilia A gene therapy

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SUPPLEMENTAL DATA

Deciphering the Ets-1/2-mediated transcriptional regulation of *F8* gene identifies a minimal *F8* promoter for hemophilia A gene therapy

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SUPPLEMENTAL METHODS

Cell culture

The epithelial-like cell line ECV-304 (ATCC® CRL-1998™) and the human embryonic kidney cell line HEK293T (ATCC® CRL-11268™) were cultured in DMEM and IMDM media (Gibco, Thermo Fisher Scientific), respectively. Each media was supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37˚C in 5% CO_2 incubator.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting (WB) analyses

Total RNA was isolated from ECV-304, HEK293T and Blood Outgrowth Endothelial Cells (BOEC), cells using the TRIzol Reagent (Thermo Fisher Scientific) and reverse transcribed using the High Capacity cDNA kit (Applied Biosystems, Foster City, CA). The presence of *F8*, Ets-1 and Ets-2 transcripts in each sample was evaluated by RT-PCR. All the primers used are listed in Supplementary table 1. For WIB analyses, proteins were extracted by lysing ECV-304 and HEK293T with RIPA buffer (50mM Tris pH7.5, NaCl 150mM, 0.1% SDS, 1% NP-40, 1% DOC) in the presence of a protease inhibitor cocktail (Roche). The concentration of whole cell lysates was determined using a BCA assay (Thermo Fisher Scientific), according to the manufacturer's instructions. A total of 30μg of protein was resolved on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF; BioRad) membranes and incubated separately overnight at 4°C with specific primary antibodies: α-ETS-1 (sc-55581; 1:500; Santa Cruz), α-ETS-2 (sc-351; 1:500 ; Santa Cruz) or α-β-actin (1:5000; Sigma). After washing, the membranes were probed with horseradish peroxidase (HRP)-conjugated

secondary antibody and resolved using the enhanced chemiluminescence (BioRad). Images were captured using ChemiDoc Imaging System and analyzed by ImageLab Software (BioRad).

Lentiviral Vector preparation

Third-generation LVs were produced according to published protocols[1]. Following collection, vector particles were titrated by transduction of ECV-304 or HEK293T cells using limiting dilutions. The GFP expressing LVs were titrated by flow cytometry analysis (FACS), while FVIII-expressing LVs were quantified as number of integrated copies by quantitative-PCR (qPCR). Primers used for qPCR are listed in Supplementary table 6.

Animal studies

In vivo experiments were performed according to an approved protocol by the Animal Care and Use Committee of UPO, Novara, Italy (approval n°492/2016-PR 10/03/2016). The C57BL/6 mice (The Jackson Laboratories, Stock No 000664) and B6;129S-F8^{tm1}Kaz/J (The Jackson Laboratory, Stock No 004424)[2] hemophilic mice (B6/129 HA) were housed under standard conditions. Both strains were tail vein injected at 8 weeks of age with $5x10^8$ or $1x10^9$ TU of LVs expressing GFP (n=3-5) or FVIII (n=4-6), respectively, under the control of 600 bp, 442 bp and 342 bp *pF8* or the ubiquitous *PGK* promoter.

Immunofluorescence analysis of mouse tissues

Liver and spleen of LV injected mice were collected, fixed in 4% paraformaldehyde (PFA) in Phosphate Buffered Saline (PBS), equilibrated in 15% sucrose overnight followed by 30% sucrose for 48h and embedded in optimal cutting temperature (OCT) medium. For each organ, 4-µm cryostat sections were cut, post-fixed in 4% PFA and blocked with a solution of PBS-0.1% Triton X-100 (PBS-T), 1% BSA and 5% goat serum. Primary antibodies were incubated in PBS-T with 2% of goat serum for 1 h in a humid chamber. Secondary antibodies were diluted in PBS-T with DAPI (4′,6-Diamidino-2-phenylindole, nuclei staining) and incubated for 30 min in dark humid chamber. Slices were washed in PBS and mounted with Mowiol®4-88 (Sigma Aldrich) 20% in TBS. Images were taken using a fluorescence microscope (Leica DM 2500) and analyzed by LASX (Leica Application Suite X). Primary and secondary antibodies are listed in Suppl Tab 5.

FVIII activity assay and anti-FVIII antibody detection

FVIII activity and anti-FVIII antibodies were monthly assessed in plasma of LV-treated B6/129 HA mice as previously described[29]. FVIII activity was expressed as a percentage of correction and

measured using the activated partial thromboplastin time (aPTT) assay generating a standard curve of serially diluted Refacto® pooled hemophilic mouse plasma. Anti-FVIII antibodies were evaluated using a direct ELISA by incubating mouse plasma in a 96-well plate coated with 5µg/ml Refacto® saturated with BSA (0.2% TBS-BSA). After the incubation with HRP-conjugated goat anti-mouse total IgG (Thermo Fisher Scientific), the reaction was developed with the addition of tetramethylbenzidine and absorbance measured at 450nm using a Victor X spectrophotometer (PerkinElmer). The plasma of LV.PGK.FVIII treated B6/129 HA mice was used as positive control.

SUPPLEMENTAL REFERENCES

1. Follenzi A, Naldini L. Generation of HIV- 1 derived lentiviral vectors. Methods Enzymol 2002;346454–465.

2. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 1995;10(1):119–121.

SUPPLEMENTAL TABLES

Table S1. RT-PCR primers

Primer name	Primer sequence	Primer use
hF8_EX19F	CCTTATTGGCGAGCATCTACA	RT-PCR
hF8_EX22R	TTGGTGCCAACAGATCCAC	RT-PCR
hEts-1_EX6F	CATATCAAGTTAATGGAGTC	RT-PCR
hEts-1_EX7R	TGTTTGATAGCAAAGTAGTC	RT-PCR
hEts-2_EX6F	GTGGAGTGAGCAACAGGTAT	RT-PCR
hEts-2_EX7-8R	CCAAAACCTAATGTATTGCTG	RT-PCR
hBactin_F	ACTACCTCATGAAGATCCT	RT-PCR
hBactin_R	TGGTACCACCAGACAGCACT	RT-PCR

Table S2. Primers for shortened F8 promoters cloning

Primer name	Primer sequence	Primer use	Lost TFBS	
pF8_-600_XhoI_F	CAGCCTCGAGGTTTTTAAAACAATAGTTGCCTAACC	pF8 deletion	----	----
pF8_-446_XhoI_F	CAGCCTCGAGCTAGAATCTTCGACAACATCCAGA	pF8 deletion	----	1 Ets-2
pF8_-342_XhoI_F	CAGCCTCGAGTGACTTCTCCATCCCTCTCC	pF8 deletion	1 Ets-1	1 Ets-2
pF8_-246_XhoI_F	CAGCCTCGAGGCCTCCCTTTTGCTACTTCA	pF8 deletion	2 Ets-1	1 Ets-2
pF8_HindIII_R	CGCAAGCTTGACTTATTGCTACAAATGTTCAAC	pF8 deletion	----	----

Table S3. Mutagenesis primers for F8 promoter Ets-core deletion

Primer name	Primer sequence	Disrupted TFBS	
pF8_E1_F	CTTCCCACTGATAAAAAGCAATCCTATCGGTTACTG	Ets-1 and Ets-2	-189 to -198
pF8_E1_R	CAGTAACCGATAGGATTGCTTTTTATCAGTGGGAAG		
pF8_E2_F	TGCTACTTCAGTTCTGTGGCTGCTTCCCAC	Ets-1	-217 to -223
pF8_E2_R	GTGGGAAGCAGCCACAGAACTGAAGTAGCA		
pF8_E3_F	CATCCCTCTCCTCCTTTAAAGGTTCTGATTAAAG	Ets-1	-310 to -319
pF8_E3_R	CTTTAATCAGAACCTTTAAAGGAGGAGAGGGATG		
pF8_E4_F	CTTGCTGCTGCCACTCAGAGGGTTGGAGTAGGCTAG	Ets-1	-395 to -401
pF8_E4_R	CTAGCCTACTCCAACCCTCTGAGTGGCAGCAGCAAG		
pF8_E5_F	ACAGTTTTAAAAACTATAAATCATACTGGCAGCAGTGTGA	Ets-2	-526 to -535
pF8_E_R	TCACACTGCTGCCAGTATGATTTATAGTTTTTAAAACTGT		

Table S4. Mutagenesis primers for Ets-1 and Ets-2 DNA binding domain deletions

Primer name	Primer sequence	Primer use
Ets-1_DBD_del_F	ACCCCTGAGGAGCTGCACGCCATGCT	Ets-DBD deletion
Ets-1_DBD_del_R	P-TGGTCCACTGCCTGTGTAGCCAGCTA	Ets-DBD deletion
Ets-2_DBD_del_F	TGCGACCTCCAGAACTTGCTGGGGTT	Ets-DBD deletion
Ets-2_DBD_del_R	P-AGGTCCACTTCCTGTGAAGCCGGCCA	Ets-DBD deletion

Table S5. List of primary and secondary antibodies used for immunofluorescence

Primary antibodies	**Host**	**Reactivity**	**Clone n°**	**Manufacturer**	**Dilution**
Anti-GFP	rabbit		polyclonal	ThermoFisher	1:1000
Anti-Lyve-1-biotin	rat	mouse	ALY7	eBioscience	1:200
Anti-F4/80-biotin	rat	mouse	A3-1	AbD serotec	1:400
Anti-CD31	rat	mouse	MEC13.3	BD pharmingen	1:50

Secondary antibodies	**Fluorophores**	**Manufacturer**	**Dilution**
Goat anti-rat IgG	Alexa Fluor 546	ThermoFisher	1:500
Goat anti-rabbit IgG	Alexa Fluor 488	ThermoFisher	1:500

Table S6. Primers for lentiviral vector integration analysis

Primer name	Primer sequence	Primer use
Wpre_F	TTGCTTCCCGTATGGCTTTC	Integration
Wpre_R	AGCTGACAGGTGGTGGCAAT	Integration
hGAPDH_Forward	AGTGGGTGTCGCTGTTGAAGT	Integration
hGAPDH_Reverse	AACGTGTCAGTGGTGGACCTG	Integration

SUPPLEMENTAL FIGURES

Figure S1. HEK293T model confirmed the importance of the Ets-responsive elements in the proximal *F8* promoter. Histograms representing fold change in luciferase activity of shortened **(A-B)** and core deleted promoters **(C-D)** at basal level or after overexpression of Ets-1. Results are expressed as mean ± SD from 3 independent experiments performed in triplicate. * $p<0.05$.

Figure S2. Mutations of E3, E4 and E5 Ets-BSs did not affect pF8 activity in ECV-304. (A-B) Histograms representing fold change in luciferase activity in ECV-304 cells of Ets core-deleted promoters not displayed in Figure 3F at basal level **(A)** or after **(B)** overexpression of Ets-1, Ets-2 or both. Results are expressed as mean ± SD from 3 independent experiments performed in triplicate. * $p<0.05$.

Figure S3. Use of a guide RNA able to target F7 promoter in comparison with the Activation system in F8 promoters. Graph showing the transactivation effects of the sgRNA F8.2 and sgRNA F7.5 on pF8-1175, pF8-600 and pF8-342 in and HEK293T cells. Results are expressed as mean ± SD from 2 independent experiments performed in triplicate.

Figure S4. *F8* shortened promoters drive GFP expression in hepatic endothelial cells after 1 month of LV delivery. Immunofluorescence analyses of C57BL/6 livers after LV.pF8-1175-GFP, LV.pF8-600.GFP, LV.pF8-446.GFP or pF8.342.GFP. **(A)** Liver sections stained for the endothelial marker (Lyve-1, in red) and the green fluorescent protein (GFP) using 400X magnification. **(B)** Liver of LV injected mice stained with the macrophage marker F4/80 (red) in combination with GFP (green). Scale bars, 50µM. Nuclei are stained with DAPI (blue).

Figure S5. GFP expression in spleens under the control of pF8 variants. Mice spleen sections, one month after delivery of LV carrying pF8 variants. In red the endothelial marker CD31 (left side) or the macrophage marker F4/80 (right side) with GFP (green). Nuclei are stained with DAPI (blue). Scale bars, 100µM.

Figure S6. GFP expression in liver and spleens under the control of pF8-342 two months after delivery. Mice liver **(A-B)** and spleen **(C-D)** sections, two month after delivery of LV carrying pF8-342. Lyve-1 and CD31 were used as endothelial markers in the liver **(A)** and spleen **(C)** respectively. F4/80 was used as macrophage marker for both spleen and liver (B-D). All tissue markers are combined with GFP (green) and nuclei are stained with DAPI (blue). Scale bars, 100µM.

Figure S1

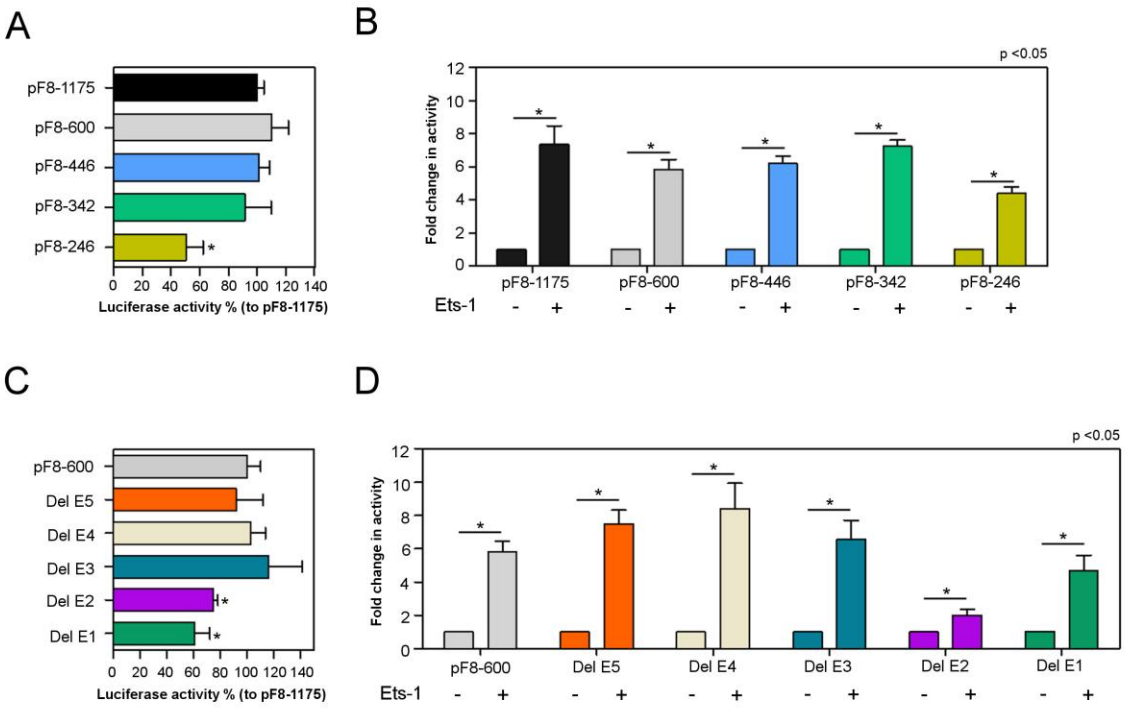

A
pF8-1175
pF8-600
pF8-446
pF8-342
pF8-246
0 20 40 60 80 100 120 140
Luciferase activity % (to pF8-1175)
B
p <0.05
Fold change in activity
pF8-1175
pF8-600
pF8-446
pF8-342
pF8-246
Ets-1
C
pF8-600
Del E5
Del E4
Del E3
Del E2
Del E1
0 20 40 60 80 100 120 140
Luciferase activity % (to pF8-1175)
D
p <0.05
Fold change in activity
pF8-600
Del E5
Del E4
Del E3
Del E2
Del E1
Ets-1

Figure S2

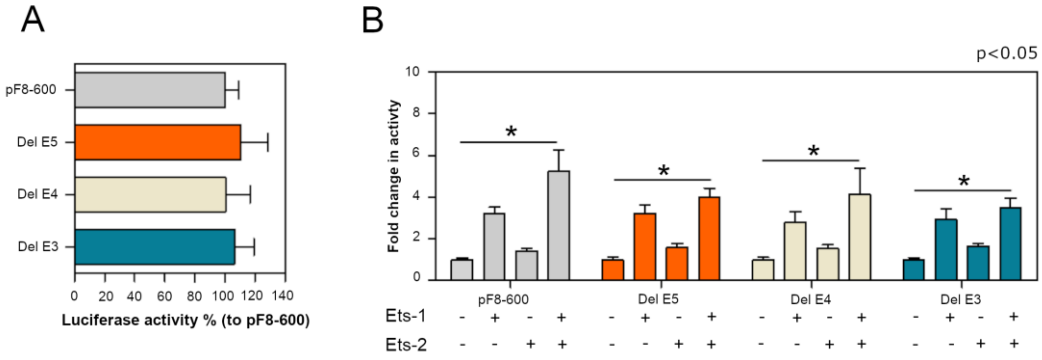

A
pF8-600
Del E5
Del E4
Del E3
0 20 40 60 80 100 120 140
Luciferase activity % (to pF8-600)
B
p<0.05
Fold change in activty
10
8
6
4
2
0
*
*
*
*
pF8-600
Del E5
Del E4
Del E3
Ets-1 - + - + - + - + - + - + - + - +
Ets-2 - - + + - - + + - - + + - - + +

Figure S3

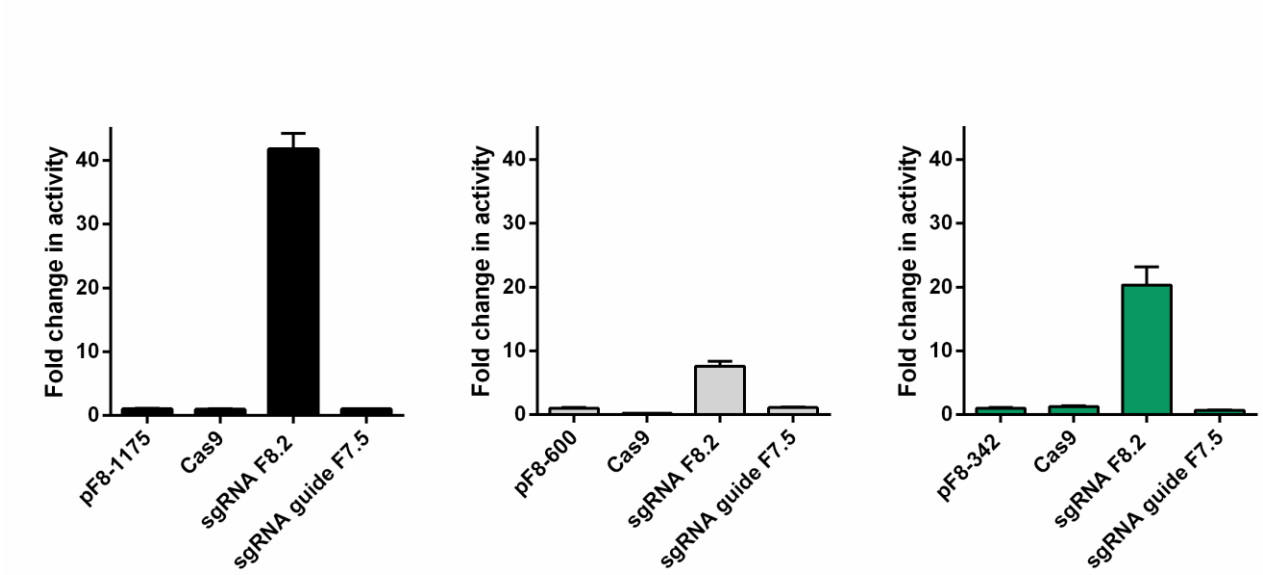

Fold change in activity
0
10
20
30
40
pF8-1175
Cas9
sgRNA F8.2
sgRNA guide F7.5
Fold change in activity
0
10
20
30
40
pF8-600
Cas9
sgRNA F8.2
sgRNA guide F7.5
Fold change in activity
0
10
20
30
40
pF8-342
Cas9
sgRNA F8.2
sgRNA guide F7.5

Figure S4

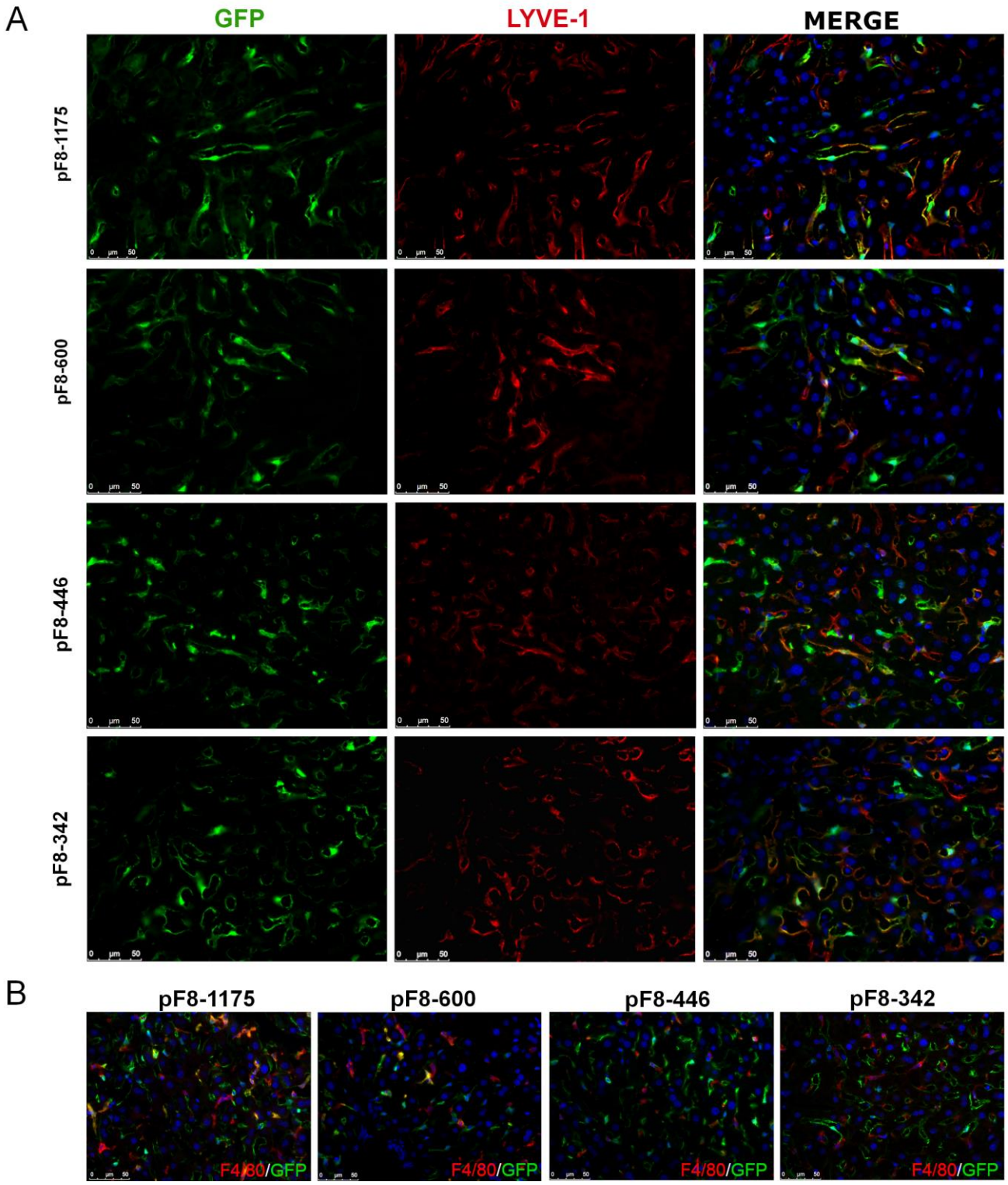

A
GFP
LYVE-1
MERGE
pF8-1175
pF8-600
pF8-446
pF8-342
B
pF8-1175
pF8-600
pF8-446
pF8-342
F4/80/GFP
F4/80/GFP
F4/80/GFP
F4/80/GFP

Figure S5

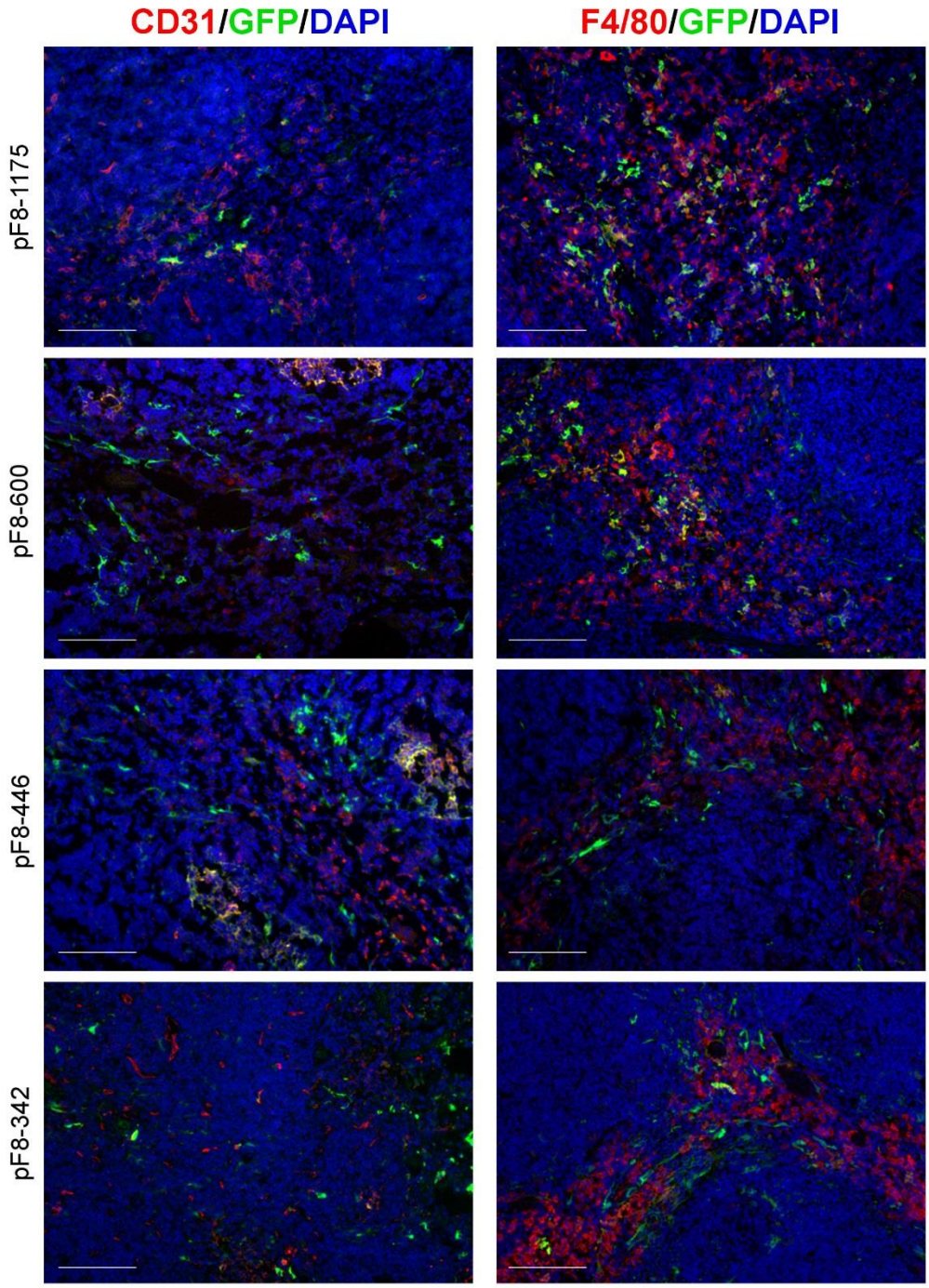
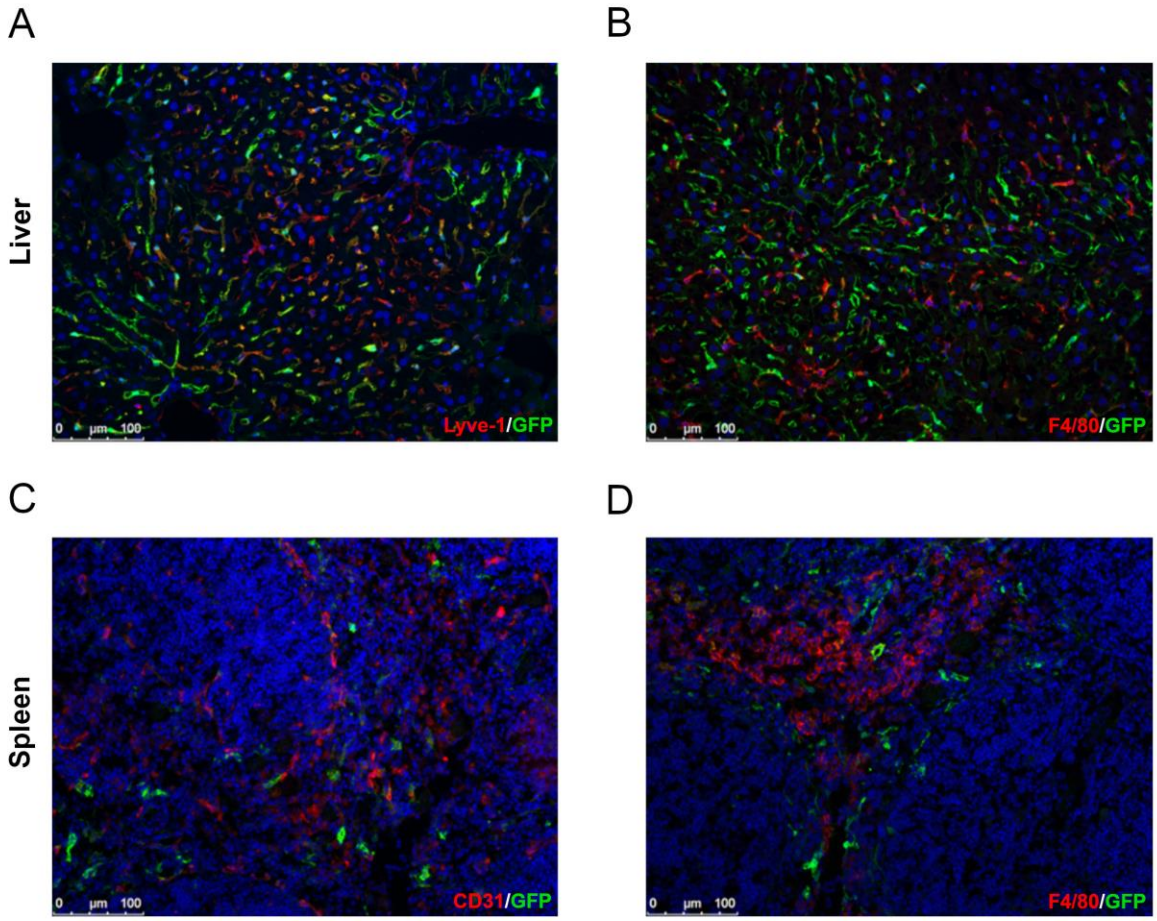


Figure S6


A
B
Liver
0 µm 100
Lyve-1/GFP
0 µm 100
F4/80/GFP
C
D
Spleen
0 µm 100
CD31/GFP
0 µm 100
F4/80/GFP