Iron deficiency anemia in cyclic GMP kinase knockout mice

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Supplemental Materials and Methods

All substances used were of the highest purity available.

Animal Welfare and Ethical Statement. All experimental procedures were conducted according to the local government's committee on animal care and welfare in München. Animal experimentation conformed to Directive 2010/63/EU. The cGKI$^{-/-}$ [1], the cGKIα- and cGKIβ- rescue mice [2] have been described. cGKI RM mice were always from the cGKIβ rescue strain, if not indicated otherwise. Mice were bred on a Sv129 background and were maintained in the animal facility of the Institut für Pharmakologie und Toxikologie, Technische Universität München and had free access to tap water and standard chow.

Mouse handling - Where indicated (+PPI), mother animals and off-spring were fed a solid pellet diet that contained 18 mg/kg esomeprazole encapsulated in MUPS® (multiple-unit pellet systems) dispersed in the normal chow by the manufacture (Altromin). PPI containing pellets were fed for at least 6 weeks. The age of the experimental animals was from 6 to 12 weeks for WT, cGKI$^{-/-}$, and cGKI RM mice. Venofer® (iron-(III)-hydroxid-sucrose-complex) was ip injected 6 x 0.8 mg Fe^{3+} per mouse every second day. Mice were sacrificed on day 13th.

Isolation of murine cells – Erythrocytes were purified in three different laboratories.
Cell preparation in laboratory A - Total blood was collected from deeply anaesthetized mice. Blood samples were obtained via left ventricular puncture. 1 ml blood was collected per animal in a syringe containing 200 µl Alsever´s solution (Sigma). Samples were transferred to 1.5 ml reaction tubes with 0.5 ml HEPES buffer (in mM NaCl 137, HEPES pH 7.4, 10, glucose 5.5, KCl 2.7, EDTA 1.0). The tubes were centrifuged at 100 x g for 10 min at 4°C. The supernatant and interphase were used for platelets and/or leukocytes preparation and the lower phase for erythrocyte isolation.
Erythrocytes preparation - Erythrocytes were resuspended in an appropriate volume (e.g. 1 ml) of HEPES buffer. The samples were then centrifuged at 200 x g for 5 min at 4°C. The supernatant was discarded and the centrifugation step was repeated 3 times. The final cell pellet was lysed in 800 µl of SDS-lysis buffer. The suspension was heated at 95°C for 10 min. Aliquots were either stored at –20°C or loaded on an 8% SDS-PAGE.
Platelet preparation - Supernatant from above was centrifuged at 200 x g for 10 min at RT. The supernatant was transferred to a clean test tube and centrifuged again at 1500 x g for 10 min at RT. The supernatant was discarded and the pellet was lysed in 100 µl SDS-lysis buffer. The samples were heated at 95°C for 10 min. Aliquots were either stored at –20°C or loaded on 8% SDS-PAGE.
Cell preparation in laboratory B - Blood was collected as described for laboratory A. Then, the samples were transferred into 500 µl PBS (*Mg- and Ca-free*). Erythrocytes from WT and cGKIα rescue mice were centrifuged differentially. Upon centrifugation at 100 x g, 10 min, 4°C, the supernatant was discarded. The pellet was resuspended in 1 ml HEPES buffer (in mM HEPES 20, NaCl 150, pH 7.4) and the centrifugation step was repeated 4 times. The supernatant was discarded and the pellet was diluted in HEPES buffer followed by determination of protein concentration. Samples were added with 6 x SDS-lysis buffer, heated for 5 min at 95°C and then stored at -20°C or loaded on a 12% SDS-PAGE.
Cell preparation in laboratory C - TER-119 positive cells were isolated by CeLLection™ Biotin Binder Kit (life technologies). 1 ml of blood was taken from heparinised WT mice (SV129 background) by puncture of the heart and washed according to the manufacturer's instructions. To each cell sample 10 µg of rat anti-mouse TER-119 biotinylated monoclonal antibody (R&D Systems) was added. After incubation for 20 min at 4°C on a rotating platform, cells were washed (PBS *(Ca^{2+}-, Mg^{2+}-free)*; 0.1% BSA; 2mM EDTA), resuspended and 25 µl of prewashed Dynabeads® were added. During 20 minutes of incubation the beads bound to the biotinylated TER-119 antibody and TER-119 positive cells could be positively isolated by a magnet. The cells were washed (PBS *(Ca^{2+}-, Mg^{2+}-free)*; 0.1% BSA)/magnetically separated at least three times to obtain a high purity of cells and resuspended in PBS (37°C). Then the Dynabeads® were removed from the erythroid cells by addition of 4 µl of Releasing Buffer (DNase I) and softly rotating and pipetting for 15 minutes. Dissolved cells were centrifuged and the obtained cell pellet was homogenized in lysis buffer (50 mM Tris-HCl, pH

7.5; 2%SDS) to release proteins. Proteins were denatured in SDS loading buffer (50mM Tris-HCl, pH 6.7; 1.25% SDS; 50mM DTT; 0.01% bromphenol blue; 10% glycerol). Each sample was boiled for 5 minutes at 95°C. Denatured proteins were separated on a 12% SDS-PAGE.

Immunoblotting of proteins - Isolated tissue was immediately frozen in liquid nitrogen and stored at -80°C until use. Extraction of the total protein was performed in lysis buffer [50mM Tris-HCl, pH 7.5; 2% SDS; protease inhibitor cocktail set V (Calbiochem)]. Proteins were denatured as described under cell preparation in laboratory C. Extracted proteins were separated on a 12% SDS-PAGE and transferred to PVDF membrane. Following antibodies were used: Rabbit anti-cGKI (1:200) [3], rabbit anti-IRAG (1:500) [3], rabbit anti-hemoglobin subunit α (1:100) (Abcam), rabbit anti-ferritin light chain (1:1,000) (Biotrend), rabbit anti-GAPDH (1:5,000) (Sigma-Aldrich), anti-PDE5 antibody (1:500) [4], anti-transferrin receptor (TfRc) (1:500) (Santa Cruz Biotechnology), anti-β-actin (1:8,000) (Abcam), and secondary anti-rabbit antibody (1:50,000) (Dianova).

Gastrointestinal blood - Haemoccult® test (Beckman Coulter) was used for detecting fecal occult blood derived from gastrointestinal bleeding. The test was performed according to the manufacturer's instructions. To collect the feces, 4- to 6 weeks old $cGKI^{-/-}$ mice, 7-12 weeks old $cGKI^{-/-}$ mice treated with PPI, and their respective wild type littermate controls were kept individually in cages for two hours and feces were collected. The same procedure was performed with cGKI RM mice (12-13 weeks old). The test was considered positive when three out of three independent measurements per mouse showed occult blood in the feces.

Histology with Perls Prussian Blue Staining - Tissues were fixed in 4% (weight/volume) paraformaldehyde in PBS overnight, dehydrated successively in an alcohol series with increasing concentrations and embedded in paraffin. For Prussian blue staining, 10µm tissue slices were incubated for 5 min in 10% (weight/volume) potassium hexacyanoferrate(II)/ ddH2O, followed by a 30 minutes incubation in 10% (weight/volume) potassium hexacyanoferrate(II) dissolved in 3.7% hydrochloric acid. Thereafter slices were rinsed in ddH2O and counter stained using 0.6% (weight/volume) safranine O in 66% ethanol. Blue-stained foci indicate ferric iron.

Determination of body and spleen weight - Mice were anesthetized with isoflurane and body weight was determined. After cervical dislocation spleen was dissected, rinsed in PBS and weighed. Ratio of spleen weight to body weight was compared between cGKI mutant mice and control littermates, each with or without PPI treatment.

Blood parameters

After heparinization (200 IU per mouse) and anesthesia with isoflurane, the heart was punctured and 50µl blood were collected in an EDTA-coated capillary and diluted 1:5 in a prefilled tube containing CELLPACK buffer and EDTA (Sysmex capillary tube). Diluted samples were used to determine blood parameters using the Sysmex XT2000iV blood cell counter as described previously [5].

Quantitative real-time PCR - Total RNA from liver was isolated using peqGOLD RNAPureTM (Peqlab Biotechnologie) according to the manufacturer's instruction. RNA purity and concentration were measured with Nanodrop 2000 Spectrophotometer (Thermo scientific). 1 µg RNA was transcribed into cDNA using SuperScript II Reverse Transcriptase (life technologies). Samples were diluted with RNase free water and quantitative real time PCR was performed with FastStart Universal SYBR Green Master Rox (Roche) and the specific primers for each target (listed in supplemental table 1). Abundance was calculated from the Ct (cycle threshold) values according to the following equation: $2^{-\Delta\Delta Ct}$, whereas GAPDH served as an internal control and "KO - PPI + NaCl" as calibrator sample.

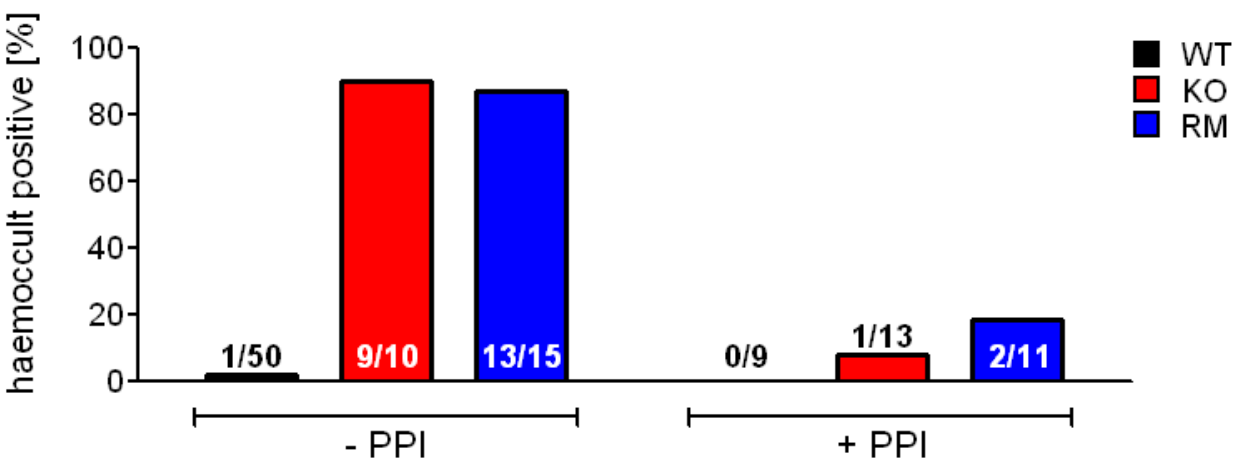
Plasma Iron - Blood was collected by puncture of the heart of heparinized and anaesthetized mice and centrifuged within 15 minutes (2000 x g). Reddish plasma samples (hemolysis) were discarded. QuantiChromTM Iron Assay (BioAssay Systems) was executed according to the manufacture's recommendations using an internal standard curve for each sample.

Interleukin-6 - Serum IL-6 measurements were performed using Quantikine murine IL-6 Immunoassay Kit (R&D Systems) according to the manufacturer's recommendations.

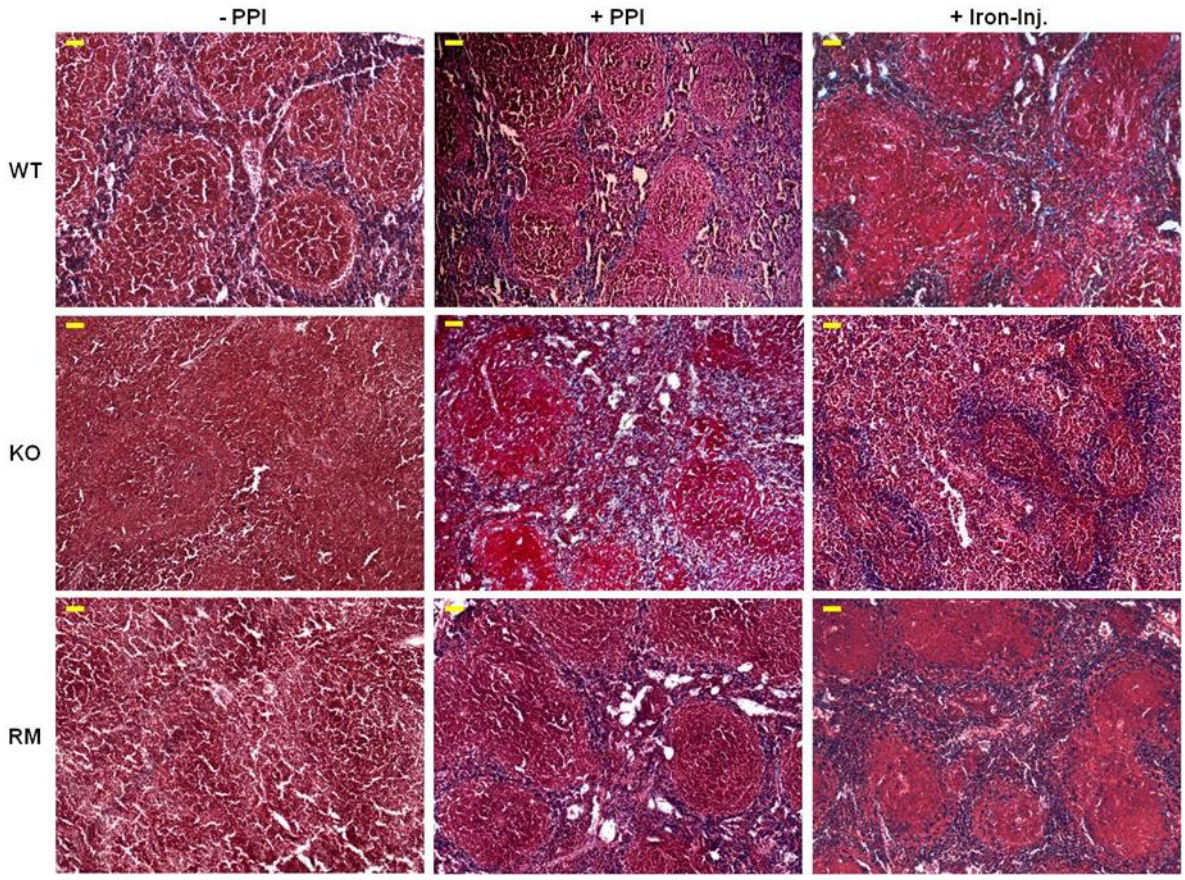
Statistical analysis - Results are presented as mean ± SEM. Statistical analysis of data was performed by ANOVA followed by Bonferroni post hoc test using Prism 5. Differences were considered significant at $p<0.05$

Supplemental Table 1
Primers for qRT-PCR and semi-quantitative PCR

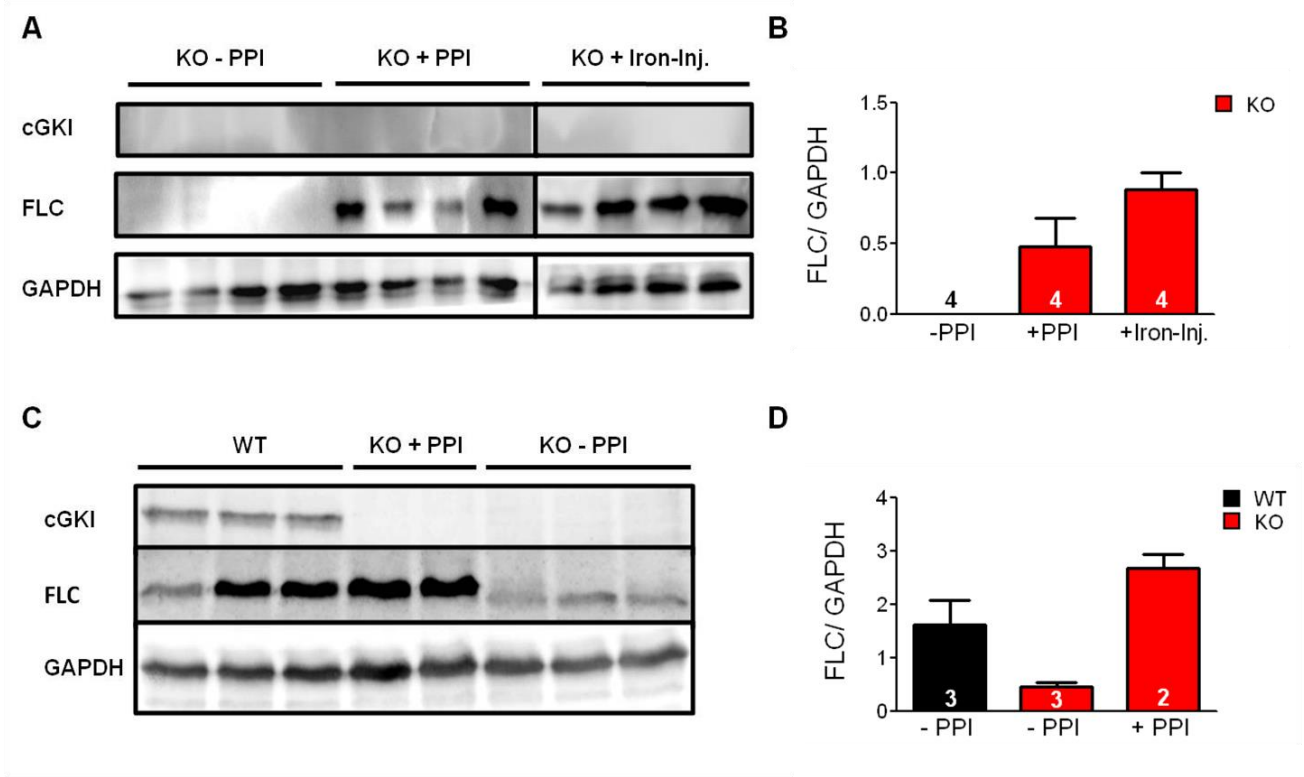
Gene	Forward (5'->3')- Primer	Reverse (5'->3')- Primer
HAMP	TGA GCA GCA CCA CCT ATC TC	ACA GCA GAA GAT GCA GAT GG
TfrRc	TAC CTG GGC TAT TGT AAG CG	TTT GAG ATC CAG CCT CAC G
GAPDH	CGG CAA ATT CAA CGG CAC AGT CA	GGT TTC TCC AGG CGG CAC GTC A



Supplemental Figure 1
Effect of PPI on intestinal bleeding.
Feces of 4 to 12 week old WT and cGKI$^{-/-}$ and cGKI RM mice were tested by the Haemoccult® test for blood. The numbers in the columns give the number of mice tested positive per total number of mice tested.



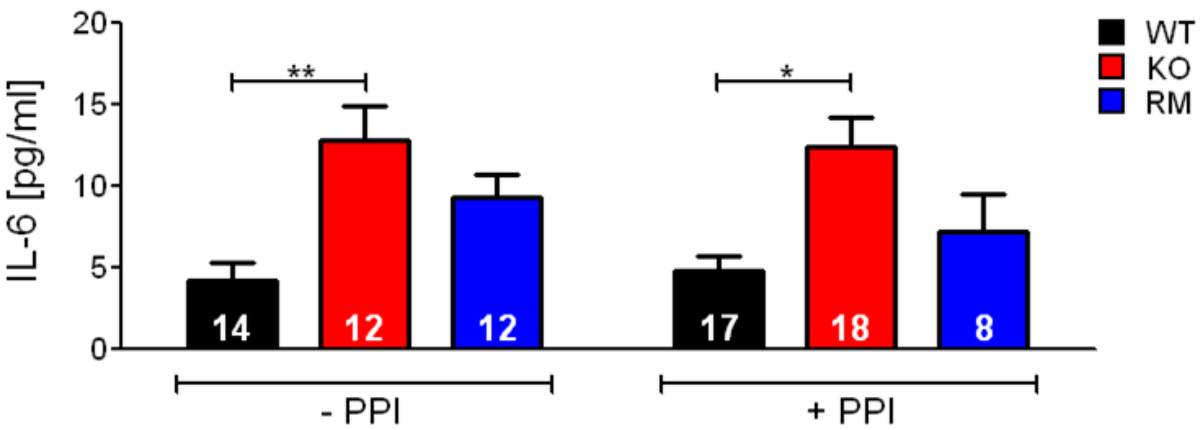
Supplemental Figure 2
Iron storage in spleen
Iron storage was visualized by Perls Prussian Blue Staining. Animals were not treated (-PPI) or treated with PPI (+PPI) or were ip injected with iron-(III)-hydroxid-sucrose-complex (see Methods). The size of the bar represents 100 µm. WT, wild type, KO, cGKI$^{-/-;}$ RM, cGKI RM.



Supplemental Figure 3

Western blot of spleen ferritin light chain in spleen (A+B) and liver (C+D)

A) cGKI$^{-/-}$ mice (KO) were not treated (-PPI) or treated with PPI (+PPI) or ip injected with Fe^{3+} (see Methods). 100 µg protein per slot of SDS denatured spleen proteins were separated on a 12% SDS-PAGE. GAPDH was used as loading control. B) Statistical summary of Western blots shown in A. C) WT and cGKI$^{-/-}$ mice (KO) were not treated (-PPI) or treated with PPI (+PPI). 120 µg protein per slot of SDS denatured liver proteins were separated on a 12% SDS-PAGE. GAPDH was used as loading control. D) Statistical summary of Western blots shown in C.



Supplemental Figure 4

Serum interleukin-6 concentration is not affected by treatment with PPI.

Serum interleukin-6 was measured without (-PPI) and with PPI treatment (+PPI). The number of mice is given within each column. **, P<0.01; *, P< 0.05

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