Ggct (γ-glutamyl cyclotransferase) plays an important role in erythrocyte antioxidant defense and red blood cell survival

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Summary

The expression of GGCT (γ-glutamyl cyclotransferase) is upregulated in various human cancers. γ-glutamyl cyclotransferase enzyme activity was originally purified from human red blood cells (RBCs), but the physiological function of GGCT in RBCs is still not clear. Here we reported that Ggct deletion in mice leads to splenomegaly and progressive anaemia phenotypes, due to elevated oxidative damage and the shortened life span of Ggct−/− RBCs. Ggct−/− RBCs have increased reactive oxygen species (ROS), and are more sensitive to H2O2-induced damage compared to control RBCs. Glutathione (GSH) and GSH synthesis precursor L-cysteine are decreased in Ggct−/− RBCs. Our study suggests a critical function of Ggct in RBC redox balance and life span maintenance through regulating GSH metabolism.

Keywords: γ-glutamyl cyclotransferase, reactive oxygen species, anaemia, glutathione, red blood cell, mouse model.

Background

GGCT (γ-glutamyl cyclotransferase) was also named C7orf24, and was reported to be upregulated in various human cancers, including bladder urothelial carcinoma,1 breast cancer,2 lung, oesophagus, stomach, bile duct and uterine cervix cancer.3 In 2008, Oakley et al. cloned the cDNA encoding human γ-glutamyl cyclotransferase enzyme activity from human red blood cells (RBCs), and this study identified C7orf24 as GGCT, an enzyme in the γ-glutamyl cycle.4 γ-glutamyl cyclotransferase catalyses the following reaction: γ-glutamyl–amino acid → 5-oxoproline + amino acid. The physiological function of this enzyme activity is not clear. Meister proposed that this enzyme is a critical component of the γ-glutamyl cycle, and is involved in glutathione (GSH) degradation and amino acid transport through the plasma membrane.5 In the γ-glutamyl cycle, extracellular GSH can be hydrolysed by membrane-bound γ-glutamyl transpeptidase (GGT) to cysteinyl-glycine and γ-glutamyl–amino acid dipeptide.6,7 In the cytoplasm, γ-glutamyl cyclotransferase cleaves the γ-glutamyl–amino acid to give 5-oxoproline and amino acid.5 Currently, the specific physiological function of the γ-glutamyl cycle has been debated.8 Recent studies also identified the GSH cytoplasmic degradation pathway through ChaC family proteins, the ChaC family of proteins function as γ-glutamyl cyclotransferases, which specifically degrade glutathione instead of other γ-glutamyl peptides.9 Till now, the physiological function of GGCT in mammals is still not clear.

To study the physiological function of GGCT, we reported the generation of the first Gct knockout mouse. Gct deletion is compatible with normal murine embryonic development.10 Here we show that young Gct−/− mice appear normal, while Gct−/− adult or old mice show splenomegaly, progressive anaemia and reduced physical activity phenotypes. The GGCT enzyme was originally purified from human RBCs, however the function of GGCT in RBC is still unknown. In the present study, we examined the in vivo function of Gct in RBC of Gct−/− mice, and found that Ggct plays a critical role in protecting RBC against oxidative stress in mice.

Materials and methods

Western blot

Spleen tissue was weighed and washed three times with cold PBS. Cell lysate buffer (20 Mm HEPES PH = 7.5; 150 Mm NaCl; 1%NP40) was added to spleen tissue and crushed using a homogeniser. The homogenate was passed through a 40 μm filter, and boiled with a protein-loading buffer. Anti-
GGCT antibody (ab198503, Abcam), Anti-β-actin antibody (AC-15, Sigma), were used for Western blot analysis. For chemiluminoenzcse, horseradish peroxidase-conjugated secondary antibodies and Western Lightining® Plus-ECL (NEL105001EA, PerkinElmer) were used.

**Gget**<sup>−/−</sup> mouse genotyping with PCR

Genomic DNA was obtained by the Mouse Direct PCR Kit (Bimake, B40013, Shanghai) from mouse tail. Gget<sup>−/−</sup> mice were genotyped with the following primers:

GGCT-ko-F: 5′-TGAGTCATAGATCTGACAGCAAGAG-3′
GGCT-ko-R: 5′-ATAACCCCTGTGAACCATCATTCA-3′

Predicted PCR product size for wild type allele is 994 bp, Gget<sup>−/−</sup> allele is 382 bp. Gget<sup>−/−</sup> mouse lines were generated by Shanghai Model Organisms Center, Inc. (SMOC). All mouse studies were carried out in strict accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the School of Life Science and Technology, ShanghaiTech University.

**Histological and haematological analyses**

Fifteen-week-old male and female mice were weighed and sacrificed. Selected organs, including spleen, liver, kidney, heart and lung, were removed and weighed to calculate an organ index (organ index = organ weight/body weight). For the histological study, spleens were fixed in 4% buffered neutral formalin, embedded in paraffin, and stained with haematoxylin and eosin. Tissue sections were examined with an Olympus VS120 microscope. Peripheral blood samples were drawn into EDTA-coated microtubes (IDEXX, 98-0010316-00, UK) by retro-orbital sinus bleeding and analysed with a Procyte Dx Veterinary Hematology Analyzer (IDEXX, B6972, UK). Blood smears were stained with Giemsa and analysed with Olympus BX53.

**Erythrocyte differentiation stage quantification by flow cytometry**

Mouse spleen and bone marrow cells were mechanically dissociated through a 70 μm strainer and washed with cold phosphate-buffered saline containing 2% fetal calf serum. Splenocyte single cell suspensions were double-stained with antibodies against fluorescein isothiocyanate-conjugated CD71 (CD71-FITC) and phycoerythrin-conjugated erythroid antigen (Ter119-PE). Flow cytometry was performed using a FACSCalibur.

**RBCs life span determination**

RBCs of wild-type or GGCT<sup>/−</sup> mice were labelled with 4 mmol/l CMFDA (Molecular Probes) which emits green fluorescence after cleavage by intracellular esterases. The labelled cells were injected into wild-type or Gget<sup>−/−</sup> recipient mice intravenously. Blood was collected at indicated time points, and CMFDA labelled cells were quantified by flow cytometry.

**Osmotic fragility assay**

RBCs were harvested using heparin-coated tubes, and then suspended in varying concentrations of NaCl. The samples were incubated at room temperature for 10 min and centrifuged at 1500 g for 10 min to remove unlysed cells and stromal cells. The absorbance of the supernatant was measured at 540 nm in a spectrophotometer. The lyses percentage of RBCs was calculated from the absorbance, and a fragility curve was generated by plotting varying salt concentrations versus haemolysis.

**Erythropoietin (EPO) quantification by enzyme-linked immunosorbent assay**

EPO level in blood plasma was determined using the mouse EPO enzyme-linked immunosorbent assay kit (Jiningshiye, A00895-2, Shanghai). Heparinised blood was collected from wild-type and Gget<sup>−/−</sup>, and blood samples were then centrifuged at 1000 g for 10 min to obtain the plasma. Of the plasma, 50 μl was taken for the experiment according to the manufacturer’s protocol.

**Metabolic cage experiment**

Wild-type and Gget<sup>−/−</sup> mice were individually housed in the sealed chambers of the Oxymax Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH, USA), each of which was equipped with an O<sub>2</sub> electrochemical sensor, a CO<sub>2</sub> infrared sensor and infrared beam activity sensors. The airflow rate was 0.5 l/min per cage. Mice were placed in the chamber one day prior to the start of measurements to allow for acclimation to the new environment. The metabolic data collected include the volume of O<sub>2</sub> consumed (V<sub>O2</sub>), volume of CO<sub>2</sub> generated (V<sub>CO2</sub>), respiratory exchange ratio (RER) (RER = V<sub>CO2</sub>/V<sub>O2</sub>) and heat produced. O<sub>2</sub> consumption and CO<sub>2</sub> production were measured over a two minute period, which was repeated every 10 min. V<sub>O2</sub> and V<sub>CO2</sub> values were normalised to the body weights of the mice (ml/kg/h). The infrared beam interruptions in both horizontal (X) and vertical (Z) directions were used to quantify the physical activity of mice. Any horizontal beam breakage was recorded as total physical activity count, as was any vertical beam breakage. During the recording, the mice were deprived of food, with free access to water.

**Metabonomics mass spectrometry**

Peripheral blood was drawn into heparin-coated tubes, and centrifuged at 4° 1000 g for 10 min. 100 μl plasma and blood cells were taken and 1 ml of MeOH: ACN (acetoni-trile) H<sub>2</sub>O (2:2:1, V/V) solvent mixture was added to the
sample and vortexed for 30 s, sonicated for 10 min and incubated for one hour at −20°C, centrifuged for 15 min at 13 000 rpm and 4°C. The supernatant was evaporated to dryness at 4°C using a vacuum concentrator. Add 100 μl of ACN: H2O(1:1/V/V) sonicate for 10 min, centrifuge for 15 min at 13 000 rpm and 4°C. Keep supernatant at −80°C prior to LC/MS analysis. Analyses were performed using a Waters Acquity I Class UPLC system connected to a Sciex tripleTOF mass spectrometer using electrospray ionisation. The compound was detected in positive and negative ion mode. Three microliters of samples were flow-injected by the autosampler onto a Waters Acquity UPLC BEH Amide column (1.7 μm, 2.1 × 100 mm). The mobile phase components consisted of (A) 20 Mm ammonium acetate, 20 Mm ammonium hydroxide and (B) CH3CN. The gradient profile used is detailed as the following: initial time, 5% A and 95% B; 0-5 min, 5% A and 95% B; 8 min, 35% A and 65% B; 9-5 min, 60% A and 40% B; 10-5 min, 60% A and 40% B; 11-5 min, 5% A and 95% B; 15-1 min, 5% A and 95% B. The flow rate was 0.45 ml/min. The mass spectrometry settings were as follows: Ion Source Gas 1 (GS1), 60; Ion Source Gas 2 (GS2), 60; Curtain Gas (CUR), 30; temperature, 500; IonSpray Voltage, 5-5 kV; Collision Energy, 40; data collection and analysis was performed using Sciex software.

**Intracellular ROS measurement**

The intracellular reactive oxygen species (ROS) were determined by carboxy-H2DCFDA (Aladdin, H131224-50 mg, Shanghai). RBCs were washed with cold PBS (PH = 7.0) and incubated with 10 μmol/l carboxy-H2DCFDA in the dark for 30 min at 4°C. Intracellular fluorescent products were measured immediately by flow cytometry.

**Erythrocyte oxidation parameters detection**

Erythrocyte ghosts were prepared according to a modification technique. In short, the haemolysis of RBCs occurs in the hypotonic solution, and ghosts can be obtained by centrifugation to remove haemoglobin and inclusions. The sulphhydryl content in the erythrocyte was measured with the ELLMAN method. The level of the carbonyl group in the protein was determined by DNPH (2,4-dinitrophenylhydrazine). The MDA (malondialdehyde) level is quantified with a MDA kit (Jianchen Bioengineering Institute, A003-1-2, Nanjing).

**Results**

**Ggt deletion in mice leads to splenomegaly and progressive anaemia**

Ggt-deficient mice were generated through embryonic stem cell targeting and blastocyst injection as described previously. Homozygous Ggt knock-out mice were genotyped by PCR (Fig S1A). The depletion of Ggt protein was confirmed by western blot analysis (Fig S1B). Although the Ggt−/− mice are viable and appear healthy, they were found to have splenomegaly (Fig 1A). The spleen was 1.8 times larger (Fig 1B) in the Ggt−/− mice than in the wild-type sibling controls. No significant difference in body weight was observed between Ggt−/− and the control littermates. Furthermore, the weight of the other major organs in Ggt−/− mice were not significantly changed compared to sibling controls (Fig S2). Haematoxylin- and eosin-stained sections of spleens from Ggt−/− mice revealed expanded red pulps (Fig 1C,D). Giemsa-staining of blood smears indicated dysmorphic red cells, such as stomatocytes in Ggt−/− mice (Fig 1E,F).

Haematological tests showed anaemia in Ggt−/− mice, as evidenced by the low erythrocyte number, low haemoglobin content, low haematocrit and increased reticulocyte count in Ggt−/− mice compared to wild-type mice (Fig 2A–C,E). Meanwhile, leucocyte counts were similar in wild-type and Ggt−/− mice (Fig S3). The anaemia phenotype is more severe in adult or older Ggt−/− mice, and the RBC differences are not significant in very young (less than three months old) Ggt−/− mice compared to sibling controls (Fig S4). Collectively, these data indicate that Ggt deficiency in mice leads to a progressive anaemia phenotype.

**Erythroid hyperplasia and effective erythropoiesis in Ggt−/− mice**

The anaemia phenotype observed in Ggt−/− mice could be due to defective RBC maturation (ineffective erythropoiesis), increased RBC destruction (haemolysis), or a combination of both processes. We analysed splenocytes by flow cytometry. A subpopulation of Ter119+ cells was distinguished based on their expression of the transferrin receptor (CD71), which decreases with erythroblast maturation. Using flow cytometry, we found that the proportions of erythroid precursor cells (Ter119+CD71+) in Ggt−/− mouse spleens were two-fold higher than that in wild-type mouse spleens (Fig 3A,B). In haematologic analysis, reticulocytes in Ggt−/− mice were higher than in wild-type mice (Fig 2E). The increased reticulocyte levels were in line with elevated plasma EPO protein in Ggt−/− mice (Fig S5). In addition, the number of erythroid precursor cells was higher in the bone marrow of Ggt−/− mice than in wild-type mice (Fig 3C,D), and the percentage of Ter119+CD71+ cells was increased 1.2-fold in the bone marrow of Ggt−/− mice, from 25-76% in wild-type mice to 28-72% in Ggt−/− mice. These data suggest that the erythroid hyperplasia phenotype of Ggt−/− mice could be due to a compensatory reaction to the decreased haemoglobin and haematocrit after Ggt deletion in mice.

**Intracellular ROS levels and oxidation damages are increased in Ggt−/− RBCs**

We measured intracellular reactive oxygen species (ROS) levels within RBCs with carboxy-H2DCFDA fluorescent dye.
Fig 1. Ggct deletion in mice leads to splenomegaly. (A) Representative images of six-month-old wild-type and Ggct−/− mouse spleen. (B) Spleen index (spleen weight/body weight) in wild-type and Ggct−/− mice. P value of Student’s t-test is shown. (C–D) Haematoxylin-and eosin-stained sections of spleen from wild-type (C) and Ggct−/− (D) mice. Ggct−/− mice show enlarged red pulp. Thick arrow indicates white pulp and thin arrow indicates red pulp. (E–F) Giemsa-stained blood smears from wild-type (E) and Ggct−/− (F) mice. Arrows indicate stomatocyte. [Colour figure can be viewed at wileyonlinelibrary.com]

Fig 2. Ggct−/− mice show anaemia phenotype. Haematological parameters of six-month-old wild-type and Ggct−/− mice are shown. (A) RBC, red blood cells; (B) HGB, haemoglobin; (C) HCT, haematocrit; (D) MCV, mean cell volume; (E) RET, reticulocytes. P values of Student’s t-test are shown. Error bars indicate mean ± SD.
Intracellular ROS concentrations measured under base-line conditions or after challenging with exogenous H$_2$O$_2$ (50 μmol/l) were elevated in Ggct$^{-/-}$ RBCs compared with wild-type RBCs (Fig 4A,B). One of the typical features of damaged RBCs is the presence of lipid peroxidation and protein carbonylation. Protein oxidation can be measured by classic biochemical methods for carbonyls that result from the reaction of side chains of lysine, proline, threonine, or arginine with ROS. In agreement with the observed elevation in ROS concentrations, oxidised protein levels in Ggct$^{-/-}$ RBCs were markedly increased (1.5-fold) as measured by 2,4-dinitrophenylhydrazine-derivatised carbonyl (Fig 4C). Since thiol groups are known to be easily oxidised by an attack of ROS, we then monitored the thiol group of cellular proteins by 5,5'-DiThiobis-2-NitroBenzaic acid (DTNB). The results show that the thiol group levels of membrane protein were decreased in Ggct$^{-/-}$ RBCs compared with wild-type RBCs (Fig 4D). In addition, we found that lipid peroxidation measured by MDA content was upregulated in Ggct$^{-/-}$ RBCs compared with wild-type RBCs (Fig 4E). These data suggests that Ggct$^{-/-}$ RBCs are more susceptible to ROS, and suffer from significant oxidative damage, as evidenced by increased protein carbonyl groups and lipid peroxidation, and decreased thiol groups in Ggct$^{-/-}$ RBCs compared to wild-type RBCs.

**Ggct$^{-/-}$ RBCs show decreased life span**

The normal life span of circulating RBCs is determined by their clearance from the peripheral circulation (predominantly by the spleen). We have shown that Ggct-deficient erythrocytes are more susceptible to ROS; we therefore examined whether a Ggct-deficiency could affect RBC survival and whether the observed anaemia was due to increased destruction of RBCs in the circulation *in vivo*. To this end, mice were infused with CMFDA labelled wild-type or Ggct$^{-/-}$ RBCs, and cell life span was determined by flow cytometric analysis of circulating labeled RBCs. We
observed that labelled \(Ggct^{-/-}\) RBCs disappeared more rapidly than wild-type RBCs (Fig 5A), suggesting a faster clearance and a shorter lifespan of \(Ggct^{-/-}\) RBC. To further demonstrate the destruction of erythrocytes in \(Ggct^{-/-}\) mice, we performed a blood cross-transfusion experiment. Wild-type mice received either CMFDA-labeled wild-type erythrocytes (WT-WT) or \(Ggct^{-/-}\) erythrocytes (KO-WT), and \(Ggct^{-/-}\) mice received either CMFDA-labeled wild-type erythrocytes (WT-KO) or \(Ggct^{-/-}\) erythrocytes (KO-KO). The result demonstrated that the KO-WT group had a higher clearance rate of infused erythrocytes than the WT-WT group, and similar results were observed when comparing the KO-KO with the WT-KO group (Fig 5B), suggesting an accelerated clearance of \(Ggct^{-/-}\) erythrocytes. In line with the increased clearance rate, we observed that \(Ggct^{-/-}\) RBCs were significantly less resistant to hypotonic lysis than wild-type RBCs (Fig 5C).

To explore the physiological consequence of \(Ggct\)-loss induced anaemia in mice, we performed metabolic cage experiments on \(Ggct^{-/-}\) mice and wild-type mice. The results showed that the physical activity of \(Ggct^{-/-}\) mice was significantly reduced compared to wild-type mice (Fig S6A,B); particularly during the dark phase (Fig S6C). Energy expenditure, measured as \(O_2\) consumption and \(CO_2\) production, was markedly reduced in \(Ggct^{-/-}\) mice (Fig S6D). Heat production, an indicator of the metabolic rate, was also reduced significantly in \(Ggct^{-/-}\) mice (Fig S6F). Mice of both genotypes exhibited a similar respiratory exchange ratio (RER) (RER = \(V_{CO_2}/V_{O_2}\)) (Fig S6E), indicating that the loss of \(Ggct\) did not alter fuel preference.

**Metabolomics analysis in wild-type and \(Ggct^{-/-}\) RBC**

We compared the contents of small molecules in wild-type and \(Ggct^{-/-}\) RBCs with metabonomics mass spectrometry. The results show that compared to wild-type mice, the content of \(GSH\) in RBCs is decreased, and the precursor molecule for \(GSH\) synthesis, such as L-cysteine, is also reduced in \(Ggct^{-/-}\) RBCs (Fig 5D). The reaction product of \(\gamma\)-glutamyl cyclotransferase enzyme activity is 5-oxoproline, and its concentration is also decreased in \(Ggct^{-/-}\) RBCs (Fig 5D). GGCT could affect cellular L-cysteine content through regulating amino acid transport during the glutathione cycle.\(^{18}\) L-cysteine is the rate-limiting substrate in glutathione synthesis,\(^{19}\) and its downregulation can lead to the downregulation of glutathione in \(Ggct^{-/-}\) RBCs.
In summary, our data indicate that a Ggct deficiency affects the metabolic balance of GSH-ROS in RBCs, results in the upregulation of the ROS level, thus affecting the lifespan and the physiological function of RBCs. Splenomegaly and the reduced physical activity phenotypes observed in Ggct/C0/C0 mice could be due to the antioxidant defect of Ggct/C0/C0 RBCs (Fig 5E).

**Discussion**

In this study, we provide the first evidence to suggest that Ggct is required for mouse RBC life span maintenance and antioxidant defense. The progressive anaemia and reduced physical activity phenotypes of Ggct/C0/C0 mice could be due to the defect in Ggct/C0/C0 RBC. This conclusion was supported by several lines of evidence. First, the rate at which labeled erythrocytes were eliminated from the circulation was markedly higher in Ggct/C0/C0 mice than in wild-type littermates (Fig 5A). Consistent with this result, reticulocytes were increased in Ggct/C0/C0 mice (Fig 2E), and the increased reticulocyte levels correlate with elevated plasma EPO protein (Fig 3E). In addition, Ter119^CD71^ erythroblasts in the spleen and bone marrow were also markedly expanded (Fig 2A–D).

The abnormal structure and deformability of the erythrocyte membrane plays an important role in the shortened erythrocyte survival in many types of haemolytic anaemia. Consistent with this interpretation, we found the level of ROS in erythrocytes of Ggct/C0/C0 mice was upregulated, resulting in the aggravation of erythrocytes’ oxidative damage. In accordance with this result, erythrocyte membrane proteins and lipids in Ggct/C0/C0 mice were oxidised, leading to a significant decrease in osmotic fragility, an indication of increased rigidity of erythrocytes. As a result, the life span of erythrocytes in Ggct/C0/C0 mice is shorter than wild-type littermates. This phenotype is similar to that observed in mice deficient
for the antioxidant enzymes, such as AMPKα1,21 Nix17 and prx II.22

GSH is synthesised in the cytoplasm, and the availability of L-cysteine is the key determinants of GSH biosynthesis.19 Before the identification of ChaC family proteins as the cytosolic pathway for glutathione degradation in mammalian cells,9 GSH was thought to be degraded exclusively in the extracellular space by membrane-bound γ-glutamyl transpeptidase (GGT) to cysteinyl-glycine and γ-glutamyl-amino acid dipeptide.23 One of the best acceptor amino acids for GGT enzymatic reaction is L-cysteine.18 In the absence of Ggt, intracellular GSH level is downregulated due to the decreased availability of intracellular L-cysteine.24,25 Based on our experimental data, Ggt deficiency also leads to decreased intracellular L-cysteine and consequently GSH downregulation. Thus membrane-bound Ggt and cytoplasmic Ggct could function together in GSH homeostasis through recycling L-cysteine.

In summary, using the Ggt−/− mouse model, we demonstrate a critical function of Ggct in the GSH metabolism, antioxidant defense and RBC life span maintenance. The progressive anaemia and reduced physical activity phenotypes of Ggt−/− mice could be due to the defects in Ggct−/− RBC.

Author contributions
ZH, XS, DB, XZ maintained the mouse lines and performed the experiments; SW participated in critical project discussions; ZH, YH, PH performed the metabolomics experiments; XSL designed, supervised the study and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Supporting Information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Ggt−/− mouse genotyping. (A) PCR-genotyping results of Ggt homozygous (−/−), Ggt heterozygous (+/−), and wild-type mouse (+/+). (B) Western blot analysis of Ggt expression in the spleen from wild-type and Ggt−/− mice.

Fig S2. Organ index. No significant changes of heart index, liver index, lung index and heart index in Ggt−/− mice compared to wild-type mice. The data statistically analysed by t-test. Error bars indicate mean ± SD.

Fig S3. Haematological parameters: analysis of leucocyte in mouse peripheral blood. (A) WBC, white blood cell. (B) NEUT, neutrophil. (C) LYMPH, lymphocyte. (D) MONO, monocyte. (E) EO, eosinophilia. (F) BASO, basophil.

Fig S4. Haematologic parameters of young (<15 weeks of age) Ggt−/− and wild-type mice. (A–E) Selected blood routine parameters. (A) RBC, red blood cells; (B) HGB, haemoglobin; (C) HCT haematocrit; (D) MCV mean cell volume; the data statistically analysed by t-test.

Fig S5. EPO quantification in wild-type and Ggt−/− mice. EPO protein was quantified from wild-type and Ggt−/− mouse plasma. P values of Student’s t-test are shown. Error bars indicate mean ± SD.

Fig S6. Ggt−/− mice show a reduced physical activity phenotype.

References
15. Dong HY, Wilkes S, Yang HS. CD71 is selectively and ubiquitously expressed at high levels in erythroid precursors of all maturation stages: a