Supporting Materials and Methods

Vector construction

The strong liver-specific promoter, designated as P3, is composed of a minimal transthyretin promoter (TTR_{min}), which contains ~200 bp region upstream of the *TTR* gene Cap site (GenBank accession number M19524.1), coupled to a *de novo* designed hepatocyte-specific *cis*-regulatory module 8, HS-CRM8[1]. The 72 bp HS-CRM8 element (double underlined in the sequence given below) is derived from the human *Serpina1* gene and contains several overlapping putative transcription factor binding sites (TFBS) including FOXA1, CEBP, HNF1, MyoD, LEF-1 and LEF-1/TCF that are strongly associated with robust liver-specific expression *in vivo* . The entire P3 enhancer-promoter sequence was the following: 5'–ACTAGTACCGGCGCGCCGGGGGAGGCTGCTGGTGAATATTA ACCAAGGTCACCCCAGTTATCGGAGGAGCAAACAGGGGCTAAGTCCACACGCGT GGTACCGTCTGTCTGCACATTTCGTAGAGCGAGTGTTCCGATACTCTAATCTCCCT AGGCAAGGTTCATATTTGTGTAGGTTACTTATTCTCCTTTTGTTGACTAAGTCAAT AATCAGAATCAGCAGGTTTGGAGTCAGCTTGGCAGGGATCAGCAGCCTGGGTTG GAAGGAGGGGGTATAAAAGCCCCTTCACCAGGAGAAGCCGTCACACAGATCCAC AAGCTCCTGTCGCGA–3'. This P3 promoter fragment containing the restriction sites *Spe*I (underlined) and *Nru*I (dashed underlined) was synthesized by GeneArt AG (Regensburg, Germany) to obtain vector pMA-P3S.

The mouse *Pah*-cDNA (ENSMUST00000020241) containing the murine 5' Kozak consensus sequence overlapping with the (underlined) translation initiation codon (5'-GCCGCCAGCATGG-3') was amplified by PCR from plasmid pAAV2-PKU5, which was used previously for viral-directed gene therapy[2], with primers containing the terminal restriction sites *Nru*I (underlined in forward primer: 5'-TTGGTCGCGAATTCTAGCTGCAGCA-3') and *Pvu*I (underlined in reverse primer: 5'-

TTGACGATCGACGCGTCACGATCTG-3'). The amplified *Pah*-cDNA fragment was ligated between the P3 promoter and the bovine growth hormone polyA signal (BGHpA) into vector pMA-P3S (from GeneArt AG, Regensburg, Germany). The entire expression cassette containing P3-*Pah*-cDNA-BGHpA was excised with *Spe*I and *Xba*I, and ligated between the *att*B and *att*P sites of plasmid pMC.BESPX (kindly provided by Dr. Kay, Stanford University, Stanford, CA, USA) to obtain a parental plasmid pMC.PKU20, a producer plasmid of minicircle.

A 'Flag' tag (amino acid sequence DYKDDDDK) was inserted at the N terminus of the mouse *Pah*-cDNA of pMC.PKU20 to generate pMC.PKU37 by PCR with the forward primer: 5'-ATGGACTACAAAGACGATGACGACAAGGCAGCTGTTGTCCTGGAGAAC-3' (underline is the sequence of the flag tag) and the reverse primer: 5'-CGTCGCGAGCCGCCAGCATGGACTACAAAG-3'.

Minicircle generation

Minicricle (MC)-DNA was generated as described by a protocol by Kay et al.[3]. Briefly, the minicircle-producer plasmid, pMC.PKU20, was transformed into the bacterial strain ZYCY10P3S2T, expressing bacteriophage ØC31 integrase and I-*Sce*I homing endonuclease, both under the tight control of the arabinose-inducible promoter p*BAD*. Bacterial cells were inoculated in 400-ml Terrific Broth (Invitrogen, Carlsbad, CA, U.S.A) containing Kanamycin (50 µg/ml, Sigma Aldrich, St Louis, MO, USA) for overnight. The next day, 1 volume of Lennox L-Broth (400-ml LB-medium, Invitrogen) containing 0.04 volume of 1N NaOH (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and 0.1% volume of 20% L-arabinose (final concentration 0.01%; Sigma Aldrich) was combined with a 400 ml overnight culture, followed by cultivating the cells at 32ºC for additional 7 hours. Cells were centrifuged and the (supercoiled) minicircle-DNA vectors were isolated from bacterial lysate by using the Qiagen

endofree plasmid purification kit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's protocol with double volume of the re-suspension, lysis and neutralization buffers. Purified MC-DNA vectors were quantified by UV spectroscopy (Nanodrop ND1000, Thermo Fisher Scientific, USA), and densitometry following 0.8% agarose gel electrophoresis.

Serum biochemistry parameters

Whole blood of animal was obtained by retro-orbital bleeding at 14 days prior injection or 1 or 14 days after hydrodynamic gene delivery. Serum was prepared by separation of coagulated whole blood in Microtainer tubes (Becton, Dickinson and Company, Basel, Switzerland). Serum concentration of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by automated analyzer UniCel DXC600 (Beckman Coulter, Nyon, Switzerland) in the Division of Clinical Chemistry and Biochemistry at University Children's Hospital Zurich, Zurich, Switzerland. Endogenous (DNA) immunostimulatory and inflammation parameters such as interleukin 12 (IL-12 p40), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-□) were measured by Cytolab (Dällikon, Switzerland).

***Pah*-mRNA *gene* expression analysis**

20-30 mg of mouse liver tissue was used for RNA isolation using QIAmp RNA blood mini kit (Qiagen AG, Switzerland) in accordance with the manufacturer's protocol. Random primed cDNA was prepared from 1 µg of total RNA using the Reverse Transcription kit (Promega, Wallisellen, Switzerland). Quantitative PCR was performed using TaqMan Gene Expression Assay of the murine *Pah* (Mm00500918_m1) and TaqMan universal master mix (Applied Biosytems, Zug, Switzerland). Standard primers and probes for murine *Gapdh* (Mm99999915_g1, Applied Biosystems, Switzerland) were used as internal controls. The

relative amounts of *Pah* expression were calculated by real-time qPCR using an ABI PRISM 7900 sequence detector (Applied Biosystems, Switzerland).

Copy number assay

Genomic DNA (gDNA) from different tissues (liver, kidney, heart, lung, muscle, spleen and brain) was isolated using DNeasy blood and tissue kit (Qiagen AG, Switzerland). In accordance with the manufacturer's protocol, 100 ng of gDNA from each sample was used as a template. A standard curve plotting cycle threshold (Ct; y-axis) against log vector copy number (x-axis) was generated using serially diluted DNA vector with various copy numbers (2×10^7 copies to 200 copies) with $y = -3.2 x + 38.2$, $R^2 = 0.996$ and $y = -3.3 x + 39.3$, $R^2 = 0.992$ for parental plasmid (PP) pMC.PKU20 and minicircle (MC) MC.PKU20, respectively, along with 100 ng non-infused control gDNA. The number of vector genomes per cell in different tissues was determined by quantitative TaqMan PCR analysis using primers and probes corresponding to BGHpA: forward primer: 5'-GCCTTCTAGTTGCCAGCCAT-3'; reverse primer: 5'-GGCACCTTCCAGGGTCAAG-3'; and probe: 5'-FAM-TGTTTGCCCCTCCCCCGTGC-TAMRA-3'. The same *Gapdh* Taqman assay as described above was included as loading control and the BHGpA signal was then normalized to *Gapdh* signal. Quantitative PCR reactions were performed by ABI PRISM 7900 sequence detector, and the data were analyzed with Sequence Detection System (Applied Biosystems, Switzerland). The copy number in 100 ng gDNA from each group was calculated according to the description by Lu et al.[4], and from Applied Biosystems http://www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf.

To test the F1 generation from MC-treated PKU mice for the presence of MC vector DNA, the same BGHpA-forward and reverse primers were used for saturating PCR.

Methylation-dependent Southern analysis

We first analysed the sequences of MC.PKU20 and pMC.PKU20 by using the MethPrimer application[5]. This analysis identified the presence of distinct CpG islands in the P3 promoter and in the *Pah* gene as well as several CpG motifs throughout the vectors. Total liver DNA was extracted using a DNeasy mammalian genomic DNA kit (QIAGEN). The isolated DNA was quantified using a NanoDrop ND1-1000 spectrophotometer (LabTech). Total liver DNA (30 □g) was then digested with *Eco*RI either alone or along with *Asc*I. (*Eco*RI cuts both the minicircle (MC)-DNA and the producer plasmid once whilst *Asc*I also cuts both vectors once but only in the absence of CpG methylation). The digested DNA was then separated on a 0.8% agarose gel (20 V, 20 mA overnight), and blotted onto nylon membranes (Hybond XL, Amersham Biosciences). A 319 bp DNA fragment derived from PCR amplification of the P3 promoter (using the following primers 5'-ACCGGCGCGCCGGGGGAG-3' and 5'-CAGGAGCTTGTGGATCTGTGTGAC-3') which is common to all plasmids, was labelled with ^{32}P (Rad-Prime labelling kit, Invitrogen) and used as a probe. The hybridization was performed in Church buffer (0.25 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS) at 65°C for 16 h.

Histological studies

Flag-tagging was required for histochemical analysis to discriminate between the transgenic minicircle-derived wild-type PAH and the endogenous PKU (enu2) PAH-p.F263S, as the (inactive) mutant protein is expressed in PKU mice. Previous studies have shown that a PAH-fusion with a large N-terminal tag such as the maltose binding protein has similar kinetic properties, including positive cooperativity, like isolated wild-type PAH[6].

Mouse livers were perfused through hepatic portal vein, while the kidneys were perfused through renal arteries with 4% PBS-buffered paraformaldehyde when the mouse was under anesthesia with 5% isoflurane. Subsequently, the organs were fixated with 10% formalin for 1 day and preserved in ethanol 70% for paraffin embedding. The specimens were sectioned into

3 μm thick slices and stained with hematoxyline and eosin for regular histo-pathological analysis. For immunohistochemistry, sections were deparaffinized and rehydrated. Antigen retrieval was performed using the citrate buffer (10 mM dosium citrate buffer, pH6.0) steam method. The sections were then blocked with normal goat serum (Vectastain ABC kit, Vector laboratories, Burlingham, CA, USA) blocking solution at room temperature for 1 hour. Next, the primary DYKDDDDK tag antibody (1:500-1:1000, Cell Signaling Technology, Danvers, MA, USA) diluted in TBST-5% normal goat serum was added to the slides and incubated at 4°C overnight. The following day, the sections were incubated with anti-rabbit SignalStain® Boost Detection Reagent (Cell Signaling Technology, Danvers, MA, USA) for 30 min at room temperature. The slides were stained using liquid DAB Substrate Chromogen (DAKO North America, CA, USA) and hematoxylin for counterstain. Sections were examined and images were taken at 2.5 and 10 fold magnifications using a Leica microscope and camera (Leica UTR 6000). The assessment of the Flag tag was analyzed by a publicly available web application, called ImmunoRatio, which calculates the percentage of positively diaminobenzidine (DAB)-stained area out of the total hematoxyline-stained nuclear areas[7].

Supplementary text and figure:

Generation and production of minicircle (MC)-DNA vectors

For MC-DNA production, we used the system developed by Dr. Kay and coworkers, which exploits an *in vivo* intramolecular recombination process[3], as we could not establish a robust purification procedure for MC-DNA when applying the originally published methods[8]. Our transgene expression cassette comprises a liver-specific promoter P3[1], the murine phenylalanine hydroxylase cDNA (*mPah*), and the bovine growth hormone polyA signal. The expression cassette was flanked by the two recombination sites *att*B and *att*P, and was inserted into the parental plasmid (PP) vector to generate pMC.PKU20 (see Supporting Figure 1A). MC-DNA vector (MC.PKU20) devoid of bacterial backbone was generated via intramolecular recombination after addition of arabionse and was purified. A representative example is shown in Supporting Figure 1B, where purified vectors from bacterial cultures before and after arabinose addition were analyzed. Our results showed that the recombined MC-DNA vector was the major product (~ 97%, lane 4 in Supporting Figure 1B) with very low amounts of PP vector or plasmid bacterial backbone remaining, a result that is comparable to previously reported data[3]. Taken together, up to 2.5 mg of MC-DNA could be purified, equivalent to ~10^{15} MC.PKU20 particles, from a 1 l bacterial culture.

Supporting Figure 1. Generation of minicircle-DNA vectors by site-specific recombination. (A) Schematic representation of minicircle (MC)-DNA production. The parental plasmid (PP) pMC.PKU20 is based on vector pMC.BESPX, and was used as a producer for the generation of MC-DNA vector. The expression cassette contains the murine phenylalanine hydroxylase (*mPah*) transgene driven by the P3 promoter and the bovine growth hormone polyadenylation signal (BGHpA). By adding arabinose to the bacterial culture media and adjusting the temperature to 32ºC, the MC-DNA vector was produced by bacteriophage ØC31 integrase-mediated recombination between the bacterial (*att*B) and phage (*att*P) attachment sites, leaving a 36 bp hybrid *attR* sequence between the 5' promoter sequence and the 3' polyadenylation signal of the transgene expression cassette. The PP *and* the excised plasmid bacterial backbone were linearized by I-*Sce*I homing endonuclease and subsequently eliminated by bacterial exonuclease activity *in cellulae.* (B) Analysis of PP and MC-DNA vectors. Lanes 1 and 3 show the supercoiled, concatemeric PP and MC-DNA vectors, respectively. Lanes 2 and 4 depict the *Hind*III/*Cpo*I double-digested PP (1.1 kb and 5.0 kb) and the linearized MC-DNA vector (2.1 kb), respectively. *Hind*III cuts within the expression cassette, while *Cpo*I cuts the plasmid backbone. M, 1 kb ladder; PP, parental plasmid; MC, minicircle; P3-promoter element; *mPah*-cDNA, mouse phenylalanine hydroxylase-complementary DNA; BGHpA, bovine growth hormone polyadenylation tail; *att*B and *att*P, bacterial and phage attachment sites, respectively; I-*Sce*I, restriction endonuclease recognition site; ColE1, DNA replication origin; Kanamycin, kanamycin resistance gene; *att*R, right hybrid sequence; *att*L, left hybrid sequence.

Supplementary References

1 Nair, N. *et al.* Computationally designed liver-specific transcriptional cis-regulatory modules and hyper-functional factor IX improve liver-targeted gene therapy for hemophilia B. *Blood* **in press**, doi:10.1182/blood-2013-1110-534032 (2014).

2 Ding, Z., Georgiev, P. & Thöny, B. Administration-route and gender-independent long-term therapeutic correction of phenylketonuria (PKU) in a mouse model by

recombinant adeno-associated virus 8 pseudotyped vector-mediated gene transfer. *Gene Ther* **13**, 587-593 (2006).

3 Kay, M. A., He, C. Y. & Chen, Z. Y. A robust system for production of minicircle DNA vectors. *Nat Biotechnol* **28**, 1287-1289, doi:nbt.1708 [pii] 10.1038/nbt.1708 (2010).

4 Lu, J., Zhang, F., Xu, S., Fire, A. Z. & Kay, M. A. The Extragenic Spacer Length Between the 5' and 3' Ends of the Transgene Expression Cassette Affects Transgene Silencing From Plasmid-based Vectors. *Mol Ther*, doi:mt201265 [pii] 10.1038/mt.2012.65 (2012).

5 Li, L. C. & Dahiya, R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* **18**, 1427-1431 (2002).

6 Knappskog, P. M., Flatmark, T., Aarden, J. M., Haavik, J. & Martinez, A. Structure/function relationships in human phenylalanine hydroxylase. Effect of terminal deletions on the oligomerization, activation and cooperativity of substrate binding to the enzyme. *Eur J Biochem* **242**, 813-821 (1996).

7 Tuominen, V. J., Ruotoistenmaki, S., Viitanen, A., Jumppanen, M. & Isola, J. ImmunoRatio: a publicly available web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki-67. *Breast Cancer Res* **12**, R56, doi:bcr2615 [pii] 10.1186/bcr2615 (2010).

8 Bigger, B. W. *et al.* An araC-controlled bacterial cre expression system to produce DNA minicircle vectors for nuclear and mitochondrial gene therapy. *J Biol Chem* **276**, 23018-23027 (2001).