Extracellular glycine is necessary for optimal hemoglobinization of erythroid cells

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Supplemental Information

Supplemental Methods

Blood analyses

Blood of newborn pups was analyzed shortly after birth. Neonates were decapitated with sharp scissors and 10-20 µl blood collected by capillary blood tube (Vacutainer™; Becton Dickinson, Franklin Lakes, NJ, USA). Similarly, blood of adult animals was collected from the saphenous vein. Samples were analyzed on a hematology analyzer (Scil Vet ABC™; scil animal care company; Gurnee, IL, USA).

Culture of primary mouse erythroblasts

Erythroid cells were isolated and cultivated as previously described.(1, 2) Briefly, cells were grown from fetal livers obtained from E12.5-13.5 embryos and cultured in serum-free StemPro-34™ medium plus Nutrient Supplement (Invitrogen-GIBCO, Carlsbad, CA) plus 2 U/mL human recombinant erythropoietin (EPO; 100 ng/mL), murine recombinant stem cell factor (100 ng/mL), the synthetic glucocorticoid dexamethasone (Dex; 10^{-6}M) and insulin-like growth factor 1 (40 ng/mL). The cell number and the size distribution of cell populations were monitored daily in an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany). Dead or differentiating cells were removed by Ficoll purification.

To induce terminal differentiation, continuously self-renewing erythroid progenitors were washed twice in PBS and seeded in StemPro-34^{TM}, containing 10 U/mL EPO, insulin ($4x10^{-4}$ IE/mL), the Dex antagonist ZK-112993 ($3x10^{-6}$ M)(3) and iron-saturated human transferrin (Fe_2-Tf; 1 mg/mL=12.5 µM=25 µM Fe; Sigma, St. Louis, MO).

Giemsa Staining

Fetal livers isolated from GlyT1$^{+/+}$, GlyT1$^{+/-}$ and GlyT1$^{-/-}$ fetuses had their morphology accessed by optical microscopy after Giemsa staining following previously described protocol (4).

Isolation of Ter119^{-}- and Ter119^{+}-cells using magnetic beads

Femora and humeri from wild type mice were collected and their bone marrows extracted. Bone marrow cells were resuspended in Iscove's medium (Invitrogen, Burlington, Ontario) with 2% bovine serum and passed through a 40 μM cell strainer (BD Falcon, Becton Drive, USA). Cells were washed in PBS containing 0.5% bovine serum, and then re-suspended in PBS with 0.2% bovine serum. Cells were incubated with Ter119-microbeads antibody (Miltenyl Biotec, Cambridge, USA) following manufacturer's instructions. Labelled cells were them loaded onto LS magnetics columns (Miltenyl Biotec, Cambridge, USA) and separated using MidiMACS^{TM} Separator (Miltenyl Biotec, Cambridge, USA) following manufacturer's instructions.

Glycine uptake and its incorporation into heme

Cells were incubated with 4-5 μCi of [2-^{l4}C]glycine (100 μCi/ml; NEN, Boston, MA, USA) for 1h, following which cells were washed three times with 1 ml PBS. Heme was extracted as previously described (5). Briefly, cells were lysed in 500 μL distilled water and subjected to one freeze-thaw cycle; 1 N HCl was then added to adjust the pH to 2. [^{14}C]Heme was extracted from hemoglobin by the addition of an equal volume of ice-cold methylethylketone (6). After centrifugation for 5 min at 1,200 x g, an aqueous lower phase containing proteins and a ketonic upper phase comprising heme were separated and placed into different tubes. To make sure that all hemoglobin heme was completely extracted, the extraction step was repeated and both

ketonic phases were pooled before the ^{14}C-radioactivity was determined with a liquid scintillation counter (Packard Cobra; PerkinElmer, Santa Clara, USA). In order to avoid quenching of scintillation, the solutions of heme were decolorized in scintillation vials with 300 µL 30% H_2O_2 and then mixed with 10 mL scintillation cocktail (Ecolite plus; MP Biomedicals; Santa Ana, California, USA). We also measured the ^{14}C-radioactivity of the aqueous phase; this plus [^{14}C]heme radioactivity represents the total cellular [2-^{14}C]glycine uptake.

Thiazole orange staining of reticulocytes

Staining of reticulocytes with thiazole orange (TO; Sigma, St. Louis, MO, USA) was performed as described by Lee *et al.*(7) The stock solution (1 mg/ml) of TO was prepared in methanol and diluted 1:20,000 in PBS shortly before measurement. 3 µL freshly drawn whole blood was transferred from an anticoagulation tube to 2 ml PBS/TO and incubated at room temperature for 30 min before analysis by flow cytometry.

Real time PCR (qRT-PCR)

Total RNA was isolated from cells using an RNeasy® kit (Qiagen, Toronto, ON, Canada) following the manufacturer's instructions. Single stranded cDNA was synthesized using the qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, USA). qRT-PCR reactions were performed using the qScript™ One-Step SYBR® Green qRT-PCR Kit, Low ROX™ (Quanta Biosciences, Gaithersburg, USA) and the following primers: GlyT1 forward 5'CTGTCTCATCCGAGGAGTCAAG 3', GlyT1 reverse 5'TGATACCCGTGAAGGCTCCT-TC 3', Globin forward 5'TGTGTTGACTTGCAACCTCAG 3', Globin reverse 5'GCAGAGGA-

TAGGTCTCCAAAGC 3', β-Actin forward 5'AGCCATGTACGTAGCCATCC 3' and β-Actin reverse 5'TGATGTCACGCACGATTTCC 3'.

qRT-PCR reactions were amplified using the 7500 Fast Real-Time PCR System (Applied Biosystems®, Streetsville, Canada) and data analysis was performed with 7500 Software v2.0.5 (Applied Biosystems®, Streetsville, ON, Canada). SEMs are based on nine samples (three technical replicates from three biological replicates) and the $2^{-\Delta\Delta CT}$ formula was used to compare samples (8).

Western blotting

Cells were harvested and lysed using Munro's lysis buffer (10 mM Hepes [pH 7.6], 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, and 0.2% NP-40). Protein content was determined using Bradford reagent (BioRad, Mississauga, Canada). 30 μg of protein was resolved on a 15% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. Membranes were blocked with blocking solution (5% milk powder or 5% albumin in TBS/0.2% Tween 20). They were then incubated overnight with the indicated primary antibodies against β–actin (Sigma-Aldrich Inc, Oakville, Canada; 1:5000) and GlyT1 (Abcam, Cambridge, United Kingdom; 1:500). After washing, membranes were incubated with the appropriate secondary antibody, mouse or rabbit horseradish peroxidase–coupled anti-IgG antibody (Jackson Laboratories, West Grove, USA) in a 1:20,000 dilution in blocking solution for 1h at room temperature. The western blot was developed using HyBlot CLTM autoradiography film (Denville Scientific Inc, Metuchen, USA). Results shown were obtained from 3 mice for each group (n=3).

Measurement of ^{59}Fe in heme and non-heme fractions

Measurements of ^{59}Fe in heme and non-heme fractions were carried out as described previously by an acid precipitation method (9, 10). Briefly, cells were collected by centrifugation (4,000 x g for 30 s), lysed in water and boiled in 1 ml 0.2 M HCL; the samples were then transferred to an ice-bath and ^{59}Fe-heme-containing proteins were precipitated with ice-cold 7% trichloroacetic acid (Bioshop Canada Inc, Burlington, ON, Canada) solution, for 2 h, and collected by centrifugation (2,800 x g for 5 min at 4°C). Precipitated proteins (containing ^{59}Fe in heme) and supernatants (containing non-heme ^{59}Fe) were collected and placed into different tubes. Measurements of ^{59}Fe radioactivity in heme and non-heme fractions were carried out in a Packard Cobra gamma counter (PerkinElmer, Santa Clara, CA, USA).

Cell membrane transferrin receptor detection

Non-differentiated and differentiated cells were washed twice with PBS containing 2% fetal calf serum and stained with fluorescence-labeled antibodies against transferrin receptor (TfR) (FITC; PharMingen, San Diego, CA, USA) (11). Surface marker expression was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Mississauga, ON, Canada).

Supplemental References

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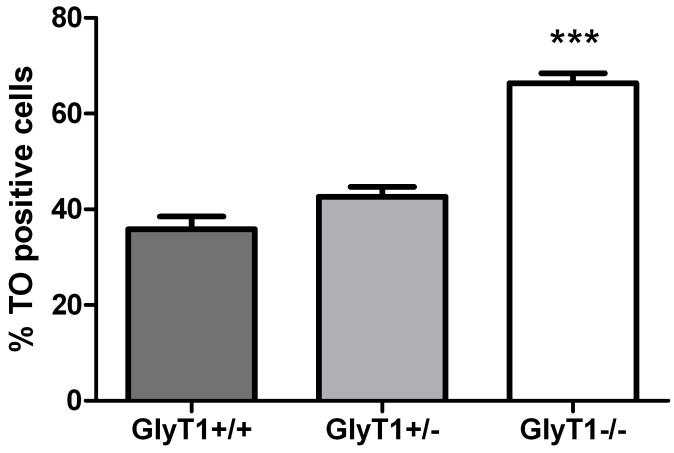
Supplemental Figure Legends

Supplemental Figure 1. Blood derived from GlyT1$^{-/-}$ newborns exhibits elevated levels of reticulocytes. (A) Blood samples from newborns were collected and stained with thiazole orange followed by flow cytometry analysis. Values are presented as percentages of reticulocytes in blood, n ≥ 5 per genotype; (B) Red blood cell distribution width (RDW) measurement obtained from newborn blood, n ≥ 14 per genotype. Statistical analysis was done by using one way ANOVA followed by Bonferroni's multiple comparison test; ***p<0.001.

Supplemental Figure 2. GlyT1$^{+/+}$ and GlyT1$^{+/-}$ erythroblasts treated with GlyT1 specific inhibitor, Sarcosine, exhibit significantly reduced ^{59}Fe-Tf incorporation into heme. Erythroid cells isolated from GlyT1$^{+/+}$, GlyT1$^{+/-}$ and GlyT$^{-/-}$ embryos were differentiated during 48 h and, in the last 24 h, incubated with 4 mM sarcosine. Statistical analysis was done by using one way ANOVA followed by Dunnett's multiple comparison test in relation to the respective GlyT$^{+/+}$ sample (n=3); **p<0.01.

Supplemental Figures

A



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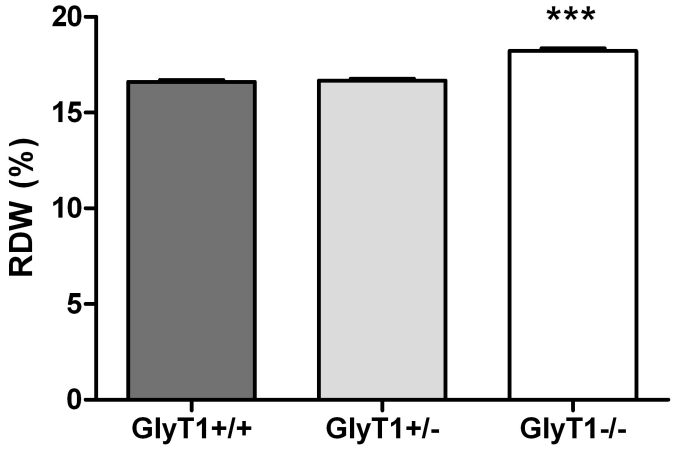
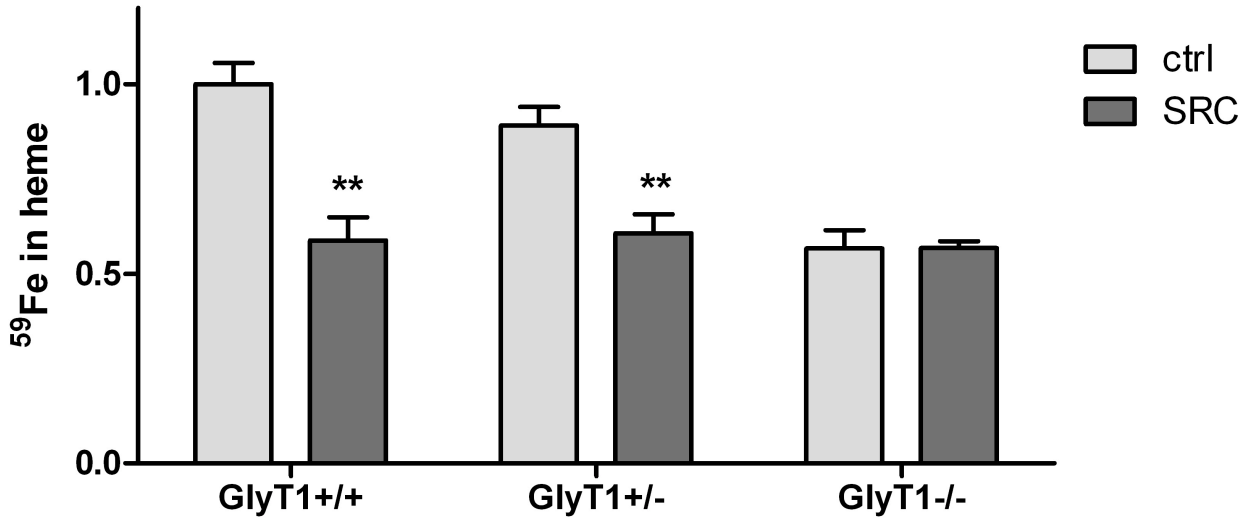


Supplemental Figure 1



Supplemental Figure 2