Supporting Information

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SI Materials and Methods

Plasmids. The coding sequences of human and mouse glycophorin A (GPA) and Kell were obtained from National Center for Biotechnology Information (NCBI) reference sequences NM_002099.6, NM_010369.3, and NM_000420.2, respectively. The Myc-tag coding sequence (GAGCAGAAACTCATCTCAGAAGAGGATCTG) was inserted between the signal peptide and the mature GPA to detect cell-surface expression. To attach the sortase tag at the N terminus of GPA, five or three glycine-tag coding sequences (GGTGGCGGAGGTGGA or GGTGGCGGA) were inserted immediately after the signal peptide. The full-length engineered human and mouse GPAs were synthesized by Genscript and subsequently were cloned into murine stem cell virus (MSCV) retroviral vectors. Human Kell (hKell) was cloned into an MSCV retroviral vector extended at its C terminus with the coding sequence for the Myc tag (GAACAAAAACTTATTTCTGAAGAAGATCTG), LPETGG (CTGCCAGAAACTGGTGGA), followed by the HA epitope tag (TACCCATACGACGTCCCAGACTACGCT).

Protein Expression and Purification. Both sortase A from *Staphylococcus aureus* Δ59 mutated for k_{cat} improvement (P94R, D160N, D165A, K190E, K196T) (1) and Ca^{2+}-independent activity (E105K, E108Q) (2) in pET30 and sortase A from *Streptococcus pyogenes* were expressed and purified as described previously (3). VHH7 (a nanobody that recognizes MHC class II) C-terminally extended with LPETGGHHHHHH in pHEN was expressed and purified as described previously (4). The design and synthesis of sortase probes have been described previously (3, 5).

Sortase-Labeling Reaction. To label the GPA N terminus with biotin, 30 μL of 500 μM *S. aureus* sortase and 1 mM K(biotin) LPRTGG peptide was preincubated in buffer [83 mM Tris·HCl (pH 7.5), 250 mM NaCl] on ice for 15 min and was added to 70 μL of ∼1×10^6 cultured cells (reticulocytes or HEK293T cells) or to up to 5×10^7 RBCs (10 μL of whole blood) in DMEM (prewashed with DMEM). To label the GPA N terminus with VHH7, 50 μL of 100 μM *S. aureus* sortase and 100 μM VHH-LPETG-His6 was preincubated in buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl] on ice for 15 min and was added to 50 μL of up to 5×10^7 RBCs in DMEM. To label the Kell C terminus with biotin, 30 μL of 500 μM *S. aureus* sortase and 1.4 mM GGGK (biotin) peptide in buffer [83 mM Tris·HCl (pH 7.5), 250 mM NaCl] was added to 70 μL of ∼1×10^6 cultured cells (reticulocytes or HEK293T cells) or up to 5×10^7 RBCs in DMEM (prewashed with DMEM). All sortase and cell mixtures were incubated on ice for 30 min with occasional gentle mixing. They were spun at 500 × *g* for 2 min at 4 °C to remove buffer/DMEM and were washed three times with 1 mL of ice-cold PBS. For sequential dual labeling of GPA and Kell, 30 μL of 333 μM *S. pyogenes* sortase and 1.33 mM K(biotin)LPETAA peptide were preincubated in buffer (83 mM Tris·HCl, 250 mM NaCl, 16.7 mM $CaCl_2$) at 37 °C for 15 min and then were added to 70 μL of 1×10^6 HEK293T cells in DMEM (prewashed with DMEM) and incubated at 37 °C for 25 min with occasional gentle mixing. Cells were spun down at 500 × *g* for 2 min at 4 °C, washed two times with 1 mL of ice-cold PBS, and were resuspended in 70 μL of ice-cold DMEM. Then 30 μL of 333 μM *S. aureus* sortase and 1.67 mM GGGK(Alexa647) peptide in 83 mM Tris·HCl (pH 7.5) and 250 mM NaCl were added to the cells, and the cells were incubated for 30 min on ice with occasional gentle mixing. Cells were washed three times with 1 mL of ice-cold PBS.

Western Blotting. To sortase-label whole blood, RBCs first were lysed with 500 μL of ammonium chloride solution (Stemcell Technologies) via 5-min incubation on ice and were spun down at 21,000 × *g* for 10 min at 4 °C. Membranes were washed once with 500 μL of PBS. Cells were solubilized in 25 mM Tris·HCl (pH7.5), 0.5% Nonidet P-40, 5 mM MgCl2, 150 mM NaCl supplemented with a cOmplete Mini Protease Inhibitor mixture tablet (Roche), vortexed, incubated on ice for 10 min, and spun at 21,000 × *g* for 10 min at 4 °C. Supernatant was incubated with SDS sample buffer and boiled for 8 min. After SDS/PAGE, proteins were transferred onto PVDF membrane and immunoblotted for biotin using streptavidin-HRP (GE Healthcare), myc tag (2040; Cell Signaling), HA tag (3F10; Roche), hemoglobin, or CD19 (3574; Cell Signaling).

Transfection. For virus production, 6 million 293T cells were split and plated on 10-cm plates 1 d before transfection in antibiotic-free DMEM with the addition of 15% (vol/vol) FBS and 2 mM L-glutamine (Invitrogen). On day 0, plasmids (10 μg) together with packaging vector (5 μg) were added to the 293T 10-cm plate with Fugene 6 (Promega). This medium was replaced 6–8 h later with new DMEM with added 15% (vol/vol) FBS, 2 mM L-glutamine, and 1× penicillin-streptomycin (Invitrogen). On day 1, the supernatant containing fresh virus was collected, filtered through a 0.45-μm filter (Millipore), and then used immediately to infect the murine erythroid progenitors. For GPA and Kell expression, HEK293T cells were plated on a 10-cm dish and were transfected with either 15 μg of G_n-myc-human/mouse GPA (h/mGPA) constructs in retroviral vectors or with 7.5 μg of hKell-LPETG-HA and 7.5 μg of 3G-myc- hGPA, both in retroviral vectors (for sequential dual sortase labeling) using TransIT transfection reagent (Mirus) according to the manufacturer's recommendations. Cells were analyzed for protein expression and were sortase-labeled 24 h after cellular transfection.

Isolation of Erythroid Progenitors from Murine Embryonic Day 14.5 Fetal Liver Cells. At embryonic day 14.5, pregnant C57BL/6J mice were killed by CO_2 administration, and the embryos were isolated. The entire fetal livers were separated carefully and placed in PBS with 2% FBS and 100 μM EDTA. Single fetal liver cell suspensions were obtained after triturating and filtering through a 70-μm filter (BD Biosciences). Mature RBCs in the fetal liver cell suspensions were lysed after incubation for 10 min with ammonium chloride solution (Stemcell). Nucleated cells were collected by centrifugation at 500 × *g* for 5 min and were resuspended in PBS. Using the BD Pharmingen Biotin Mouse Lineage Panel (559971; BD Biosciences) and BD Streptavidin Particles Plus-DM (557812; BD Biosciences), we purified lineage-negative fetal liver cells, which were enriched for erythroid progenitors (>90%).

Viral Infection and Culture of Murine Erythroid Progenitors. After purification, lineage-negative fetal liver cells were prepared at a concentration of 10 million cells/mL Then 10 μL of the cells were plated in each well of a 24-well plate, together with 1 mL virus-containing supernatant with 5 μg/mL Polybrene (Sigma-Aldrich). The plate was spun at 500 × *g* for 90 min at 37 °C. Immediately after spin-infection, the virus-containing supernatant was aspirated and replaced with erythroid maintenance medium (StemSpan-SFEM; StemCell Technologies) supplemented

with 100 ng/mL recombinant mouse stem cell factor (SCF) (R&D Systems), 40 ng/mL recombinant mouse IgF1 (R&D Systems), 100 nM dexamethasone (Sigma), and 2 u/mL erythropoietin (Amgen). The next morning, the infection rate was examined by flow cytometry by checking the percentage of GFP^+ cells, which typically was >95%. The cells then were cultured for 48 h in epoetin-only erythroid differentiation medium [Iscove modified Dulbecco's medium containing 15% (vol/vol) FBS (Stemcell), 1% detoxified BSA (Stemcell), 500 μg/mL holo-transferrin (Sigma-Aldrich), 0.5 U/mL epoetin (Epo; Amgen), 10 μg/mL recombinant human insulin (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), and 1× penicillin-streptomycin (Invitrogen)].

Flow Cytometry. We collected the desired cells, washed them once by PBS, and resuspended them at a density of 5–10 M/mL in PBS with 1 μg/mL propidium iodide for FACS sorting or analysis. During enucleation analysis, the cells were first stained with Anti-Mouse TER-119 antibody (14–921; eBioscience) and Hoechst (Sigma) for 15 min at room temperature. To detect the surface expression or labeling of Myc-tag, HA-tag, or biotin labeling, the cells were stained with anti-Myc antibody (3739; Cell Signaling), anti-HA antibody (NC9843881; Thermo Fisher Scientific) or anti-biotin antibody (12-9895-82; eBioscience), respectively, for 30 min at room temperature.

Viral Infection of Bone Marrow Cells. Bone marrow in femur and tibias from C57BL/6J mice was isolated using a 23-G needle and was cultured in six-well plates at a density of 2×10^6 cells/mL for 18 h in DMEM supplemented with 15% (vol/vol) FCS, 2 mM L-glutamine (Invitrogen), 1× penicillin-streptomycin (Invitrogen), 20 ng/mL IL-3 (Peprotech), 50 ng/mL SCF (Peprotech), and 50 ng/mL IL-6 (Peprotech). Cells were infected by incubating 4×10^6 cells in 500 μL of retrovirus-containing medium, 500 μL DMEM, and 5 μg/mL Polybrene, spinning the cells at $500 \times g$ for 1.5 h at room temperature, and further incubating them in a CO_2 incubator for 5 h. Cells were returned to the above IL-3/SCF/IL-6–supplemented medium and incubated further for 16 h.

Irradiation Procedure and Transplantation of Mouse Fetal Liver Cells. B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ mice (Jackson Laboratory) were subjected to total body irradiation with 1,050 cGy in a Gammacell 40 irradiator chamber (Nordion International Inc.) 1 d before transplantation. Mouse fetal liver cells were harvested and prepared as described above. After retroviral infection, cells were cultured in erythroid maintenance medium for 18 h. Alternatively, mouse bone marrow cells were harvested, and retrovirus was transduced as described above. Infected cells then were washed two times in sterile PBS and were resuspended in sterile PBS at a density of 2–5 million cells/mL. Then 100 μL of these mouse stem and progenitor cells were injected retro-orbitally into the lethally irradiated mice. Mature RBCs were extracted from the irradiated mice for analysis and sortagging starting 4 wk after injection.

Cytospin Preparation and Immunofluorescence. After mature RBCs and in vitro-differentiated reticulocytes were sortagged with biotin, they were washed twice in cold 1× PBS. Then 50,000 sorted, biotinylated mature RBCs or unsorted, in vitro-differentiated reticulocytes were centrifuged onto poly-L-lysine–coated slides for 5 min at 400 rpm (Cytospin 3; Thermo Shandon). Samples were air dried, fixed in 4% paraformaldehyde for 30 min at room temperature, and blocked for 1 h in blocking buffer (2% BSA + 2% donkey serum in PBS). Cells then were incubated with primary antibodies: [phycoerythrin (PE)-conjugated anti-biotin (1:1,000) (12-9895-82; eBioscience) and allophycocyanin (APC)-conjugated anti-Ter119 (1:100) (14-5921; eBioscience)] in blocking buffer overnight at 4 °C, followed by three washes in cold 1× PBS. Finally, cells were mounted with mounting medium containing DAPI (Prolong Gold Antifade; Invitrogen) to visualize nuclei in all immunostaining experiments. Visualization was carried out using a Zeiss LSM 700 Laser Scanning Confocal Microscope.

Transfusion and Survival of Engineered RBCs. After mature RBCs were sortagged, they were washed twice in 1× PBS and resuspended in RPMI medium. These sortagged RBCs were collected by centrifugation at $500 \times g$ for 5 min and labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) in HBSS (Life Technologies) for 8 min. Equivolumes of 10% (vol/vol) FBS in HBSS then were added to quench the reaction. RBCs were washed, counted, and resuspended in sterile HBSS for injection. Then 2.5 billion CFSE-labeled RBCs (± 300 μL mouse blood) were injected i.v. into recipient $CD45.1^+$ mice. Normal RBCs served as a control. Only ~20–50% (vol/vol) of RBCs expressed engineered hGPA or hKell; the rest of the RBCs were normal and served as an internal control by monitoring the CFSE signal. The first blood sample (20 μL) was collected retro-orbitally 1 h after transfusion and was labeled as day 0. The subsequent 20-μL blood samples were collected every 3 days until day 28. The blood samples were stained with anti-biotin antibody conjugated with PE (eBioscience), so that eRBCs linked with biotin had strong PE signal during flow cytometry analysis. However, RBCs with hKell-biotin had very low red fluorescent intensity after staining with anti-biotin antibody conjugated with PE, and it was difficult to detect the fluorescence in the presence of strong green fluorescent signal from CFSE. Therefore the hKell-RBCs were sortagged with biotin and transfused into mice without CFSE staining. The hKell-RBCs were monitored by their inherent GFP signal and the weak PE signal from anti-biotin antibodies conjugated with PE. The control RBCs and hGPA-RBCs were transfused into a total of six mice at two separate times. The hKell-RBCs were transfused into a total of three mice, one of which died for an unknown reason.

B-Cell–RBC Binding Assay. B cells were isolated from wild-type C57BL/6 mice and from mice knocked out for MHC class II using Dynabeads Mouse CD43 (Untouched B cells) (Life Technologies) according to the manufacturer's recommendations. Cells from 1–1.5 spleens were incubated with 5 μg of biotin-labeled anti-murine CD19 antibody (BD Pharmingen) for 30 min on ice and were washed once with 500 μL of ice-cold PBS. Cells were resuspended in 500 μL of PBS along with 70 μL of prewashed magnetic Dynabeads Myone streptavidin T1 (Life Technologies), were incubated on a rotating platform for 1 h at 4 °C, and were washed with 500 μL of ice-cold PBS. Resuspended cells (500 μL of PBS) were incubated further with $\sim 2 \times 10^7$ RBCs expressing 3G-myc-hGPA and were sortase-labeled with VHH7 for 1 h at 4 °C, washed four times with 1 mL of ice-cold PBS, and finally were incubated with SDS sample buffer and boiled for subsequent SDS/PAGE analysis.

Engineering of Terminally Differentiated Human RBCs. Plasmids used to engineer in vitro-differentiated human RBCs were created by cloning the GGG-containing human GPA (hGPA) into HIV/MSCV lentiviral vector with the addition of eGFP at its C terminus to make 3G-myc-hGPA-EGFP. Lentivirus was produced by cotransfection of 293T cells with pVSV-G envelope plasmids and pDelta 8.9 packaging vectors. Granulocyte-colony stimulating factor (G-CSF)–mobilized $CD34^+$ peripheral blood stem cells were differentiated in vitro (18 d) into hemoglobin-containing reticulocytes using the method described previously (6). On day 3 of culture, differentiating cells were infected by incubating 5×10^5 cells in 2 mL of lentivirus-containing medium in the presence of Polybrene and were spun at $500 \times g$ for 1.5 h at room temperature, followed by further incubation in a CO_2 incubator overnight. The next day, the infected cells were washed

twice and put into fresh differentiation medium. At day 18, the enucleated reticulocytes were collected and subjected to sortase-labeling with a biotin-containing probe as described above. Analyses of the resulting engineered human reticulocytes were carried out by flow cytometry using the following antibodies: PE-conjugated anti-biotin (1:1,000) (12-9895-82; eBioscience), Hoechst (Sigma), and APC-conjugated anti-human CD235a (Glycophorin A) APC (eBiosciences) (1:100) (17-0087-42; eBioscience).

Creation of Mouse-Kell–LPETG Mice Using CRISPR/Cas9 Technology. A single-guide RNA (sgRNA) sequence for the CRISPR/Cas9-dependent double-strand break to stimulate homologous direct repair (HDR) was designed on the last exon of the mouse Kell (mKell) locus as shown in Table S1, and the oligo DNA used as a template to introduce LPETG into the mKell C terminus was designed as shown in Table S2. Potential off-target effects of the target sequence were explored using the NCBI *Mus musculus* nucleotide BLAST. Cas9 mRNA was prepared as described in ref. 7. The T7 promoter and the mKell-C terminus–targeting sgRNA coding sequence without the protospacer-associated motif (PAM) were added just in front of the sgRNA generic tail (the part from hairpin region to the terminal motif) by PCR amplification using pX330 vector as a template and the primers mKell T7-sgRNA forward and sgRNA generic reverse, as shown in Table S3 [KOD Xtreme (VWR International, LLC): 95 °C for 2 min; 35 cycles of 98 °C for 10 s, 60 °C for 30 s, 68 °C for 30 s; 68 °C for 2 min; hold at 4 °C]. The T7-sgRNA template PCR product for in vitro transcription was gel purified by column (Promega), and the purified PCR product was used as a template for vitro transcription using the MEGAshortscript T7 kit (Life Technologies). Both the Cas9 mRNA and the sgRNA were purified using the MEGAclear kit (Life Technologies) and were eluted in RNase-free water. Fertilized zygotes were collected from oviducts of superovulated females, and Cas9 mRNAs, sgRNA, and template oligo DNA were injected into the cytoplasm at the pronuclear stage. The injected zygotes were transferred at the two-cell stage into the oviduct of pseudopregnant females. To genotype the mice, cells from mouse tails were lysed at 55 °C for 12 h using tail lysis buffer, and genomic DNA was purified from the lysates by isopropanol precipitation. Genomic DNA in the vicinity of the sgRNA target was amplified by PCR using the primers mKell-sequencing forward and mKell-sequencing reverse, as shown in Table S3 [KOD Xtreme (VWR International, LLC): 95 °C for 2 min; 35 cycles of 98 °C for 10 s, 50 °C for 30 s, 68 °C for 30 s; 68 °C for 2 min; hold at 4 °C], and then were gel purified. The purified PCR products were sequenced using the same forward primer.

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2. Hirakawa H, Ishikawa S, Nagamune T (2012) Design of Ca2+–independent Staphylococcus aureus sortase A mutants. *Biotechnol Bioeng* 109(12):2955–2961.
3. Guimaraes CP, et al. (2013) Site-specific C-terminal and internal loop labeling of proteins using sortase-mediated reactions. *Nat Protoc* 8(9):1787–1799.
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7. Wang H, et al. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153(4):910–918.

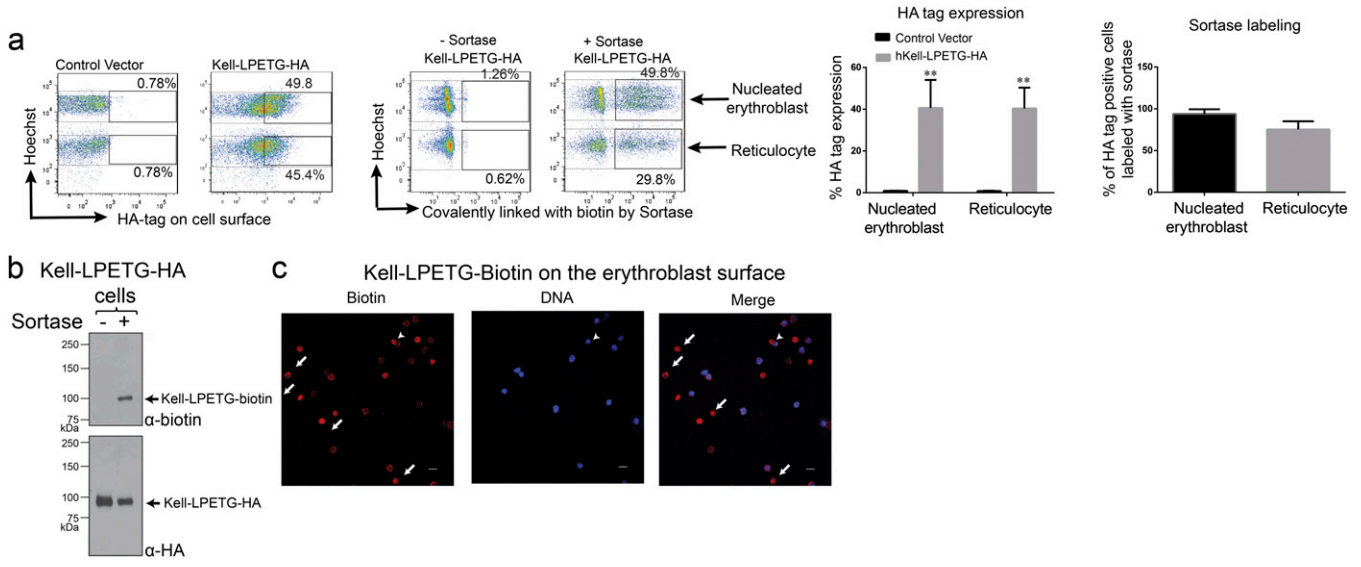

Fig. S1. Expression and sortase-mediated C-terminal labeling of Kell on the surface of in vitro-differentiated erythroblasts. (*A*) Flow cytometry of in vitro-differentiated erythroblasts for the presence of hKell-LPETG-HA and sortase-labeled hKell-LPETG-biotin on the cell surface by staining with α-HA– and α-biotin–tagged antibodies. The percentage of erythroblasts and reticulocytes with an HA tag on the cell surface and the percentage of HA-tag$^+$ cells sortagged with a biotin probe were determined from three independent experiments and are shown as mean ± SD; $^{**}P < 0.01$. (*B*) Evaluation of in vitro-differentiated erythroblasts for sortase-labeling by incubating control or hKell-LPETG-HA erythroblasts with sortase and the biotin-containing probe followed by immunoblotting for biotin and Kell. (*C*) Immunofluorescence shows biotin (labeled with red) at the C terminus of hKell on the surface of differentiated erythroblasts. Nuclei were stained by Hoechst (blue). Arrows indicate reticulocytes, and the arrowhead indicates enucleating erythroblasts.

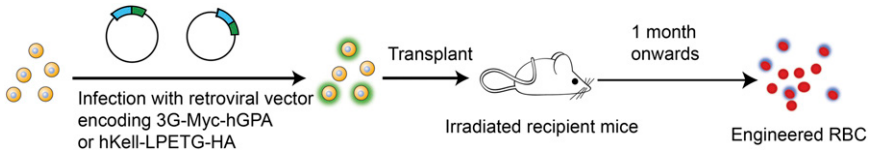

Fig. S2. Scheme of engineered RBC production by mouse bone marrow/fetal liver cell transplantation. Isolated murine bone marrow cells or fetal liver cells were transduced with a retrovirus carrying hKell-LPETG-HA or 3G-myc-hGPA. Lethally irradiated mice were reconstituted with transduced bone marrow or fetal liver cells. At 1 mo and thereafter, a significant percentage of mature RBCs are derived from transduced donor progenitors.

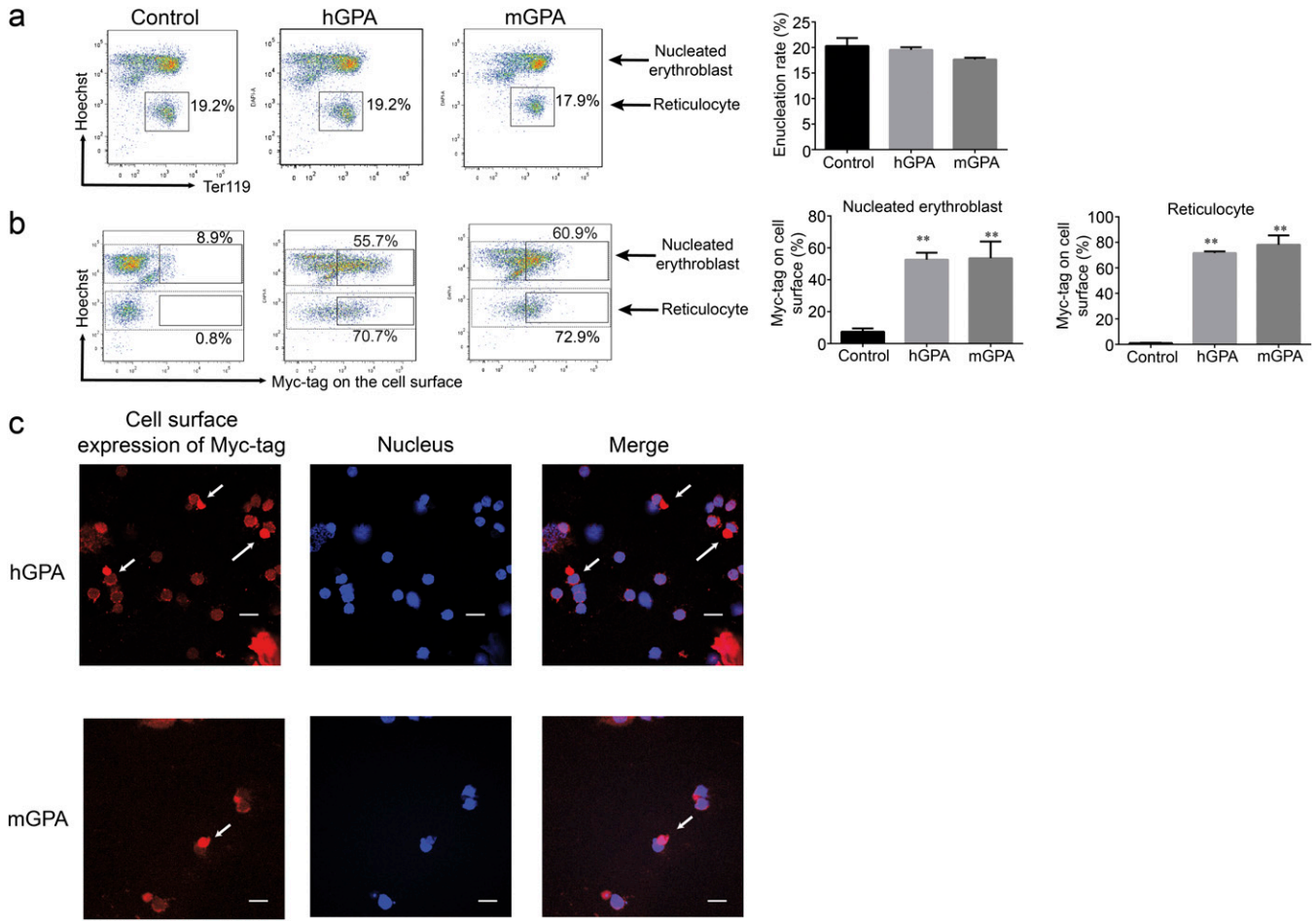

Fig. S3. The overexpression of modified hGPA and mGPA containing a myc-tag at the N termini does not affect in vitro differentiation of mouse erythroid progenitor. (*A*) Flow cytometry analysis of the differentiation capacity of in vitro-differentiated murine fetal liver-derived progenitor cells infected with retroviral constructs containing modified hGPA or mGPA constructs extended at the N terminus with a myc-tag: myc-hGPA, and myc-mGPA. Differentiation capacity in these cells is assessed by the expression of Ter119 and enucleation, i.e., nuclei expulsion, resulting in $Ter119^{+}$ reticulocytes. The percentages of reticulocytes produced by erythroid progenitor cells infected with myc-hGPA or myc-mGPA (~20%) are comparable to those in cells infected with empty (control) vector, indicating that these constructs do not disturb erythroid terminal differentiation. Enucleation rates were quantified in independent experiments and are shown as mean ± SD. (*B*) Evaluation of murine terminally differentiated erythroblasts, i.e., nucleated erythroblasts and reticulocytes, for the surface expression of myc-hGPA or myc-mGPA. More than 60% of these terminally differentiated cells expressed the desired modified GPA proteins as measured by flow cytometry using α-myc–tagged antibodies. The percentage of erythroblasts and reticulocytes with a myc tag on the cell surface was determined from three independent experiments and is graphed as mean ± SD; $^{**}P < 0.01$. (*C*) Immunofluorescence images further confirm the surface expression of myc-tagged (labeled with red) and the enucleation capacity (nuclei are stained in blue) of the in vitro terminally differentiated erythroblasts.

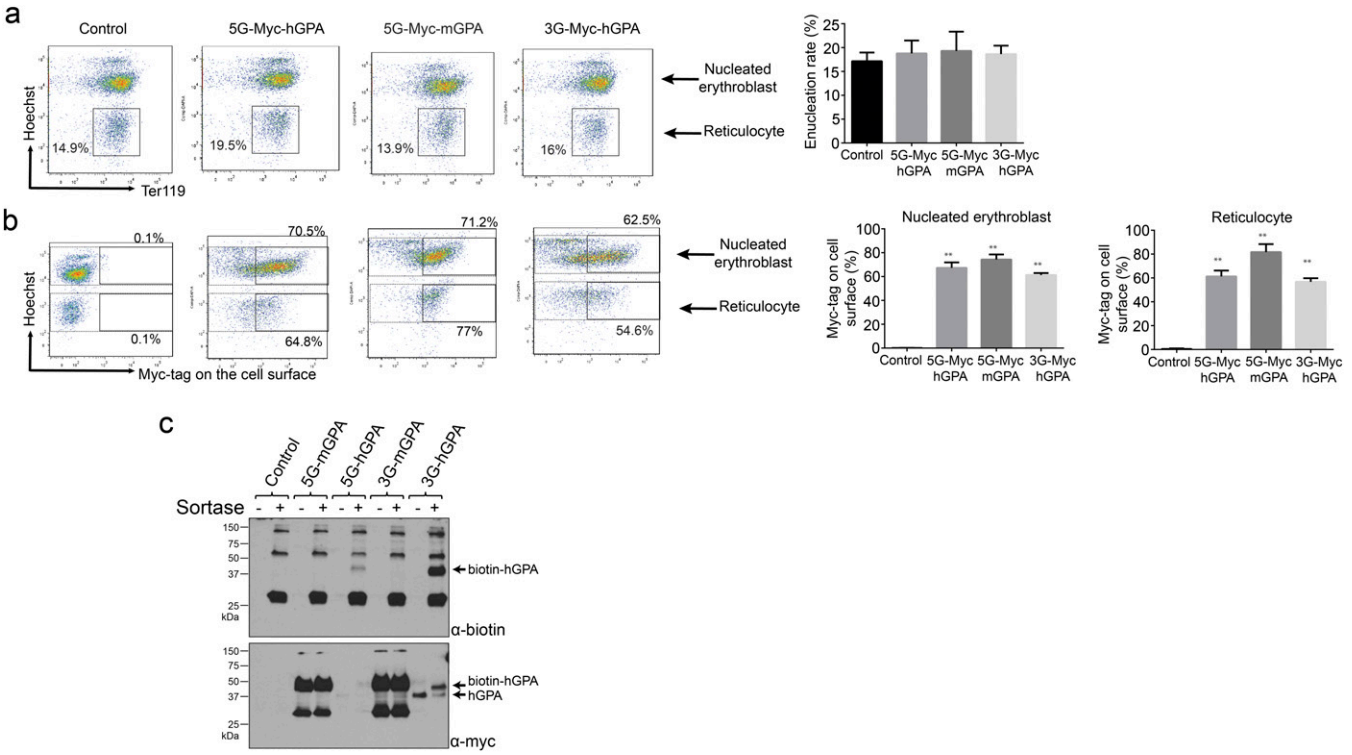

Fig. S4. The overexpression of engineered hGPA and mGPA containing a myc tag with multiple (three or five) glycines at the N terminus does not affect the in vitro differentiation of mouse erythroid progenitors. Only cells expressing engineered hGPA with a myc tag and three glycines at the N terminus can be biotin-labeled efficiently by sortagging. (*A*) Flow cytometry analysis on the differentiation capacity of in vitro-differentiated murine fetal liver-derived progenitor cells infected with retroviral constructs containing modified hGPA or mGPA constructs extended at the N terminus with a myc tag and three or five glycines (3G or 5G, respectively): 5G-myc-hGPA, 5G-myc-mGPA, and 3G-myc-hGPA. The differentiation capacity in these cells is assessed by the expression of Ter119 and enucleation (i.e., nuclei expulsion), resulting in Ter119$^+$ reticulocytes. The percentages of reticulocytes produced by erythroid progenitor cells infected with 5G-myc-hGPA, 5G-myc-mGPA, and 3G-myc-hGPA (~17.5%) are comparable to those in cells infected with empty (control) vector, indicating that these constructs do not disturb erythroid terminal differentiation. The enucleation rate was quantified in three independent experiments and is graphed as mean ± SD. (*B*) Evaluation of murine terminally differentiated erythroblasts (i.e., nucleated erythroblasts and reticulocytes) for the surface expression of 5G-myc-hGPA, 5G-myc-mGPA, and 3G-myc-hGPA. More than 50% of these terminally differentiated cells expressed the desired modified GPA proteins as measured by flow cytometry using α-myc–tagged antibodies. The percentage of erythroblasts and reticulocytes with a myc tag on the cell surface was determined from three independent experiments and is graphed as mean ± SD; $^{**}P < 0.01$. (*C*) HEK 293T cells were transfected to express 5G-myc-hGPA, 5G-myc-mGPA, 3G-myc-mGPA, and 3G-myc-hGPA. These cells then were incubated with a biotin probe with or without sortase, and total cell protein was immunoblotted for the myc tag and biotin. The immunoblot stained for biotin shows successful biotin conjugation only to the hGPA constructs 3G-myc-hGPA (strong band) and 5G-myc-hGPA (weak band) with a much higher efficiency for 3G-myc-hGPA. α-Myc–tagged immunoblotting further confirms the biotin probe conjugation, as indicated by a shift in hGPA molecular mass upon biotin conjugation.

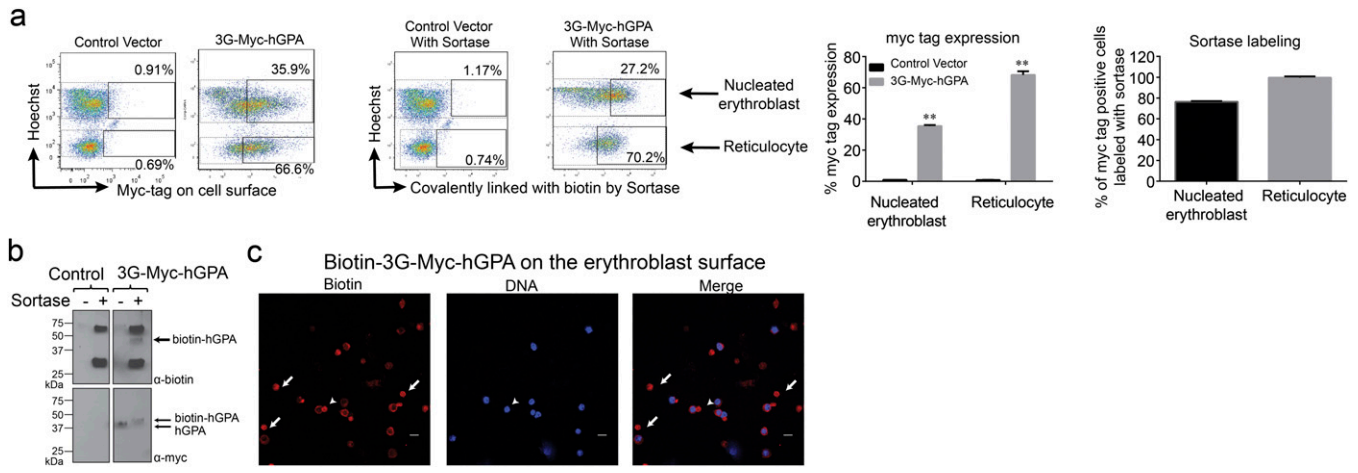

Fig. S5. Expression and sortase-mediated N-terminal labeling of GPA on the surface of in vitro-differentiated erythroblasts. (*A*) Flow cytometry analysis of in vitro-differentiated erythroblasts for the presence of 3G-myc-hGPA and for sortase-labeled biotin-3G-myc-hGPA on the cell surface by staining with α-myc– and α-biotin–tagged antibodies. The percentage of erythroblasts and reticulocytes with a myc tag on the cell surface and the percentage of myc$^+$ cells sortagged with a biotin probe were determined from three independent experiments and are graphed as mean ± SD; $^{**}P < 0.01$. (*B*) In vitro-differentiated erythroblasts were evaluated for sortase labeling by incubating control or 3G-myc-hGPA erythroblasts with sortase and the biotin-containing probe and immunoblotting with α-myc–tagged and α-biotin antibodies. The shift in molecular mass of hGPA upon biotin conjugation in the α-myc blot indicates complete modification of hGPA. Biotin-conjugated GPA is denoted by an arrow in the α-biotin immunoblot. (*C*) Immunofluorescence shows biotin (labeled with red) at the N terminus of hGPA on the surface of differentiated erythroblasts. Nuclei were stained by Hoechst (blue). Arrows indicate reticulocytes, and arrowheads indicate enucleating erythroblasts.

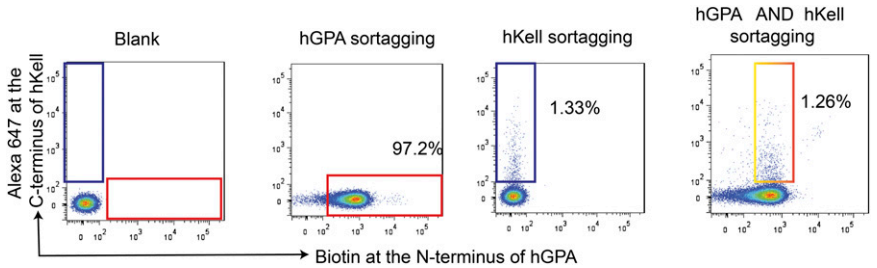

Fig. S6. Sortase-mediated dual labeling at the N terminus of hGPA and the C terminus of hKell on the surface of mature RBCs. Mature RBCs expressing hKell-LPETG-HA and 3A-myc-hGPA were incubated with sortase A from *S. pyogenes* and the K(biotin)LPETAA probe for N-terminal GPA labeling (*Center Left*), or with sortase A from *S. aureus* and the GGGC(Alexa-647) probe for C-terminal Kell labeling (*Center Right*), or with both sequential label with both sortase enzymes and their corresponding probes (*Right*). Flow cytometry analysis of mature RBCs for the presence of both hKell-LPET-Alexa-647 and biotin-myc-hGPA indicates successful dual labeling.

	Normal Range	Wild-type n=2		Kell-LPETG (+/-) n=3		Kell-LPETG (-/-)	
		Mean	error	Mean	error	Mean	error
RBC	6.5-10.5	9.76	0.34	7.98	0.81	8.82	0.43
HGB	11.8-14.8	14.55	0.07	11.9	1.47	13.3	0.68
HCT	36.0-52.4	54.05	1.34	41.7	4.66	45.28	2.27
MCV		55.4	0.57	52.2	1.11	51.4	1.06
MCH		14.9	0.42	14.9	0.3	15.1	0.42
MCHC		26.9	0.57	28.2	0.26	29.38	0.31

Fig. S7. RBC parameters of heterozygous and homozygous mKell-LPETG transgenic mice. HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume.

Table S1. Sequence of sgRNA

sgRNA	5′ to 3′ sgRNA template sequence with PAM
mKell CT-targeting sgRNA	CTCTGCCCGCTGCAAGCTCTGG

PAM is underlined.

Table S2. Template oligo DNA for HDR

Template oligo DNA for HDR	5′ to 3′ Oligo DNA sequence
mKell-LPETG template oligo DNA	TGAGCAATACTCCAGATTTTGCCAAACATTTTCATTGTCCACGTGGGACCCTTCTGAATCCCTCTGCCCGC-TGCAAGCTC**GGAGGATCAGGAGGATCATTACCAGAGACAGGAGGA**TGGTAAAACTTGGCTACCAAAGAG-ACTGATGTAAATGCATGGGCTGCTTGTGAGTCCATCCTTGAAGTCAAAATAAATCT

The LPETG-encoding sequence is shown in bold.

Table S3. Primers list

Primers	5′ to 3′ primer sequence
mKell T7-sgRNA forward	*TAATACGACTCACTATAG*CTCTGCCCGCTGCAAGCTCgttttagagctagaaatagcaag
sgRNA generic reverse	TAAGTTATGTAACGGGTAC
mKell sequencing forward	ATCTAACCCATCCCTATCACCCTATGG
mKell sequencing reverse	ATGGAGATGTAGCTGATGAGCAGC

The T7 promoter is in italic type, the sgRNA target template is in uppercase letters, and the noncapital: 5′-generic sequence of the sgRNA tail is in lowercase letters.