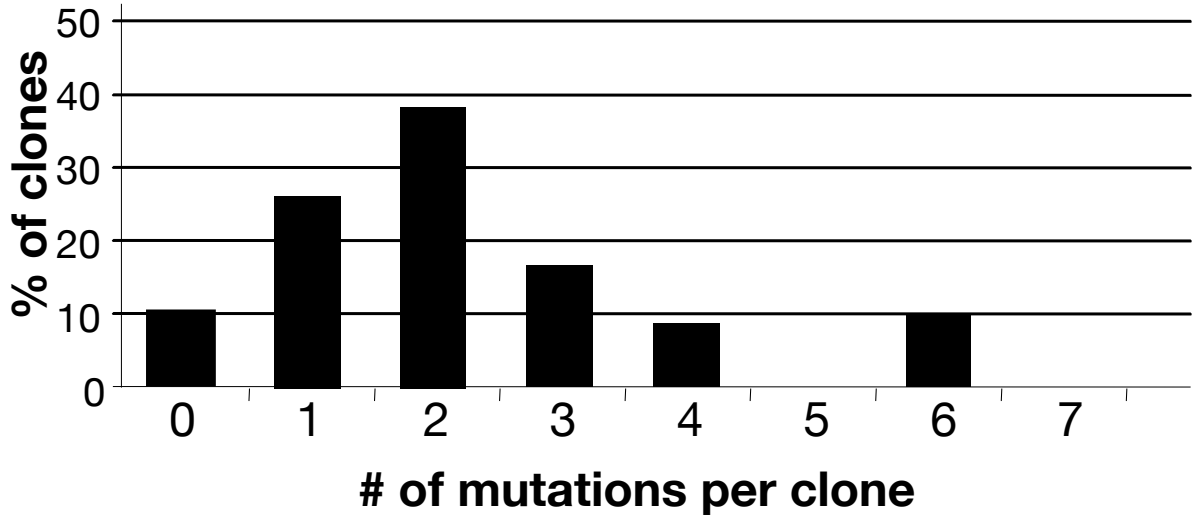
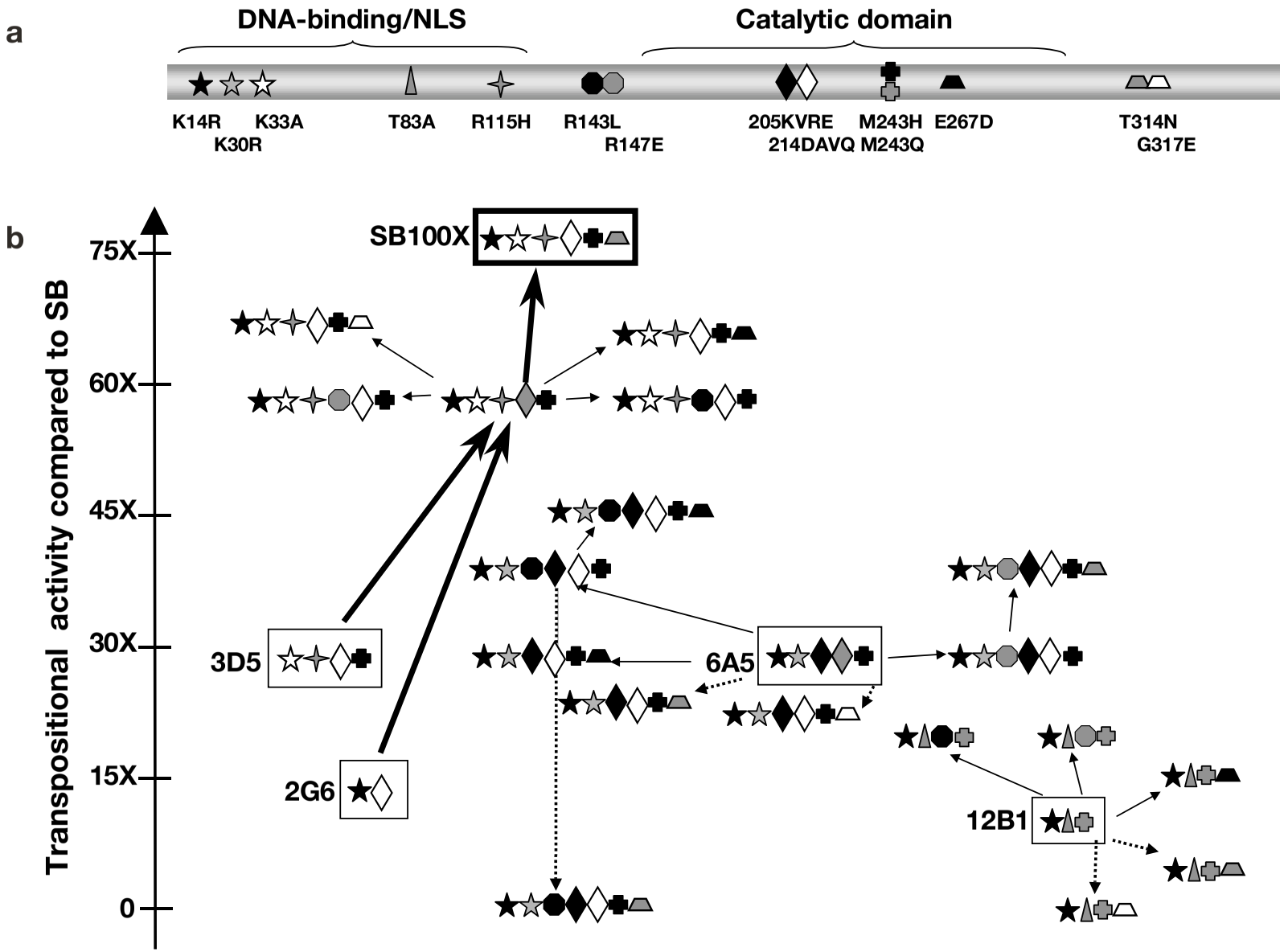
Supplementary Information

Molecular Evolution of a Novel Hyperactive Sleeping Beauty Transposase Enables Robust Stable Gene Transfer in Vertebrates

Lajos Mátés, Marinee K. L. Chuah, Eyayu Belay, Boris Jerchow, Namitha Manoj, Abel Acosta-Sanchez, Dawid P. Grzela, Andrea Schmitt, Katja Becker, Janka Matrai, Ling Ma, Esmira Samara-Kuko, Cony Gysemans, Diana Pryputniewicz, Csaba Miskey, Bradley Fletcher, Thierry VandenDriessche, Zoltán Ivics and Zsuzsanna Izsvák

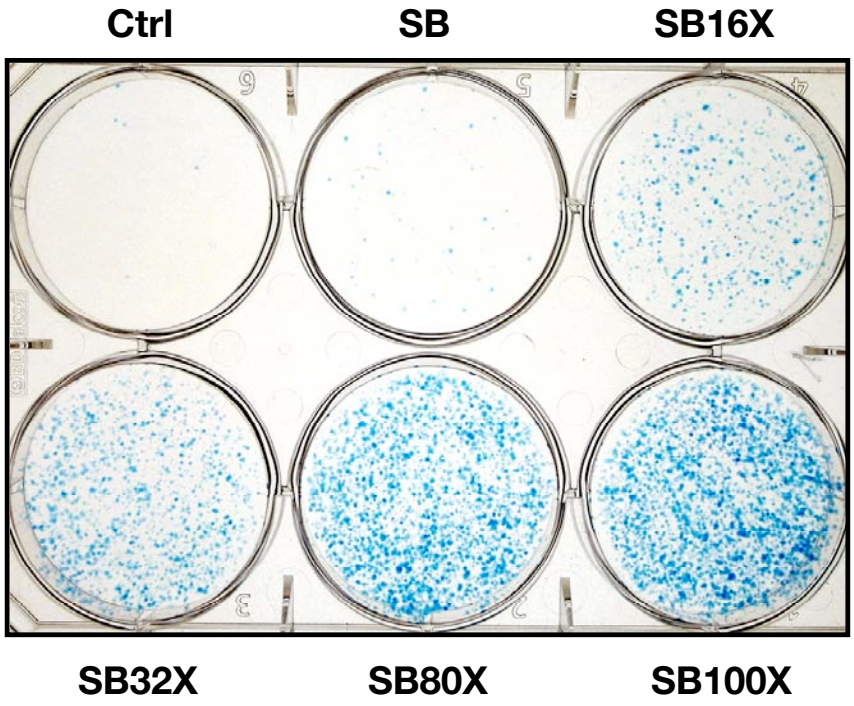


Supplementary Figure 1. Clonal distribution of the hyperactive library. The clonal distribution of the variants was determined by sampling 45/2000 clones.



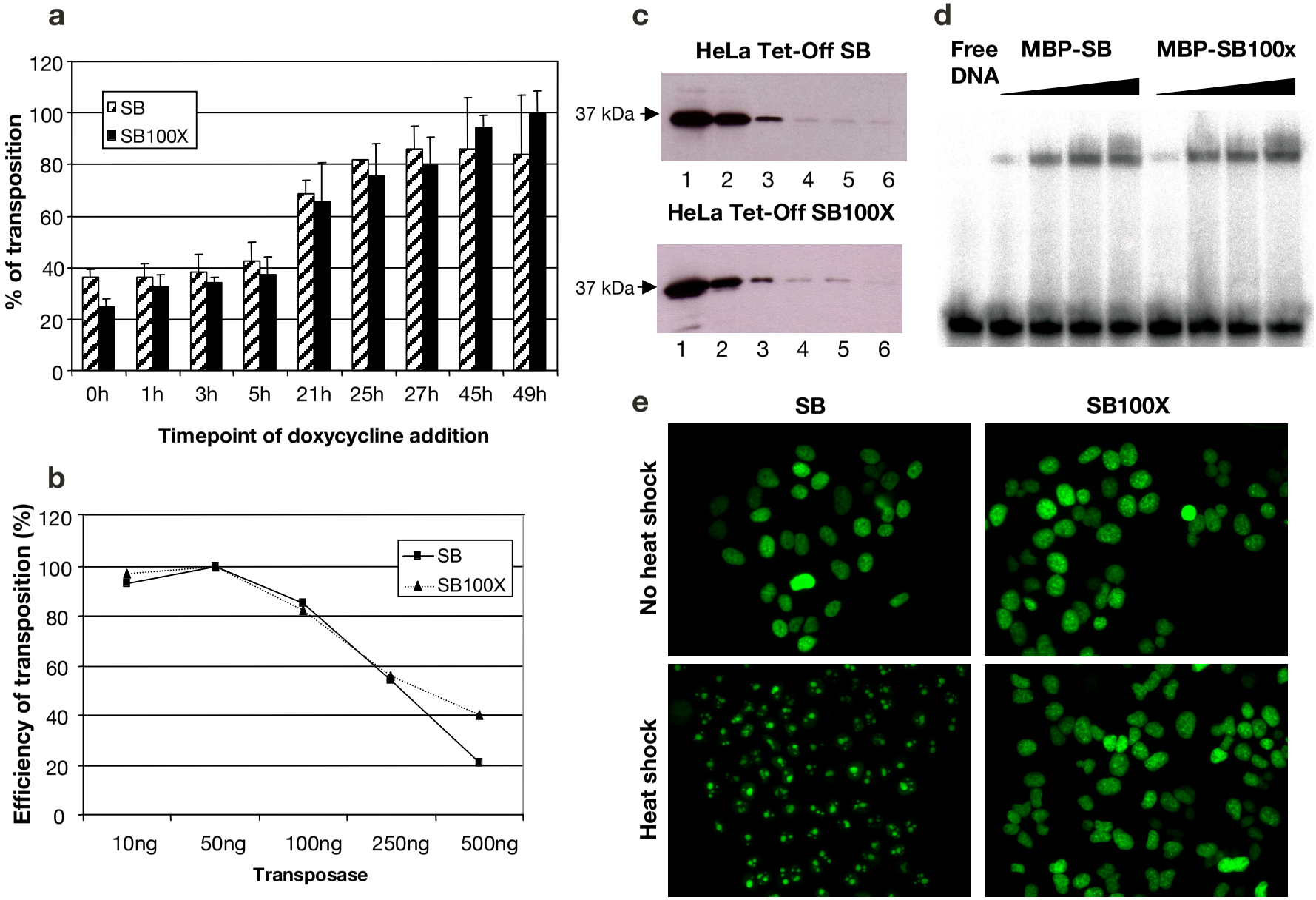
Supplementary Figure 2. The strategy of generating hyperactive transposases of higher order combinations. (a) The location of "friendly" mutations identified by the large-scale genetic screen. (b) Manual combination of four hyperactive clones showing 10-25-fold higher activities than SB (thin boxes) with "friendly" mutations. 2G6 could be successfully combined with 3D5, elevating the hyperactivity to 60-fold. Even "friendly" mutations behaved unpredictably in some combinations. For example, the G317E mutation significantly increased the hyperactivity on a 2G6/3D5 background (60- to 70-fold), but was deleterious in combination with 12B1. Similarly, the T314N mutation had a negative effect on 6A5 (20- to 25-fold), but further enhanced the hyperactivity of 2G6/3D5 (60- to 80-fold). Arrows represent an increase (solid arrow) or a decrease (dashed arrow) in transpositional activity. Thick arrows highlight the successful strategy that yielded SB100X.

Nature Genetics: doi:10.1038/ng.343



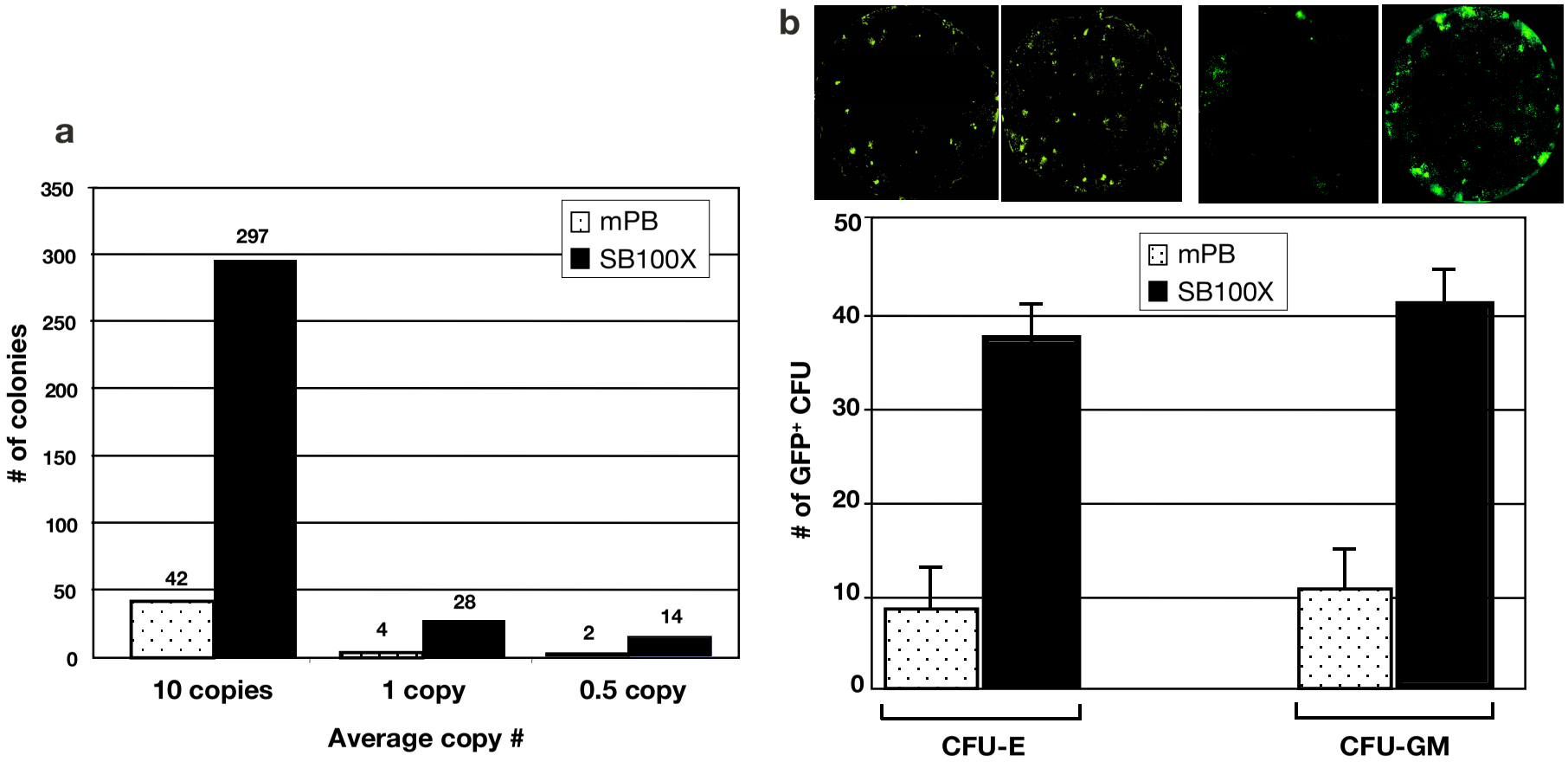
Supplementary Figure 3. Comparison of different hyperactive versions of SB in transfected human HeLa cells. The transfected amount of the transposon and transposase expression plasmids were 5 and 50 ng, respectively.

Nature Genetics: doi:10.1038/ng.343



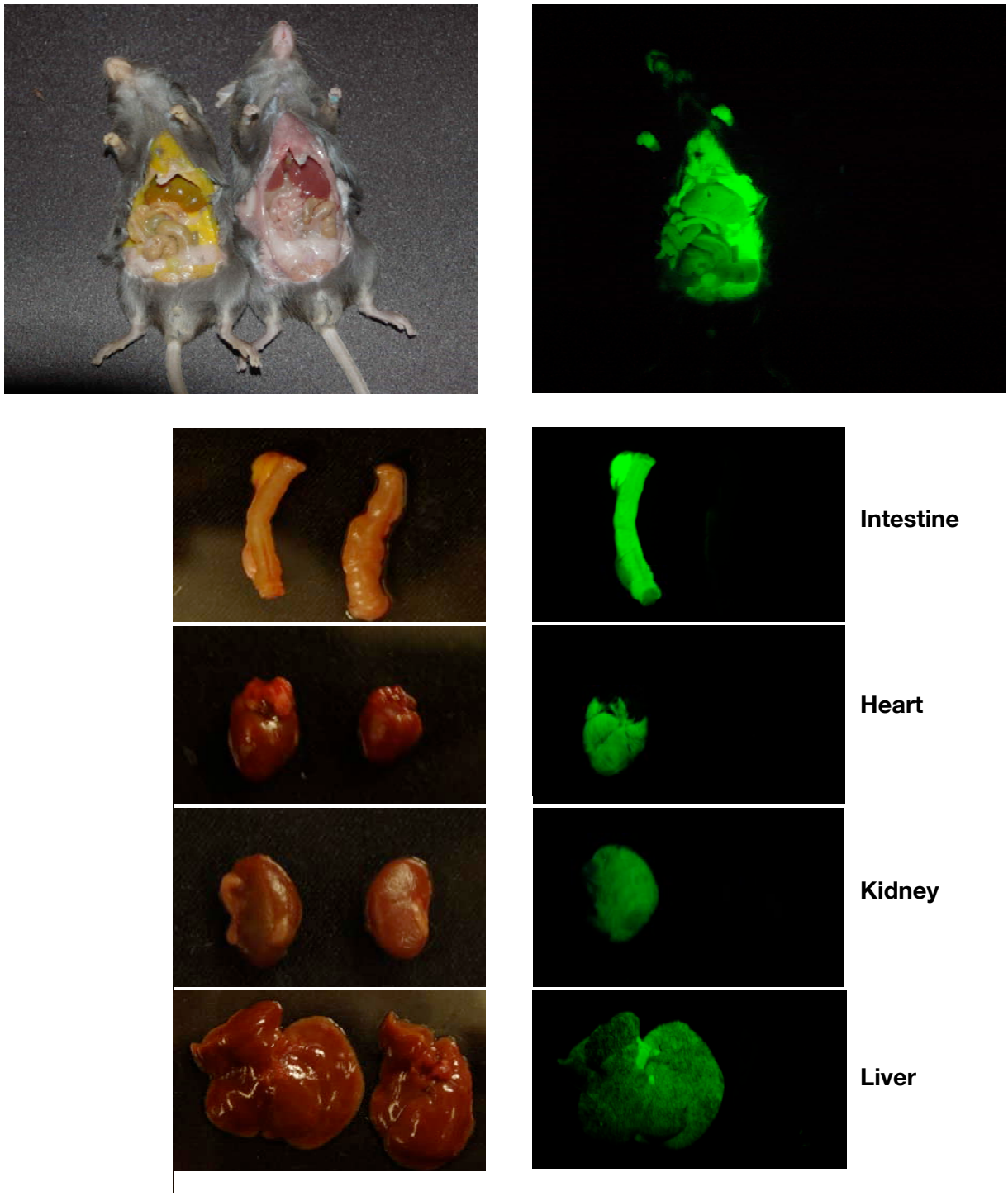
Supplementary Figure 4. Characterization of the SB100X transposase in HeLa cells. (a) Transposition assay is performed in HeLa-derived Tet-Off lines stably expressing the SB or SB100X transposases under the regulatable Tet-promoter. Cells were transfected with a puromycin-resistant transposon plasmid at time point 0. Doxycycline was added at indicated time points to shut down transposase expression. Antibiotic selection was started after 49 h. After one week of selection colonies were counted. Samples without doxycycline were set to 100% and other samples were calculated in relation to this value. (b) Overproduction inhibition profiling. Transposition assay using 50 ng transposon and increasing amounts of the transposase. Conditions with 50 ng SB or SB100X were set to 100%. (c) Protein stability assay. Western blot (polyclonal anti-SB) from HeLa Tet-Off SB or HeLa Tet-Off SB100X lines after different incubation times of doxycycline that shuts down transposase expression. 1) No doxycycline added. 2)-6) samples were taken after the cells were incubated in doxycycline for 1-4 days or for 6) one week. (d) Comparison of DNA-binding activities of SB and SB100X. EMSA conducted with increasing amounts of purified MBP-SB and MBP-SB100X on labeled oligo corresponding to the inner binding site of left ITR, as described (Zayed et al., 2003). (e) Sensitivity to heat shock. HeLa cells containing stably integrated Venus-transposase fusion constructs were incubated on a 43°C thermo-block for 30 min.

Nature Genetics: doi:10.1038/ng.343

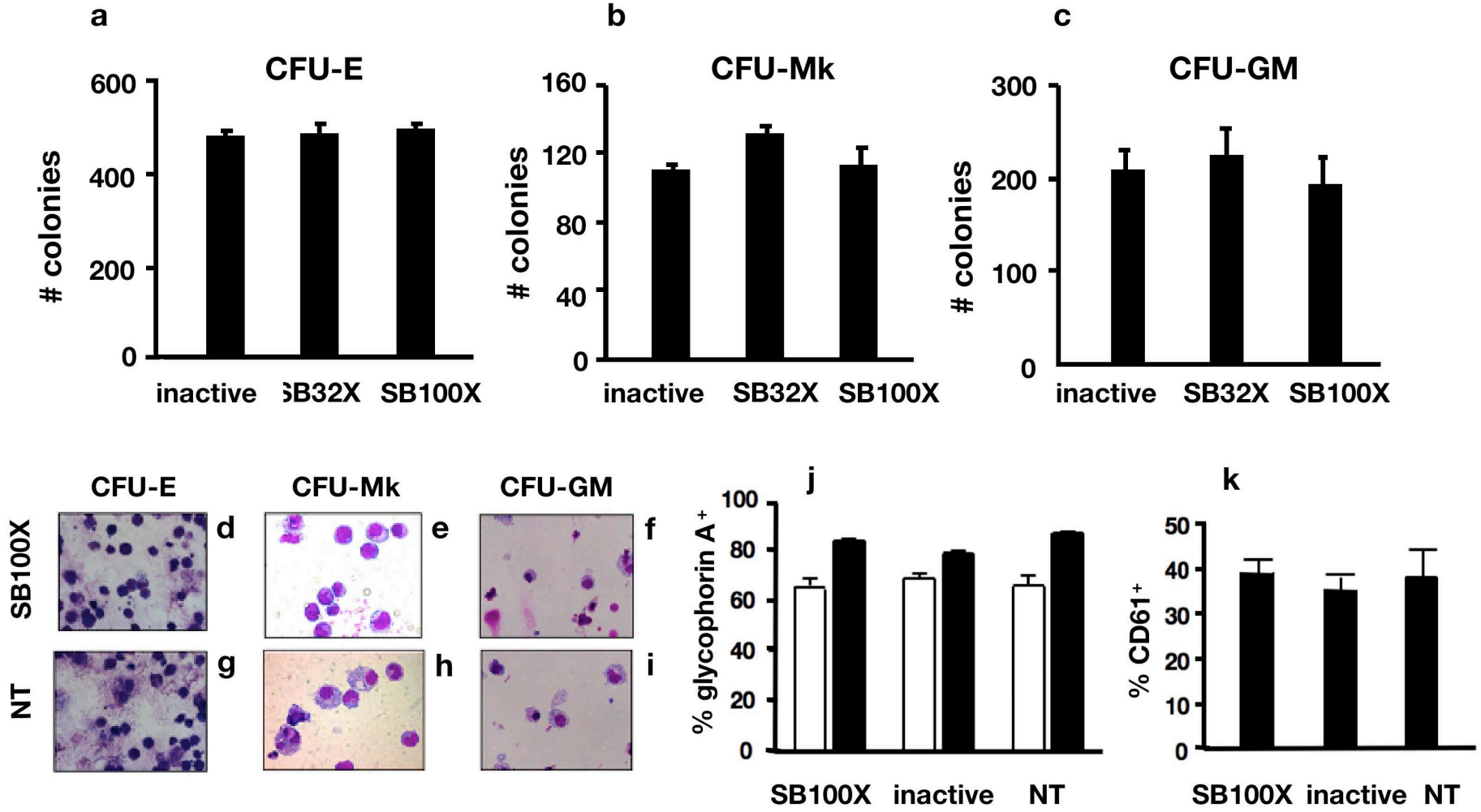


Supplementary Figure 5. Comparison of codon-optimized *piggyBac* and SB100X transposases. (a) Comparison of SB100X and codon-optimized *piggyBac* (mPB) transposases regarding efficacy of stable transgene integration at minimized transposon (Tp) plasmid dosages in HeLa cells. 600,000, 60,000 and 30,000 pT2/SVNeo and pXL-BacII/SVNeo plasmid molecules have been transfected into 300,000 HeLa cells, corresponding to a theoretical 10, 1 and 0.5 copy per transfected HeLa cell, calculated by a 20% transfection efficiency. The transfected amounts of the pCMV-SB100X and pCMV-mPB transposase expression plasmids were 50 ng and 100 ng respectively, based on earlier optimizations (data not shown). The total DNA amounts have been filled up to 500 ng at each experimental point using the irrelevant pFV4a plasmid. (b) Comparison of the transposition efficiency of the *piggyBac* and *Sleeping Beauty* (SB100X) transposon system in $CD34^+$ cells. Cord blood-derived $CD34^+$ cells were nucleofected with mPB or SB100X transposons along with a construct encoding the respective transposase [10 μg pXL-BacII/CAGGS-GFP and 5 μg pCMV-mPB; 10 μg pT2/CAGGS-GFP and 5 μg of pCMV-SB100X, based on earlier optimizations (data not shown)]. The total numbers of GFP^+ CFUs in the erythroid (CFU-E) and granulocytic/monocytic/macrophage (CFU-GM) lineages were compared. The mean value of GFP^+ CFUs +/- standard deviation of three independent experiments are shown. Mosaic images of the culture plate reflect the overall GFP^+ colonies in each condition.

Nature Genetics: doi:10.1038/ng.343

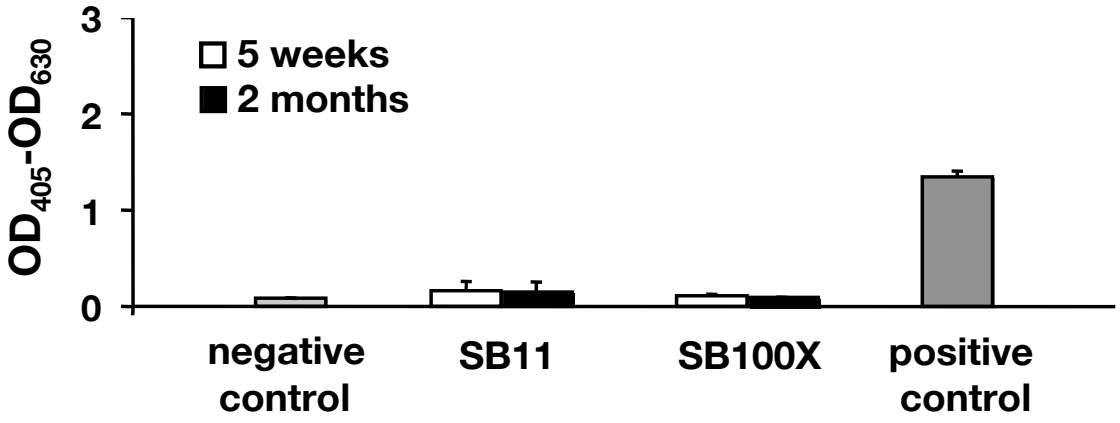


Supplementary Figure 6. Ubiquitous expression of the transgene in adult transgenic mice. The left panels show images taken at normal light, whereas the right panels show expression of the transgenic Venus marker in various organs of a transgenic animal.



Supplementary Figure 7. Phenotypic characterization of *in vitro* differentiated CD34⁺ cells transfected with hyperactive SB. Phenotypic characterization of *in vitro* differentiated CD34⁺ cells transfected with hyperactive SB. Cord blood-derived CD34⁺ cells were nucleofected with 10 μg of pT2HB-CAG-GFP and 5 μg of pCMV-SB100X, pCMV-SB32X or inactive control. The total number of CFUs using clonogenic assays after differentiation in the erythroid (CFU-E) (a), megakaryocytic (CFU-MK) (b) and granulocyte/monocyte/macrophage (CFU-GM) (c) lineages of was compared. The mean values of CFUs +/- standard deviation of three independent experiments are shown. Cytological comparison of Giemsa-stained pCMV-SB100X transfected (d, e, f) vs. non-transfected cells (g, h, i) after differentiation along the CFU-E (d, g), CFU-MK (e, h) and CFU-GM (f, i) lineages. (j, k) Cytofluorimetric (FACS) analysis of lineage specific cell surface markers in pCMV-SB100X transfected *vs*. non transfected (NT) HSCs: % glycophorine A expression (j) at day 7 (white bars) and day 10 (black bars) based on CFU-E clonogenic assays; CD61 expression (k) after 14 days of differentiation based on CFU-Mk clonogenic assays (k).

Nature Genetics: doi:10.1038/ng.343



Supplementary Figure 8. Analysis of anti-FIX antibody response. Anti-FIX antibodies were measured by ELISA in plasma from mice subjected to hepatic gene delivery with FIX-transposons and expression plasmids encoding SB100X, SB11 or inactive transposase. Plasma from non-injected mice and mice injected with rFIX and adjuvant were used as controls.

Mutations	Activity	SB 11	SB 16X	SB 32X	SB 80X	SB 100X
K13A	**1.7x**[1]		X			
K13D	**4x**					
K14R	**4x**				X	X
K30R	**2x**					
K33A	**4x**		X	X	X	X
T51N	**wt**					
T52V	**wt**					
L64A	**1.7x**[1]					
E69A	**3x**[1]					
L72A	**2x**[1]					
T83A	**2x**[1]		X			
L91A	**3x**[1]					
G95A	**2x**[1]					
I100L	**1.1x**					
N111G	**4x**					
R115H	**1.6x**[2]			X	X	X
N125K	**wt**					
T136R	**2x**[3]	X				
R143L	**2x**					
R147E	**2x**					
H187Y	**3x**					
G189A	**3x**					
AA199SS	**wt**					
T203V	**4x**					
A205K	**wt**					
AHKD205,7,8,10KVRE	**2x**[4]		X			
RKEN214-217DAVQ	**4x**[4]		X	X	X	X
N217H	**4x**					
M243H	**2x**			X	X	X
M243Q	**1.3x**[3]	X				
V253L	**2.5x**					
VVA253-255HVR	**2x**[3]	X				
K262R	**1.5x**					
E267D	**2x**					
S270A	**2x**					
A283R	**wt**					
R288A	**2x**					
L303F	**wt**					
H312P	**wt**					
T314N	**2.5x**					X
G317E	**1.5x**					
		3x	**16x**	**~32x**	**~80x**	**~100x**

Activity

Supplementary Table 1. List of 41 transposase versions used in this manuscript. The table on the left lists 41 individual mutations and their respective transpositional activity ranging between 100-400% hyperactivity. The marked hyperactive mutations have been previously reported[1-4]. The table on the right lists the presence of individual hyperactive amino acid replacements in the hyperactive transposase versions used in this manuscript, and their cumulative effects on transpositional acivities as determined in single-copy transposon remobilization assays.

1. Yant, S.R., et al., *Mutational analysis of the N-terminal DNA-binding domain of sleeping beauty transposase: critical residues for DNA binding and hyperactivity in mammalian cells.* Mol Cell Biol, 2004. **24**(20): p. 9239-47.
2. Zayed, H., et al., *Development of hyperactive sleeping beauty transposon vectors by mutational analysis.* Mol Ther, 2004. **9**(2): p. 292-304.
3. Geurts, A.M., et al., *Gene transfer into genomes of human cells by the sleeping beauty transposon system.* Mol Ther, 2003. **8**(1): p. 108-17.
4. Baus, J., et al., *Hyperactive transposase mutants of the Sleeping Beauty transposon.* Mol Ther, 2005. **12**(6): p. 1148-56.

Supplemental Table 2: Transposon insertion sites recovered from transfected CD34$^+$ cells *ex vivo* and *in vivo*
Junction fragments rescued by splinkerette PCR, transposase: SB100X

Source	Clone	Junction with IR sequence in bold	E-value	Location, Homology
Human Cd45$^+$ cells, *in vivo*	1	**CAACTGTA**TAAAGTTATATACTCTCCCCAGAGCCTTGA GACACCCTCTATACTCCCAGTAAAAGAAAAAAGAAAAT GCAATTAGCATG	7e-37	Chr5, not in gene, ref|NT_006576.15|Hs5_6733
	2	TAAATCTTTATGTTCACTTTAAACCTGCTCACAGCCTG GTTCATCTGGGCTCGCTGTTTTGGGCGTGGAGGATAAC ATCTGACCCAGTAGCTATGTGACTATCCCAGGAGTTGG TTTTCAGTTTGACCAACAGCCATCACCTTGGCTACRRA GATAAGTGAGAAATGAGTGAAACAGACCCGCTCTGTTA ACTCTTTCCTCCCACTCTTGAAAAAGGTATCTCTAGAC CCAGATAGTTTCACTGGAGACGTCTGCTCATAGTTAAA GAAATATCAACACCAATTATACGTAATCTCCAGAAAAC AGAAGTAGGAGCACTTCTGGACTGATGARGCCAG**TACA GTTG**	0.0	NA, Alternate assembly, ref|NW_001841237.1|HsUn_WGA3412_ 36
	3	TGACTTGCTGGTGTAAAGTTAGCTGTCCAGACCACGAT TGCTCTAGGAGGATTACTTGGGACACAGTCCTGGGATC CACTTGTTCAGAAAAACAATGGCACTGAGAAGGAACGC AAAGGAACAAAGATCAAAGGCTGCCATCTCTCAAAGAC CTTGGTTCTCGGCAGAACAAACCAGCCTGAGTCAAAAT TGTGAAGTCACCTGCAGAAGGCCTTTCCTCTCTTCTTT ATGCCTTGCTCCTCTAATGTGTTCTCTTGATAGGCATT TACTATTTTCACGGTGACCCCAAGTTCTTTGGGCCACC CTATTTAGTGGCAATTCTGACCCATTCGCTTCATACAT TGCTAGGTGACTAGTTAGTCAAGAGCTTAGATGTC**TAC AGTTG**	0.0	Chr1, not in gene, ref|NT_004487.18|Hs1_4644
Megakaryocytes, *in vitro*	4	TGGCTGGGTTTTTCTCTTCCTTCAGATGTCTGTGTAAA AGC**TACAGTTG**	3e-14	Chr10, not in gene, ref|NT_008583.16|Hs10_8740
	5	AAGTACTGCATTTCCTATGCAACAAAAGGAAAAATAAA AAATTGCTAATGCTATGGAAGCTGTGTCATCATTTGCT TTACAGCATGCCTGGGTGATTGAGTGACAGTCACGGGT GTGGACTGGAGGCTCCCACAGAAGATGGTCTTTATGCA CCATGGGGTTTGTCAGGCACATAGCATCCTTGGCTGTG TTCTGATGGGCAGTGTTACTTTTTTCACCTACTTGGAC AAATACAGGATTTGACACTCACATAGTGTTTACTGTGT GCCTGATATTGTTCTGAGTGCTGTA**TACAGTTG**	7e-148	Chr1, not in gene, ref|NT_032977.8|Hs1_33153
Erythroid lineage, *in vitro*	6	CCTTGAATAGTCAATCTGGTTGGAGAATGGGAGGAAGT GGCATAAGGTAAGGTTAGTATCAGATCTTCAGTGTCCC TGTATGTCTCCCTAAGGAATCTGGACCTCAGGCAGCAG GCTAGGGAAACACTGAAATATTTTAAGCAAGGAAGTTA TGCAGATGATCATAGTTTTATTTTGGAATAATAAATCT GTCTGTAGTGTGAAGAATTAATTGAAGCACAGGGGACT ATCTGAGGTCAGGACTACCAGCTGGATAACTTGAAATC TATTTGAGTAAGAAGAAATGAACTAGGGCAATGACAGT AAAGATAAA**TACAGTTG**	1e-161	Chr4, not in gene, ref|NT_006316.15|Hs4_6473
	7	GGTTCTCCTATGGCCACGCACCTCGTGGCGGCTGGCTG GCCTCTTAAGCTGTCTCTGAGATTCACGCTTCACATGC TATTGGCTCACAGCGTTTGTATCAGGAAATGCTGGCCC TATTCCCCGTTTTCCTCTTTGAACTTGACTCTCCTGCC TATTGCATTACTATCTTATTTATGTTATTTGTGTTTGA CAGTGCAAACCTCTACCCATCTTTTTATTTTCAATGTT	9e-137	Chr2, not in gene, ref|NT_005120.15|Hs2_5277

Granulocyte lineage, *in vitro*	8	CTTTTATGGGCAATTTCAAGCTTAGAATTTCGAATATA AAATATCAGACAGTGTCCAAACTAGAAAGAGAGGATAC ACTCAAAAGCGTTTACCCAAGGAGAACTGAATGAAGGG GCCAAAGAAGGCGTCATCAGGGTTAATGGAACCAAAAA GGGAAAGTGTCACACCCAAGGATTAGCAATAGTGGGAA GCTATTACCACCTCTGCTGCAGGGGCAGAGACCAGGGA GAAATGAACACATGGAAAAGGCACTAACCACCGGAAGC AGGGGCCGGCGAACTATGGCCCGGGAGTCTGCCACCTG TTT**TACAGTTG**	8e-158	Chr11, not in gene, ref|NT_009237.17|Hs11_9394
	9	GGCAGGAGAATCACTTGAACCTGGGAGGCGGAGGTTGT AGTGAGCCGAGATCGCACCATCGCACTCTAGTCTGGGC AATAAGAGCGAAACTCCGTCTAAAAAAAAAAAAAAGAAA GAAAAAGAAATTAGGCTTTGGTAGAATCAGCTGTGGCA TCAGGAGGAAAGCCTAACCAGTGATGAATTCAATCTTA AAATTGCTGCTTAGTTCAAGATTAACCCGATACTCAGT GAAATATGAATAATCACTTTCCTACTTTGGCAGCCACA CATAGAAGAGATTGCGCATGCCCGGAAATAAAAACAAA GCAAAAGAGTTGCGTAAGTCAGTCTTCACTGTTATCCA TGTACTACGTTCAGTA**TACAGTTG**	0.0	Chr1, not in gene, ref|NT_004487.18|Hs1_4644
	10	TGGATTAAGTTCACCTAGAAAGTCTGTATTTTCTGAAA AGTAACCCATCCAGTAACATTTTTATGTGCAAATAACT GCAAGGGCACAATATGTGTTTTTAAAGTTTTAAAATCA ATCTTTGGAGAGTGATGGATTTAAACTAGCTTTACACG CATGCAATTACCCTTCAGCCAGCTTTCAAGAGTACCCC CAACCCATTTCAATTTAACATCCTCTAACCCCCATGGA TATTAAGACATTGTGTCTGTGCCATCAATTTTCGAATT TCTTGTGAACTGAAGAATTCAAGCATGGCAGAGACAAT CTTCCCTAGGACACCCTGTTACTCATCATTTCTCCAGT GTATGTTCATGCTGATCCTTAGTTTGGCTCAATAGAGC TAAA**TACAGTTG**	0.0	Chr17, not in gene, ref|NT_010783.14|Hs17_10940

Supplementary Table 3: Transposon insertion sites recovered from CD20$^+$ and CD33$^+$ lineages *in vivo*. (*) Represent common integration sites in the myeloid (CD33$^+$) and lymphoid (CD20$^+$) lineages. The transposon inverted repeat sequences are typed in bold.

Clone	CD33$^+$ samples	E-value	Location, Homology	CD20$^+$ samples	E-value	Location, Homology
1*.	CTAGTTTAACTACCCGGTTTGAAGAAACA ATTCATGAAACAGGCTCAGCAAATAGAAA AACTGTCACAGCCACG**TACAGTTG**	1e-29	Chr12, not in gene, ref|NT_009714.16|Hs12_9871	CTAGTTTAACTACCCGGTTTGAAGAAAC AATTCATGAAACAGGCTCAGCAAATAGA AAA**TACAGTTG**	9e-31	Chr12, not in gene, ref|NT_009714.16|Hs12_9871
2*.	**CAACTGTA**TATGGTGATAGGAAGTGGTCT AG	3e-3	Chr6, galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase, ref|NT_007299.12|Hs6_7456	ACTAGACCACTTCCTATCACCATA**TACA GTTG**	1e-3	Chr6, galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase, ref|NT_007299.12|Hs6_7456
3*.	**CAACTGTA**CCTAGTAAGCTTTAATTCAAA CTCAAAACTTCTAATTGAAAGTGGTTGCT GTTGTTGTGATTCCATTGCTTTTTCTAGG TACTATACCTTCATCCTACCTAG	1e-44	Chr3, not in gene, ref|NT_005612.15|Hs3_5769	**CAACTGTA**CCTAGTAAGCTTTAATTCAA ACTCAAAACTTCTAATTGAAAGTGGTTG CTGTTGTTGTGATTCCATTGCTTTTTCT AGGTACTATACCTTCATCCTACCTAGAA TTGCTTTTGAAGTTTTTCTGCCTTGCCT TTGATTGAACAGAATTTACAATAAAAGA TTTTCTACATCATCAAGGCTTCCACAGA GTTCAAATGAAAACACTTTTTAGGGGTA GCTGTTAACTGCCTACTTTTATAGTCTC TTGATTGAAAGCTACTGCTCTAAATTTA	2e-136	Chr3, not in gene, ref|NT_005612.15|Hs3_5769
4.	**CAACTGTA**TACCATACAGACCCCAACGAG GTATTTCTTCCCTCGATTTCTGCTATTAA TCCTCCTCATAATTAAATAGGACC	1e-30	Chr2, erythropoietin 4 immediate early response, ref|NT_005403.16|Hs2_5560	CCTAGTAAGCTTTAATTCAAACTCAAAA CTTCTAATTGAAAGTGGTTGCTGTTGTT GTGATTCCATTGCTTTTTCTAGGTACTA TACCTTCATCCTACCTAG**TACAGTTG**	2e-44	Chr3, not in gene, ref|NT_005612.15|Hs3_5769
5.	CAGTATTACATAAGCAGGAATAAAAGAGA CATTGAGTGGAATGTCTCGCTCAAATATA AATAACCTTATAAATACAATCTTTACTTC TCAGAGTCCTAAAATCTTCTTGGTTAGTC TGGTCTTCCTTGTTGATCAAGGTTCTCGT GACTAAATGGCATTAGTTTGTGTTCTCAC AGATAGGATAGGAAAAGGAATTATACATA TTGATTATGTACTATATGTCAGACCACTT ATGTA**TACAGTTG**	2e-117	Chr18, not in gene, ref|NT_010966.13|Hs18_11123	TCAGGGCCTCTTATGGCCACTTCTGTAG TGCAGTGATTTTTCAGACTGCAGGTTGC AGGTCATGCAAATCATTTTGAAGGTCCT GAGCAGCATTTCTAAAATGGAAATAGAA CAGAACAGAAGACAAATCATTTTGAAGG TCCTGAGCAGCATTTCTAAAATGGAAAT AGAACAGAACAGAAGAGAGCAGAGTGTC TCAGAGTTCATAAAGCGAAGTCATTTTG TGAAACTTA**TACAGTTG**	3e-56	Chr16, nucleotide-binding oligomerization domain containing 2, ref|NT_010498.15|Hs16_10655
6.	**CAACTGTA**TATATATATATAAAATACACA CACGTATGTCATCCCATACATATATAGGA TATATGTAATTATATATATATAAAATACA CACACGTATGTCATCCCATACATATATAG GATATATGTAATACATGTATACATACATA CATATATACACACACACATACATACATTC CACACATTCACTAAACATGTATACATACA TACATATATACACACACACATACATACAT TCCACACATTCACTAAGGACATAGCTTTT TGAAGGTTCTGAATTCATGCAGAGGTGTT GGTTTTAGCTCTCTGTCTGGACATAGCTT TTTGAAGGTTCTGAATTCATGCAGAGGTG TTGGTTTTAGCTCTCTGTCTTGCGTGAGC CCAGGACTCAGTCGTATATCCTGAGGGCA TTGAAATCCACACCCCTA	3e-55	Chr4, not in gene, ref|NT_016354.18|Hs4_16510			
7.	CTAGACTCCATTTTATTAAGTTAACTATG T**TACAGTTG**	6e-07	Chr13, not in gene, ref|NT_009952.14|Hs13_10109			

Supplemental Table 4: Transposon insertion sites recovered from transfected hepatocytes
Junction fragments rescued by splinkerette PCR, transposase: SB100X

Source	Clone	Junction with IR sequence in bold	E-value	Location, Homology
Mouse liver, *in vivo*	1.	TGTATATATATGTGTGTGTATATA**GACAGTTG**	1e-04	Chr16, not in gene, ref|NT_039625.7|Mm16_39665_37
	2.	TGTGTTGTATATAAATAACCCTGGTATTGA**TACAGTTG**	6e-08	Chr9, not in gene, ref|NT_039474.7|Mm9_39514_37
	3.	**CAACTGTA**GTATGATGTAACTACCCGGGGAAACATTTT GAAACATCTTAAATCTTTACACAAAGGGTA	2e-25	Chr19, not in gene, ref|NT_039687.7|Mm19_39727_37
	4.	**CAACTGTA**TCTAATGGCCAAGAAGCACCTAAAGATATG TTCAACATCCTTAGTCA	1e-17	Chr19, not in gene, ref|NT_039687.7|Mm19_39727_37
	5.	**CAACTGTA**CCTTGTGATTGACCTGCTTACA	0.001	Chr3, not in gene, ref|NT_039240.7|Mm3_39280_37
	6.	**CAACTGTA**TACCCAGAAGAAGTTCCAACTGGTAATAAG AACACA	2e-11	ChrX, not in gene, ref|NT_039706.7|MmX_39746_37
	7.	**CAACTGTA**TTTATTTGGAGGGGACTGCAGGAATGTCA	2e-07	Chr5, not in gene, ref|NT_109320.4|Mm5_108190_37
	8.	**CAACTGTA**GGTGGGGTTTAGAATCCTTGGGCTTTGGAC TAGGAGGAAGAGAAAGGAGAAGGAACCCAGCCA	4e-27	Chr1, not in gene, ref|NT_078297.6|Mm1_78362_37
	9.	**CAACTGTA**GGTTGTTGCCCTGTGGGTGATTCACCACCT CTCTTATTGATGTCCCAGGTCA	1e-20	Chr8, esterase 1, ref|NT_078575.6|Mm8_78640_37
	10.	TATAGATGGTGAACTTTG**TACAGTTG**	0.14	Chr10, not in gene, ref|NT_039492.7|Mm10_39532_37
	11.	GTTTGTGGTGCTTAGACA**TACAGTTG**	0.14	Chr6, not in gene, ref|NT_039353.7|Mm6_39393_37
	12.	TGGGATCATTTGTTTAGAGTCWAAGGTTCTGGCGATGT GGACATTTGCTATATTCATAGCTTT**TACAGTTG**	3e-26	Chr4, not in gene, ref|NT_039260.7|Mm4_39300_37
	13.	**CAACTAGTA**TGCTGTCACTCAGACATGCA	0.013	Chr12, not in gene, ref|NT_039551.7|Mm12_39591_37
	14.	**CAACTGTA**AGAGTTGATGCAAACCAAGGCACCTGAACA TCATG	8e-11	Chr14, not in gene, ref|NT_039606.7|Mm14_39646_37
	15.	**CAACTGTA**CATATTCATACAGAAGTCAGAGTTTGAAGC TCTGTGTTTCCA	7e-15	ChrX, not in gene, ref|NT_039706.7|MmX_39746_37
	16.	**CAACTGAT**CTTCCATCCTACTGTTTCTTAGCCATTTGT CAGTCATAAATATATCCGGATAACTTGCTTTATTTATT ATGGAATAAAGAAGAACCTTGAAGATTGCA	1e-47	Chr6, zinc finger protein 638, ref|NT_039353.7|Mm6_39393_37
	17.	**CAACTGTA**TGTGTGGGCTTTTTGTACCACTTGAGACCT GGGTGTGAGCCAATTGTCCCTCCACA	5e-23	Chr12, tumor suppressing subtransferable candidate 1, ref|NT_039548.7|Mm12_39588_37
	18.	**CAACTGTA**TAATAGTCTGGCCATACTAAGAGAAGAGGA GGCATAAGAGGCAGACCGTCAGTCA	2e-22	Chr11, serine racemase, ref|NT_096135.5|Mm11_95772_37
	19.	**CAACTGTA**TATATACAAAGCATACACATACAAGTACAC ACACA	8e-11	Chr2, potassium inwardly-rectifying channel J3, ref|NT_039206.7|Mm2_39246_37
	20.	**CAACTGTA**GAAATATCTGATGCTGTCTTCT	0.001	Chr2, not in gene, ref|NT_039207.7|Mm2_39247_37
	21.	**CAACTGTA**TGTGGGTTCCTATTGCTTATGTGTCATG	7e-07	Chr5, not in gene, ref|NT_039299.7|Mm5_39339_37
	22.	GAGCAACGCATTTTTCTGGATACCAGTGGCTGCCAGCC TCACCCCT**TACAGTTG**	4e-17	Chr11, ATP-binding cassette transporter sub-family A member 9, ref|NT_165773.2|Mm11_163287_37

Supplemental Table 4: Transposon insertion sites recovered from transfected hepatocytes
Junction fragments rescued by splinkerette PCR, transposase: SB100, continued

Source	Clone	Junction with IR sequence in bold	E-value	Location, Homology
Mouse liver, *in vivo*	23.	TGTACAGTTACCCAGTTACAGCCACCTACAACTCCAGT TCTAGGCC**TACAGTTG**	4e-17	Chr14, zinc finger protein 198, ref|NW_001030560.1|Mm14_118400_36
	24.	TGCACACTGGCCAAGCAATCTCCTCA**TACAGTTG**	9e-06	Chr6, coiled-coil domain containing 129, ref|NT_039353.7|Mm6_39393_37
	25.	TGCTGGAGTGAGGCCCCGTTCCAACCCTAAAATTATTT TGTTGC**TACAGTTG**	5e-16	Chr18, phosphodiesterase 6A, cGMP-specific, rod, alpha, ref|NT_039674.7|Mm18_39714_37
	26.	TGTGCATTGGTGTTTTGCCTGCATA**TACAGTTG**	3e-05	Chr2, activating transcription factor 2 isoform 2 and 1, ref|NT_039207.7|Mm2_39247_37
	27.	TGTAAAGAGTTCAGTTTTG**TACAGTTG**	0.035	Chr10, SEC63-like, ref|NT_039492.7|Mm10_39532_37
	28.	CCTGTGTGAAATTGTTATCC**TACAGTTG**	0.013	Un, ref|NT_166465.1|MmUn_163639_37
	29.	TGGTAGCTCACAATCATCTGTAGCTGCCTTAGTACTAG GGAAATCTGACATCTTCTGGCCTTTA**TACAGTTG**	1e-27	Chr3, absent, small, or homeotic discs 1, ref|NT_039240.7|Mm3_39280_37
	30.	TGTATGTGTACAGTGCTGTTGATGGATGTTCATCAGCA CATTACA**TACAGTTG**	1e-16	Chr14, not in gene, ref|NT_039606.7|Mm14_39646_37
	31.	TGTTCTTGGTCCAGTAACTGGCAATCAGGAACTGTT**TA CAGTTG**	2e-11	Chr17, not in gene, ref|NT_039649.7|Mm17_39689_37
	32.	TGCCAAAGAAGACACCTGGCAACTGCCA**TACAGTTG**	7e-07	Chr6, not in gene, ref|NT_039353.7|Mm6_39393_37
	33.	TGGAAACACAGAGCTTCAAACTCTGACTTCTGTATGAA TATG**TACAGTTG**	7e-15	ChrX, not in gene, ref|NT_039706.7|MmX_39746_37
	34.	TGGTAAGGGTTTTGACCTCAAACACT**TACAGTTG**	9e-06	Chr17, not in gene, ref|NT_039649.7|Mm17_39689_37
	35.	TGCTGAAGCCAATTTCTTCAAATTCCTCACATTATTGA **TACAGTTG**	2e-12	Chr6, not in gene, ref|NT_039340.7|Mm6_39380_37
	36.	TGCATGTAAACTAATGAGTCGGTGGTACAGTTCTAGCG CTGGGCA**TACAGTTG**	1e-16	Chr18, not in gene, NT_039674.7|Mm18_39714_37
	37.	TGGAGCTCAAAGGTCTGATCCACAGCAGAGCGAAACCA CAAAGTAAAGTAAAATGCGTTGGACAAAAGTGAGTGGG GATGTGTGTGTATG**TACAGTTG**	5e-43	Chr1, not in gene, ref|NT_078297.6|Mm1_78362_37
	38.	TGGTGGAACTTGAAGTTCTAGCTG**TACAGTTG**	1e-04	ChrX, not in gene, ref|NT_039718.7|MmX_39758_37
	39.	TTCTCACAGGAAGCATT**TACAGTTG**	0.54	Chr18, not in gene, ref|NT_039674.7|Mm18_39714_37
	40.	TGGAAAATGATGAAAACCACACTGTAGAACA**TACAGTT G**	6e-08	Un, ref|NW_001032119.1|MmUn_114810_36
	41.	**CAACTGTA**AATAAAGCTATTCGCTGACTTGTTAAACCA	6e-08	Chr6, not in gene, ref|NT_039340.7|Mm6_39380_37
	42.	**CAACTGTA**AATAGATTTGTAACATTCA	0.035	Chr8, not in gene, ref|NT_078575.6|Mm8_78640_37
	43.	**CAACTGTA**GGTTGTAAAAGCTGATTACA	0.013	Chr3, not in gene, ref|NT_039240.7|Mm3_39280_37
	44.	**CAACTGTA**TGTTTAATAAACACTGTTC	0.12	Chr14, hypotetical protein, ref|NT_039606.7|Mm14_39646_37
	45.	**CAACTGTA**TGTCACTTCATTGCTGCA	0.14	Chr2, not in gene, ref|NT_039206.7|Mm2_39246_37
	46.	TGCTGACAGGAGCCTGATG**TACAGTTG**	0.35	Chr14, not in gene, ref|NT_039606.7|Mm14_39646_37
	47.	TGTCATTGCTAATGATGGCAGCTCTGTCCTTAAGAAGT **ACAGTTG**	6e-12	Chr10, receptor expression enhancing protein 3,

		TGAATGTATG**TACAGTTG**		ref|NT_039433.7|Mm7_39473_37

Supplemental Table 4: Transposon insertion sites recovered from transfected hepatocytes
Junction fragments rescued by splinkerette PCR, transposase: SB100, continued

Source	Clone	Junction with IR sequence in bold	E-value	Location, Homology
Mouse liver, *in vivo*	49.	TGAAGACAAACACCCGAATGTGTCTAAGAGAGAGTTTC TAGAC**TACAGTTG**	2e-15	Chr14, not in gene, ref|NT_039606.7|Mm14_39646_37
	50.	TGTGTTTAGGAAATTGTATCT**TACAGTTG**	0.004	Ambiguous
	51.	TGAGAAGATGGTCAGTAGAAA**TACAGTTG**	0.004	Chr14, heparan sulfate 6-O-sulfotransferase 3, ref|NT_039606.7|Mm14_39646_37
	52.	TGGTAATTTGTTATGACACAGCAGAGAGGTAATGCAAC AGCTATATCATTTATGTATAATGTGTGTCTACTTTTGC ACCGACAGCAAAGTTGAGTTGACAGAGGCTCCCATA**TA CAGTTG**	5e-56	Chr18, not in gene, ref|NT_039674.7|Mm18_39714_37
	53.	TGCCTTACTAGTAAATACTCATTTTCATCCTCAAGAAA AATGATGTGCTTATAGTGTGCTTATTTA**TACAGTTG**	7e-29	Chr12, not in gene, ref|NT_039551.7|Mm12_39591_37
	54.	TGAGTCTTTGTAAGGAAATGCTGGTAGAAGCATCATAT GATACCGAAGCTCCCCCTGGTGGAGGGTAAGGTGATGC ACTTGCATTTACTGT**TACAGTTG**	1e-43	Chr3, not in gene, ref|NT_039240.7|Mm3_39280_37
	55.	TGACAAACGGTTGACTAACATTCCAGCACAGCACAGAC **TACAGTTG**	6e-12	Chr14, not in gene, ref|NT_039606.7|Mm14_39646_37
	56.	**CAACTGTA**CCTGTTTAATCACCACCTCATACACTAAGT GAGCTTATAGTATTATACA	6e-19	Chr17, not in gene, ref|NT_039649.7|Mm17_39689_37
	57.	**CAACTGTA**ACACCTTCTGGCACTATGAGGGCTAACCAG TGGGGAAGAACCCTAAGGCTTAACTCCGGATTGATTTC TCTGTGTCA	2e-35	Chr9, not in gene, ref|NT_039472.7|Mm9_39512_37
	58.	**CAACTGTA**TATACTGTGAGTGTTGCAGATTTCTACTTA CTGTTTGTGACACTGTGAGCCATATCACA	9e-25	Chr6, contactin 3, ref|NT_039353.7|Mm6_39393_37
	59.	**CAACTGTA**CATTTTTAGCTGAAGTTGCCCTCTTTTTCA TCTCTGCCAGTGTGTGTCA	6e-19	Chr9, not in gene, ref|NT_039472.7|Mm9_39512_37
	60.	**CAACTGTA**TGTGCTTAAGATGCTAGTTTGTGTACA	3e-04	Chr14, not in gene, ref|NT_039606.7|Mm14_39646_37

Supplementary Materials and Methods

Characterization of the transposase gene library. We transformed the library into *E. coli* DH5α competent cells, and isolated and fully sequenced 45 reassembled transposase genes. Our most conspicuous finding was that all the 45 genes were full-length without insertions or deletions. Only two point mutations were detected in the 1023-bp transposase coding region that had been introduced by the shuffling process itself. However, neither of them caused any amino acid change. After aligning the sequences to the original SB transposase, we assessed the clonal distribution of the 41 mutations that were included in the shuffling. 31 of the 41 mutations introduced into the shuffling were recovered in the 45 sequenced clones (data not shown). The clonal distribution of mutations in the library is shown in **Supplementary Fig. 1**.

The clones were tested in transposition assays in HeLa cells as described earlier but adapted to a 96-well format. All the tests have been done as duplicates. For reference, we used SB16[1] on all the plates. All clones that showed similar or higher activity as compared to the SB16 control have been chosen for further analyses. We further verified the activity of the best 20 clones on 6-well plates (5 ng transposon/50 ng transposase per well). 7 of the 20 retested clones showed clearly higher activity as compared to SB16. The best two clones, 6A5 and 3D5, exhibited ~25-fold higher activity as compared to the original SB transposase.

Heat-shock treatment and fluorescence acquisition. HeLa cells containing stably integrated Venus (YFP-variant)-transposase fusion construct driven by the CAGGS promoter were grown on 19-mm glass coverslips coated with 0.1% gelatine/PBS. To induce heat shock, cells were incubated on a 43°C thermo-block for 30 min. Immediately after stress treatment, cells were washed with PBS and fixed in 4% paraformaldehyde/PBS at room temperature for 15 min. Fluorescence images were taken under an Olympus IX81 wide field microscope equipped with CC12 digital camera and the Cellˆ F imaging software.

Nature Genetics: doi:10.1038/ng.343

Integration site analysis. Transposon integration sites were recovered by splinkerette polymerase chain reaction (PCR) as described previously[2], with some modifications. Genomic DNA from transfected cells or tissue was extracted by anion-exchange chromatography (Qiagen Blood & Tissue Kit) according to the manufacturer's instructions (Qiagen, Hilden, Germany), followed by digestion with NlaIII. The splinkerette linker was made by heating equimolar amounts of the primers: 5'-CCTCCACTACGACTCACTGAAGGCAAGCAGTCCTAACAACCATG-3' and 5'-GTTGTTAGGACTGCTTGGAGGGGAAATCAATCCCCT-3' (Invitrogen) to 80°C for 5 min and allowing to anneal by slow cooling to room temperature. Twelve µl of splinkerette linker (30 mM) was then ligated overnight at 16°C to the concentrated NlaIII-restricted genomic DNA (27.5 µl) using 2 µl T4 DNA ligase (Fermentas, St. Leon-Rot, Germany) and 4.5 µl of 10x T4 Ligase Buffer. From the ligation reaction 2 µl were then subjected to the primary PCR reaction in a final volume of 100 µl using pimer pairs 5'-CCTCCACTACGACTCACTGAAGGGC-3' and 5'-GCTTGTGGAAGGCTACTCGAAATGTTTGACCC-3' in 15 cycles of 95°C for 20 s, 70°C for 30 s and 72°C for 30 s, followed by 17 cycles of 95°C for 20 s, 64°C for 30 s and 72°C for 30 s. A secondary, nested-PCR was performed by using the primary PCR products diluted 1/250 using primers 5'-GGGCAAGCAGTCCTAACAACCA-3' and 5'-CCACTGGGAATGTGATGAAAGAAATAAAAGC-3'. Nested PCR involved 35 cycles of 95°C for 20 s; 63°C for 30 s and 72°C for 30 s. Both primary and nested PCRs incorporated a hot-start at 95°C for 1 min and a final extesion at 72°C for 5 min. The Expand High Fidelity PCR System (Roche Applied Science, Vilvoorde, Belgium) was used.

Transposon-genomic DNA junctions from $CD33^+$ and $CD20^+$ lineages were determined using linear amplification-mediated PCR (LAM-PCR) as described earlier[3], with the following modifications. 10-40 ng of purified genomic DNA (Qiagen Blood & Tissue Kit) was subjected to 40 cycles of linear amplification PCR in a final volume of 50 µl with the primer 5'-biotin-tgtaaacttctgacccactggaattg-3', using the following conditions: 94°C for 1 min, 59°C for 30 s and 72°C for 1 min 30 s and the products were captured on magnetic streptavidin beads (Dynabeads

kilobaseBINDER Kit, Invitrogen). After second strand synthesis the DNA samples were digested with BfaI or Sau3AI (New England Biolabs) to create compatible ends for linker ligation. Annealing the oligonucleotide pairs 5'-GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC-3' and 5'-phosphate-TAGTCCCTTAAGCGGAG-3'-amino, or 5'-CGAATCGTAACCGTTCGTACGAGAATCGCTGTCCTCTCCAACGAGCCAAGG-3' and 5'-GATCCCTTGGCTCGTTTTTTTTTGCAAAAA-3' gave rise to the BfaI- and Sau3AI-specific linkers, respectively. Ligation reactions were performed using 50 pmol of the corresponding linkers overnight at room temperature. The ligation products were collected on a magnetic separator and resuspended in 10 µl TE. 2 µl of ligation product was amplified in the primary PCR reaction in 50 µl total volume using the transposon specific primer 5'-GAATTGTGATACAGTGAATTATAAGTG-3' with either the BfaI-linker specific primer 5'-GTAATACGACTCACTATAGGGC-3', or the Sau3AI-linker specific primer 5'-CGAATCGTAACCGTTCGTACGAGAA-3', using the following conditions: 94°C for 3 min, 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, followed by 72°C for 5 min. One µl of the PCR product was further amplified in a nested round of PCR using the transposon specific primer 5'-ACAAAGTAGATGTCCTAACTGACT-3' in combination with either the BfaI-linker specific nested primer 5'- AGGGCTCCGCTTAAGGGAC-3' or the Sau3AI-linker specific nested primer 5'-tcgtacgagaatcgctgtcctctcc-3'. Conditions for the nested PCR reactions were: 94°C for 3 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by 72°C for 5 min.

PCR products were purified by Qiagen PCR extraction kit (Qiagen, Hilden, Germany) and characterized by electrophoresis on a 1.25 % agarose gel. The purified PCR product was cloned into pGEM-Teasy vector (Promega, Madison, USA) and the DNA sequence was determined by standard sequencing technology (ABI3730XL Applied Biosystems, Foster City, California). The resulting sequences were subjected to BlastN analysis against the mouse or human genome using the ENSEMBL database (www.ensemble.org).

Quantitative PCR. Genomic DNA was extracted using DNAeasy Tissue Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) and quantitative (q)PCR was performed in ABI7500 FAST (Sequencer Detector Unit from ABI) using ABI (Perkin Elmer) Q-PCR Master Mix and TaqMan probe and primers specific for the Neo^R or MGMT marker genes in the transposon (Forward primer: 5'-TCGACCACCAAGCGAAACA-3'; Reverse Primer: 5'-CGACAAGACCGGCTTCCA-3'; Probe: CGCATCGAGCGAGCACGTACTCG or TaqMan gene expression (TaqMan) probe Hs00172470_m1, Applied Biosystems). TaqMan qPCR was performed in accordance with the manufacturer's recommendations (50°C for 10 min, 95°C for 2 min and 40 cycles of 95°C 15 sec, 60°C 1 min), using a serially diluted transposon plasmid as standard.

Excision product assay. To determine transposition efficiency, genomic DNA was subjected to an excision product (EP) assay, as described previously[4,5]. This EP assay measures the accumulation of the recircularized plasmid that remains after transposon excision. The main advantage of this assay is that regardless of where the transposon integrates, the EP from each event is the same and leaves a definitive "footprint" permitting quantification of the total transposition events. A SYBR-green PCR (Applied Biosystems, Foster City, CA) was conducted on a ABI7500fast sequence analyzer (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Briefly, 50 ng of genomic DNA was added to a 25 μl reaction mixture with 1X SYBR-Green master mix and 200 nM primers (5'- CTGGAACAACACTCAACCCT-3' and 5'-CACACAGGAAACAGCTATGA-3') that yield a 430-bp EP-specific band for the liver samples. The PCR reaction was initiated after a 10 min incubation at 95°C followed by 42 cycles (95°C for 15 s, 58°C for 30 s and 72°C for 30 s). The PCR reaction was initiated after a 10 min incubation at 95°C followed by 42 cycles (95°C for 15 s, 61°C for 30 s and 72°C for 30 s). To generate a standard curve that mimicks an EP product, we used a pT2-HB plasmid from which the SB transposon was removed after restriction with SacI and XhoI followed by religation. The resulting plasmid was serially diluted and spiked wih genomic DNA from non-treated, naïve mice. The gene copy number was subsequently normalized

per 100 cells, and an independent GAPDH-verification was carried out as described[5]. PCR products were verified by electrophoresis on a 1% agarose gel to verify the EP size.

Overproduction inhibition assays. Overproduction inhibition properties have been compared in a transposition assay in which plasmids expressing SB or SB100X were cotransfected with pT2/SV40-Neo transposon donor plasmid into HeLa cells. The amount of pT2/SV40-Neo was 50 ng, while the amount of transposase coding plasmids was increased stepwise in a 50-500 ng range. All the transfections were filled up to the same 850 ng total DNA amount using the pFV4a(CAT) filler plasmid. The transfections were carried out in 6-well tissue culture plate using the Fugene6 (Roche) transfection reagent, according to the instructions of manufacturer. Two days after transfection the cells were trypsinized, and 1/100 of the cells were replated onto 10 cm dishes in selection medium containing 1 mg/ml G418.

Sandwich transposon system for studying the genomic remobilization of SB out of single-copy transposon donor sites. To establish single-copy genomic donor sites suitable for monitoring SB remobilization, we created a sandwich transposon arrangement of *piggyBac* (PB) and SB transposons supplemented with marker genes necessary for monitoring the excision and reinsertion of the SB transposon. First we modified the coding sequence (CDS) of the puromycin resistance gene at Ala(106), Thr(107) and Val(108) positions from GCC ACC GTC to GC*T* AC- G*TA*. The modification creates a recognition site for the SnaBI endonuclease (underlined) and also mimics the *TA* target site duplication (italic) at SB insertion sites. An SB transposon fragment, lacking the first C and last G nucleotides, was inserted into the SnaBI site, thereby mimicking an SB transposon insertion into the puromycin CDS. After excision of the SB element from the donor site the puromycin CDS is reconstituted, and contains only silent mutations by both possible repair products (*TA*CAG*TA* or *TA*CTG*TA,* where CAG or CTG are the transposon footprints, and *TA* are target site duplications) created by the host NHEJ repair system. This arrangement allows selection for

genomic excision events without background. The SB transposon carried the neomycin marker gene that allows monitoring the reinsertion of the excised SB transposon into the genome. The whole complex donor site construct was inserted into HeLa cells using PB as an outer transposon. Clones carrying single insertion events mediated by PB transposition were selected using genomic Southern blot analysis.

Clonogenic assays. Megakaryocytic clonogenic assays (CFU-MK) were performed by adding 50 µl of Stemline medium (Sigma-Aldrich, USA) supplemented with 100 ng/ml SCF, 20 ng/ml IL-6, 100 ng/ml IL-3, 20 ng/ml Flt3-L and 100 ng/ml TPO to the 100 µl of electroporated CD34+ cell suspension. Fifty µl of the final cell suspension was then added to 450 µl of megakaryocyte differentiation medium corresponding to Myelocult H5100 (Stemcell Technologies, Vancouver Canada) supplemented with 25 ng/ml TPO, 25 ng/ml hSCF, 10 ng/ml hIL-6, 10 ng/ml hIL1band seeded over 3 wells in a 24-well plate, at a concentration of $5x10^4$ cells per well. At day 6 post-transfection, medium was changed by centrifuging the plate briefly, discarding the supernatant and adding fresh megakaryocyte differentiation medium. At day 10, CFU-MK colonies were counted using a fluorescence inverted microscope (Olympus, Japan). GFP expression and scoring of CFU-MK colonies was monitored using the Olympus fluorescence microscope. In addition, confocal microscopy was conducted at 488 nm with an Axiovert 100M, LSM510 (Zeiss), using the AxioPlan 2 LSM 510 version 2.8 software.

Granulocyte/monocyte/macrophage clonogenic assays (CFU-GM) were performed by adding 30 µl of the final cell suspension to 270 µl of granulocyte/monocyte/macrophage differentiation medium corresponding to semi-solid Methocult GF H4534 (Stemcell Technologies, Vancouver Canada) composed of 1% methylcellulose (4000 cps), 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml rhSCF, 10 ng/ml rhGM-CSF, 10 ng/ml rhIL-3 in Iscove's Modified Dulbecco's Medium (MDM). The cell

suspensions were seeded over 3 wells in a 24-well plate at a concentration of $5x10^4$ cells per well. At day 14, GFP^+ and total CFU-GM colonies were scored as mentioned above.

Erythroid clonogenic assays (CFU-E) were performed by adding 30 μl of the final cell suspension to 270 μl of erythoid differentiation medium corresponding to semi-solid Methocult SF^{BIT} H4436 (Stemcell Technologies, Vancouver Canada) composed of methylcellulose, fetal bovine serum, bovine serum albumin, 2-mercaptoethanol, L-glutamine, rhSCF, rhGM-CSF, rhIL-3, rhIL-6, rhG-CSF, rh Epo in Iscove's MDM. The cell suspensions were seeded over 3 wells in a 24-well plate, hence containing $5x10^4$ cells per well. At day 7, colonies were counted which typically contained about 70% glycophorin A^+ cells, a characteristic marker of erythroid cells. GFP^+ CFU-E colonies were monitored as mentioned above. Cytologic analysis was performed by Giemsa staining.

Cytofluorimetric and cytologic analysis. Differentiation into erythroid, megakaryocytic and granylocytic/monocytic/macrophage cells was validated using phycoerythrin-conjugated antibodies specific for glycophorin-A and CD61 (Becton Dickinson, New Jersey, USA) using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ). Analysis was performed using the Cell Quest software (Becton Dickinson, Franklin Lakes, NJ). After two weeks of differentiation in megakaryocytic and granulocytic differentiation medium and one week in erythroid differentiation medium, individual colonies were picked, cells were washed twice with PBS resuspended in 50 μl PBS containing 5% BSA and transferred to cytospin slides and spun in a cytocentrifuge at 800 rpm for 5 minutes. Slides were immersed in Wright-Giemsa stain in a Coplin jar for 30 min, and rinsed briefly in running deionized water and air dried before evaluation for cellular composition and morphology. Human cell engraftment in NOD-SCIDγc null mice was also ascertained by cytometric analysis. Peripheral blood (PB) analysis was started at week 8 by collecting blood from the retro-orbital venous plexus in 3.8 % trisodium citrate. At 4-5 months after transplantation, the mice were killed, and the femurs were removed and flushed with PBS to harvest cells. Eighty μl of whole blood and bone marrow

(BM) cells were collected, diluted in 120 μl of PBS with 2% FBS and mixed with human IgG FcR blocking reagent (Becton Dickinson) to block nonspecific staining. The mix was then incubated with anti-human CD45 antibodies for 30 min at 4°C. The mixture was depleted of erythrocytes by adding 2 ml of lysis buffer (BD PharMingen, San Diego, CA) and incubated at room temperature for 15 min followed by washing. Human white blood cells were examined by staining with PE-conjugated anti-human CD45 antibody (BD Pharmingen).

Detection of FIX. Blood of recipient mice was collected by retro-orbital bleeding in 3.8% sodium citrate. The levels of human FIX in the plasma were assayed using a human FIX-specific ELISA (Asserachrome/Diagnostica Stago, Parsippany, NJ, USA). The detection limit of the ELISA is <1% FIX (50 ng/ml).

Anti-FIX antibody response. Anti-IgM and IgG neutralizing antibodies against hFIX were measured by ELISA. Briefly, transparent bottom 96-well plates were coated overnight at 4°C with 3 I.U. human rFIX. After 24 h the plate was washed with 200 μl TBS per well, blocked with blocking buffer (pH 7.2) and incubated for 5 h at room temperature. 100 μl of the test plasma samples diluted in TBS + 0.5% BSA were incubated at 4°C overnight. After 24 h, the plate was washed 3 times with 200 μl washing buffer. 100 μl/well pNPP substrate was added and the plate was incubated for 15 min in the dark at room temperature. Reaction was stopped with 25 μl 3N NaOH and OD values were read at 405 nm. Plasma from mice injected with 2 μg rFIX and complete Freund's adjuvant (CFA) was used as positive control. Plasma from non-injected mice was used as negative control.

REFERENCES

1. Baus, J., Liu, L., Heggestad, A.D., Sanz, S. & Fletcher, B.S. Hyperactive transposase mutants of the Sleeping Beauty transposon. *Mol Ther* **12**, 1148-56 (2005).
2. Ivics, Z., Hackett, P.B., Plasterk, R.H. & Izsvák, Z. Molecular reconstruction of *Sleeping Beauty*, a Tc1-like transposon from fish, and its transposition in human cells. *Cell* **91**, 501-10 (1997).
3. Schmidt, M. et al. High-resolution insertion-site analysis by linear amplification-mediated PCR (LAM-PCR). *Nat Methods* **4**, 1051-7 (2007).

4. Liu, G., Aronovich, E.L., Cui, Z., Whitley, C.B. & Hackett, P.B. Excision of Sleeping Beauty transposons: parameters and applications to gene therapy. *J Gene Med* **6**, 574-83 (2004).
5. Bell, J.B. et al. Preferential delivery of the Sleeping Beauty transposon system to livers of mice by hydrodynamic injection. *Nat Protoc* **2**, 3153-65 (2007).