Supporting Information

Föller *et al.* 10.1073/pnas.0708940105

SI Materials and Methods

Blood and Plasma Parameters. RBC number, HCT, MCV, and hemoglobin concentration were determined using an electronic hematology particle counter (type MDM 905 from Medical Diagnostics Marx). These measurements were confirmed by the measurement of hematocrit by centrifugation at 15,000 × *g* for 3 min, measuring the hemoglobin concentration photometrically at 546 nm after adding of 20 μl of blood to 3 ml of a hemoglobin transformation solution (Dr. Lange AG) and by counting the RBCs manually after 1:400 dilution with Heyem's solution (Fluka) in a Neubauer chamber. Using the hematocrit, hemoglobin and RBC count obtained this way, the MCV, MCH, and MCHC were calculated. The RDW was determined from images of blood smears taken with a CCD camera (Axiocam, Zeiss) at a magnification of ×400 (Axioskop, Zeiss). From 1,829 to 3,444 individual erythrocytes of each animal ($n = 3$ to 4) the size was determined using an image analyzing system (MCID). The obtained standard deviations and the means of the erythrocyte size were used to calculate the coefficient of variation as a measure for the RDW. Relative reticulocyte numbers were determined using the Retic-COUNT reagent (BD) according to the manufacturer's instructions.

The plasma concentration of erythropoietin, haptoglobin, and transferrin was determined using immunoassay kits according to the manufacturer's instructions (erythropoietin: R&D systems; others: Kamiya). Vitamin B12 and folate were determined by competitive immunoassays according to clinical standards.

Analysis of Spleens. Single-cell suspensions of freshly isolated spleens were stained for surface markers and intracellular cytokines and analyzed by flow cytometry according to standard procedures. Antibodies to Ter119, CD41, CD3, CD4, CD8, B220, IL-2, IL-4, IL-10, IL-17, IFN-γ, TNF, and appropriate isotype controls labeled with FITC or PE were obtained from BD. Annexin V-FITC staining was performed using an Apoptosis Detection Kit (BD). For intracellular cytokine staining, isolated splenocytes were stimulated with PMA (15 ng/ml) and ionomycin (1.5 μg/ml) (both Sigma) for 4 h in the presence of Brefeldin A (Golgi Plug, BD). Cells were fixed in 2% formaldehyde, washed and permeabilized with 0.5% Saponin/0.5% BSA and incubated with the indicated antibodies. After washing, 50.000 or 100.000 events were counted on a FACS Calibur (BD). For investigating the proliferative capacity of splenocytes, 1×10^5 cells per well were cultured in medium or stimulated with LPS (1 μg/ml). Cell proliferation was examined by measuring DNA synthesis using [^{3}H]thymidine incorporation (1.25 μCi/ml) for 12 h before harvesting and counting using a MicroBeta device (Perkin–Elmer). Except for analysis of Ter119, CD41, and annexin V, cells were treated with ammonium chloride to lyse RBCs before further experiments.

Western Blot Analysis. Erythroid cells were isolated from bone marrow and peripheral blood by immunomagnetic selection using magnetically labeled anti-Ter119 MicroBeads and magnetic cell sorting (MACS, Miltenyi Biotec) (1). Proteins were separated on a SDS gel, transferred to a PVDF membrane and stained with a polyclonal rabbit antiserum to cGKI (2) or with an antibody against thrombospondin-1 (TSP-1) (Lab Vision). As a positive control for both cGKI as well as TSP-1 expression, platelet-rich plasma was also loaded.

Analysis of Phosphatidylserine Exposure and Intracellular Ca^{2+} in Peripheral Erythrocytes. Erythrocytes were washed two times in Ringer solution (in mM: 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 Hepes, 5 glucose, 1 $CaCl_2$, pH = 7.4). Then, erythrocytes at a final hematocrit of 0.4% were incubated in Ringer solution at 37°C in the absence or presence of drugs as indicated. After incubation, FACS analysis was performed essentially as described (3, 4). Cells were stained with Annexin-V-Fluos (Roche) in Ringer containing 5 mM Ca^{2+} at a 1:500 dilution. After 20 min, samples were measured by flow cytometry (FACS-Calibur). Annexin-V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. For intracellular Ca^{2+} measurements, erythrocytes were loaded with Fluo-3/AM (Calbiochem) in Ringer solution containing 5 mM $CaCl_2$ and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 20 min under shaking and washed twice. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer solution. Then, Ca^{2+}-dependent fluorescence intensity was measured in FL-1.

Measurement of the Clearance of Fluorescence-Labeled Erythrocytes *in Vivo*. Erythrocytes (obtained from 200 μl of blood) were fluorescence-labeled by staining the cells with 5 μM carboxyfluorescein-diacetate-succinimidyl-ester (CFSE) (Molecular Probes) in PBS and incubated for 30 min at 37°C. After washing twice in PBS containing 1% FCS the pellet was resuspended in Ringer solution (37°C) and 100 μl of the CFSE-labeled erythrocytes were injected into the tail vein of the recipient mouse. After two days, blood was retrieved from the tail veins of the mice and CFSE-dependent fluorescence intensity of the erythrocytes was measured in FL-1 as described above. The percentage of CFSE-positive erythrocytes was calculated in % of the total labeled fraction determined 5 min after injection.

Measurement of Erythrocyte Flexibility and Osmotic Resistance. Freshly drawn blood (20 μl) was suspended in 2 ml PBS containing Dextran (MW 60000, Serva) in amounts yielding a viscosity of 24.4 or 10.4 mPa*s (measured with a cone-plate viscosimeter, DVIII+ Rheometer, Brookfield Engineering Laboratories). The osmolarity of these solutions was adjusted to 310 mosm/liter. The red cell/test solution suspension was transferred into a laser defractometer (Myrenne) and the percent elongation of the erythrocytes was recorded at shear stresses between 0.31 and 61 s^{-1} (24.4 mPa*s solution) or 0.13 and 26 s^{-1} (10.4 mPa*s solution). For determination of the osmotic resistance of erythrocytes, 1 μl of blood was added to 200 μl of PBS solutions of decreasing osmolarity. After centrifugation for 5 min at 500 × *g* the supernatant was transferred to a 96-well plate and the absorption at 405 nm was determined as a measure of hemolysis. Absorption in isoosmolar PBS was defined as 0% hemolysis and absorption in pure distilled water was defined as 100% hemolysis.

Magnetic Resonance Imaging of Spleen Volume. Longitudinal magnetic resonance imaging (MRI) was performed with a dedicated 7 Tesla *in vivo* animal MRI system Clinscan (Bruker BioSpin), equipped with a 300 mT/m gradient system and operated by the software platform Syngo (Siemens). After blood withdrawal via tail veins, mice were anesthetized with 1.5–2% isoflurane (in 0.8 l/min oxygen) and placed inside the MR scanner containing a 35 mm quadrature whole body mouse transmitter/receiver coil. Respiration was recorded by an animal monitoring and gating

system (SA Instruments) to trigger MR image acquisition. A full 3D T_2 weighted sequence (T_R: 3500 ms; T_E: 355 ms; 3 averages) was acquired within 15–18 min, leading to an image dataset with a field of view of 56 mm × 42 mm × 23 mm and a voxel size of 0.22 mm × 0.22 mm × 0.22 mm (matrix size: 256 × 192 × 104). Image analysis was performed with PMOD (PMOD Technologies). Spleen volumes were determined by following the spleen boundaries visible in transversal sections of the MR images. To account for variations in spleen volume estimation, every dataset was evaluated in triplicate, and averaged spleen volumes were used for further calculations.

1. Kina T, *et al.* (2000) The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. *Br J Haematol* 109:280–287.
2. Feil S, *et al.* (2005) Distribution of cGMP-dependent protein kinase type I and its isoforms in the mouse brain and retina. *Neuroscience* 135:863–868.
3. Andree HA, *et al.* (1990) Binding of vascular anticoagulant alpha. (vac alpha) to planar phospholipid bilayers *J Biol Chem* 265:4923–4928.
4. Lang KS, *et al.* (2003) Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ* 10:249–256.

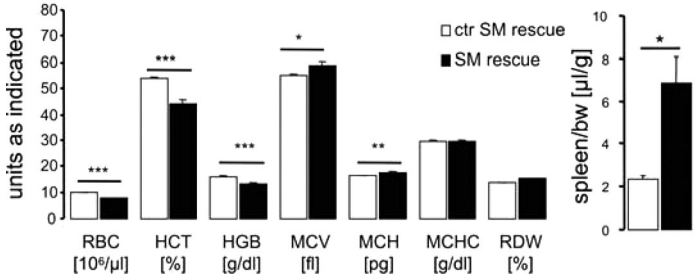

Fig. S1. Anemia and splenomegaly in 10-week-old cGKI SM rescue mice (SM rescue, black bars) as compared to their control littermates (ctr SM rescue, open bars). Left diagram, counts of RBC, HCT, HGB concentration, MCV, MCH, MCHC, and RDW. Right diagram, spleen/body weight (bw) ratios. The data were obtained from a litter-matched group of mice ($n = 4–5$). *, ** and *** indicate significant differences between genotypes with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. The genotypes of the ctr SM rescue mice were cGKI$^{+/L}$;SM-I$\alpha^{+/-}$ or cGKI$^{+/L}$;SM-I$\beta^{+/-}$.

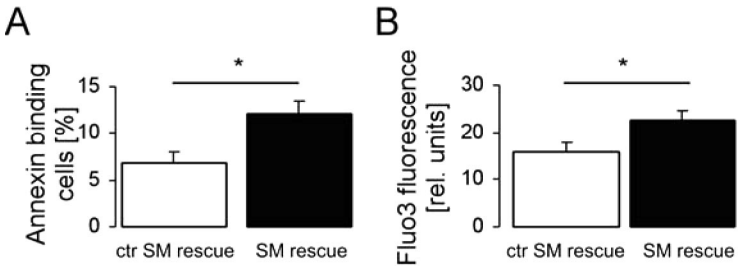

Fig. S2. Increased eryptosis and intracellular Ca^{2+} level in erythrocytes isolated from 10-week-old cGKI SM rescue mice (SM rescue) as compared to their control littermates (ctr SM rescue). (*A*) Surface exposure of PS as determined by annexin V-binding and (*B*) measurement of intracellular Ca^{2+} by Fluo-3 fluorescence after incubation in Ringer solution at 37°C for 48 h ($n = 5–6$; *, $P < 0.05$). The genotypes of the ctr SM rescue mice were $cGKI^{+/L}$;SM-I$\alpha^{+/-}$ or $cGKI^{+/L}$;SM-I$\beta^{+/-}$.

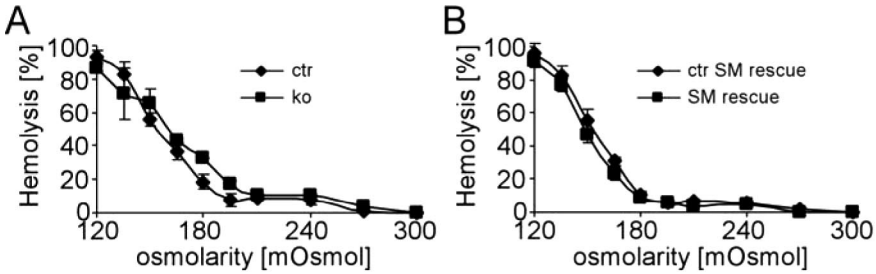

Fig. S3. Osmotic resistance of cGKI-deficient erythrocytes. Erythrocytes were isolated from (*A*) conventional cGKI knockout mice (ko, boxes) and their controls (ctr, diamonds) or (*B*) cGKI SM rescue mice (SM rescue, boxes) and their controls (ctr SM rescue, diamonds). Mice were 10 weeks old. The genotypes of the ctr mice were $cGKI^{+/+}$ or $cGKI^{+/L-}$. The genotypes of the ctr SM rescue mice were $cGKI^{+/L-}$;SM-I$\alpha^{+/-}$ or $cGKI^{+/L-}$;SM-I$\beta^{+/-}$.