SUPPLEMENTARY APPENDIX

Resveratrol accelerates erythroid maturation by activation of FoxO3 and ameliorates anemia in beta-thalassemic mice

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SUPP LEMENTARY DATA

SUPPLEMENTAL MATERIALS AND METHODS

Drugs and chemicals. NaCl, KCl, Na_2HPO_4, Na_3VO_4, KH_2PO_4, $MgCl_2$, NH_4HCO_3, MOPS, HEPES, TRIS, N-ethylmaleimide (NEM), choline cloride, benzamidine, β-mercaptoethanol, glycine, bromphenol blue, trypsin, sodium dodecil sulphate (SDS) and glycerol were obtained by Sigma/Aldrich (St Louis, MO, USA); protease inhibitor cocktail tablets were from Roche (Basel, Switzerland); Triton X-100, ECL-Plus, Percoll were purchased from GE Healthcare (Little Chalfont, UK); 40% Acrylamide/Bis Solution, 37.5:1 was from BIO-RAD (Hercules, CA, USA).

In vitro erythropoiesis from CD34$^+$ cells from peripheral circulation of normal and β-thalassemia-intermedia subjects

Cell culture, phenotypic and cell sorting strategy.

Light-density mononuclear cells were obtained by centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient, as previously described (11, 17). The CD34$^+$ cells were positively selected by anti-CD34-tagged magnetic beads (Mini-MACS columns; Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. The recovery was more than 90% CD34$^+$ cells, as determined by flow cytometry. CD34$^+$ cells were grown at a density of 10^5 cells/mL in alpha-minimal essential medium (α-MEM; GIBCO, Grand Island, NY) supplemented with 100 U/mL penicillin-streptomycin, 2 mmol/L L-glutamine, 10^{-6} mol/L hydrocortisone, 10^{-3} g/L nucleotide, $25x10^{-3}$ mg/L gentamicin, 10^{-4} mol/L 2-mercaptoethanol, 1% deionized bovine serum albumin [BSA] (all from Sigma, St Louis, MO), 30% fetal bovine serum (FBS, GIBCO, Grand Island, NY), 1 μg/mL Cyclosporin A (Sigma, St Louis, MO). The following recombinant cytokines were added to the media: 3 U/mL recombinant human (rH) erythropoietin (rHuEPO, Janssen-Cilag, Milan, Italy), 20 ng/mL rH stem cell factor (SCF, PeproTech, London, UK), 10 ng/mL rH interleukin-3 (IL-3, PeproTech, London, UK). In control cell cultures, cell samples were collected at day 7, 9, 11 and 14 of culture for cell counting and determination of cell viability. In β-thalassemic cell cultures, cell samples were collected at days 7 and 14 for cell counting and determination of cell viability. Cell morphology was analyzed on cytospin smears stained with May-Grunwald-Giemsa.

Resveratrol (5 μM; Sigma Aldrich, St. Louis, MO, USA) was added at days 1 and 7 of phase 1 of the cell cultures, when a change in liquid culture medium is required to induce terminal differentiation of erythroid precursors. Then, resveratrol was added to culture media at days 9, 11 and 13 of phase 2 of the cell cultures to ensure the continuous presence of active compound during

later stages of erythroid development since its effects have been described as persisting for only 48 hours (8).

The erythroid cell antigen profile was analyzed using the recently reported cytofluorimetric strategy with the following surface markers: CD71 (anti-CD71 fluorescein isothiocyanate (FITC)-conjugated; BD Biosciences, San Jose, CA, USA), glycophoryin A (GPA, phycoerythrin (PE)-conjugated anti- CD235a; BD Biosciences, San Jose, CA, USA) and CD36 (anti-CD36, allophycocyanin (APC)-conjugated; BD Biosciences, San Jose, CA, USA) (22). All the analyses were performed with the flow cytometer FACSCanto™ (Becton Dickinson, San Jose, CA, USA). Data were stored and processed using FACSDiva software (Becton Dickinson Immunocytometry System, San Jose, CA, USA). The biparametric scatter plots were analyzed with FlowJo software version 7.6.4 (Tree Star, Ashland, OR, USA). Unstained cells were used as a negative control. CFU-E, Pro-E Int-E and Late-Erythroblasts were sorted as reported by Merry-Weather Clarke et al. using a FACSAria™ II instrument (Becton Dickinson, San Jose, CA, USA) (23). Cell apoptosis was determined on sorted CFU-E cells by double-staining the cells with FITC-conjugated Annexin-V and PI. The human Annexin-V-FITC Apoptosis Detection Kit (Bender Medsystems, Vienna, Austria) was used, according to the manufacturer's instructions.

Quantitative-Real time PCR. For quantitative real time (qRT)-PCR mRNA was isolated and reverse transcribed into high-purity cDNA using μMACS One-step cDNA Kit according to the manufacturer's instructions (Miltenyi Biotech). We started from either 250,000 sorted human CFU-E cells or sorted basophilic erythroblasts or polychromatic erythroblasts from bone marrow of both mouse strains with and without resveratrol. 1/50th of the reactions were added to appropriate wells of the PCR plates. qRT-PCR was performed by SYBR Green PCR Master Mix (Applied Biosystems) by using Applied Biosystems Model 7900HT Sequence Detection System. Detailed methods and primer sequences are available on request and Table 1S and 2S. All PCR reactions were performed in triplicate. Relative gene expression was calculated by using the $2^{-\Delta Ct}$ method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches the detection threshold (24). The ΔCt was computed by calculating the difference of the average Ct between the X-gene and the internal control GAPDH. The data are presented as mean ± the standard deviation (SD).

Immunofluorescence assay for FoxO3a and immunoblot analysis of FoxO3a. 250.000 sorted CFU-E cells at day 7 were obtained from control and at days 7 and 11 from β-thalassemic cultures. Cells were cytospun onto glass slides and fixed with 4% paraformaldehyde. After washing with PBS, cells were permeabilized using 3% bovine serum albumin, 0.25% Triton X-100 and then

incubated in 1% bovine serum albumin overnight with the anti-FoxO3a (Millipore, Temecula, CA USA). Cells were washed and stained with Alexa Fluor 594 anti-rabbit IgG antibody (Molecular Probes). Nuclei were stained with Vectashield-DAPI (Vector Laboratories). Images were captured using Nikon EclipseE600 microscope. Quantification was performed using J software. Whenever indicated sorted CFU-E were separated in cytosol and nuclear fractions as previously reported (25).

Immunoblot analysis of sorted erythroid precursors. 1 x 10^6 sorted CFU-E cells at 7 days of culture normal and at 7 and 11 days of culture of β-thalassemic cultures were solubilized as previously described (11, 17). Proteins were separated by monodimensional electrophoresis and transferred to membrane for immunoblot analysis with specific antibodies against Akt (Millipore, Temecula, CA USA), phospho-Akt (p-Akt, Millipore, Temecula, CA USA); FoxO3a (Millipore, Temecula, CA USA), Tubulin (University of Colorado, Boulder, CO USA) and Histone-H3 (Cell Signaling) were used as loading control. Whenever indicated erythroid population as CD36+, CD71 + cells were sorted to carry out the analysis of band 3 expression. The cells were solubilized as reported above and analyzed by immunoblot using anti-band 3 (IVF12, ML Jennings, University of Arkansas for Medical Science, USA) and anti-actin (Sigma, St Louis, MO) as loading control. We carried out the densitometric analysis of the scanned images of unsaturated films (ImageJv 1.28 software).

Immunofluorescence assay for Foxo3a. 250.000 sorted CFU-E at each time point for control and β-thalassemic cultures were cytospun onto glass slides and fixed with 4% paraformaldehyde. After washing with PBS, cells were permeabilized using 3% bovine serum albumin, 0.25% Triton X-100 and then incubated in 1% bovine serum albumin overnight with the anti-FoxO3a (Millipore, Temecula, CA USA). Cells were washed and stained with Alexa Fluor 594 anti-rabbit IgG antibody (Molecular Probes). Nuclei were stained with Vectashield-DAPI (Vector Laboratories). Images were captured using Nikon EclipseE600 microscope. Quantification was performed using J software.

Studies on mouse models

Preliminary experiments showed that low dose resveratrol has limited hematological effects on older β-thal mice or for shorter periods of treatment of 1 or 2 months. Mouse behavior and weight were monitored monthly.

Hematological parameters and red cell indices. Blood was collected by retro-orbital venipuncture in anesthetized mice using heparined microcapillary tubes. Hematological parameters were evaluated on a Bayer Technicon Analyser ADVIA. Hematocrit and hemoglobin were manually determined as previously described (16, 29-32).

Cytofluorimetric analysis of mouse bone marrow precursors and immunoblot analysis of sorted murine erythroblasts. The cyto fluorimetric analysis of erythroid precursors from bone marrow of both wild-type and was carried out as previously described using CD44-FITC (eBioscience, San diego, CA USA), TER-119 APC (eBioscience, San diego, CA USA) strategy (24, 33). Population II and III corresponding to basophilic and polychromatic erythroblasts ($1X10^6$) were sorted from bone marrow of both wild-type and β-thalassemic mice with and without resveratrol supplementation as previously reported (20, 33). Cells were used for RT-PCR analysis (see above). In sorted polychromatic erythroblasts Prdx2 expression was evaluated by immunoblot analysis using the specific antibody against Prdx2 C-terminal as gently gifted by prof. Chae HZ, Chonnam National University, Korea).

Red cell survival. Red cells from wild-type and β thalassemic mice treated with or without resveratrol were labeled with CFSE (10μM; Molecular probe, Invitrogen) in PBS, BSA 0.5% fro 20 min at 37°C. After quencing with PBS in presence of FBS 1%, red cells washed 3 times with sterile PBS and resuspended at 2×10^9 cells in 250 μL of sterile PBS. Wild-type and β thalassemic mice were injected intravenously with the CFSE-labeled red cells, the kinetics of disappearance of labeled cells from circulation was measured by flow-cytometry (34).

Red cell ghost preparation and immunoblot analysis. Red cell ghosts from red cells of resveratrol treated and untreated mouse groups were prepared as previously described (30, 35). Whenever PRDX2 was evaluated in one-dimensional (1D) SDS-PAGE analysis, 100 mM of NEM was added to the lyses buffer to avoid possible artifacts due to PRDX2 oxidation after cell lysis. For mono-dimensional electrophoresis proteins from ghosts and cytosol fraction were solubilized in non-reducing sample buffer (SB: 50 mM Tris pH 6.8, 2% SDS, 10% glycerol, a few grains of bromphenol blue). Gels were transferred to nitrocellulose membranes for immuno-blot analysis with specific anti-Peroxiredoxin-2 antibody (AbCam, Cambridge, UK) and anti-actin (Sigma Aldrich, USA). Blots were developed using chemiluminescence reagents (ECL, Amersham).

Red cell membrane carbonyl groups. Carbonyl- groups of red cell membranes were determined as reported with slight modifications (36). Approx. 20-50 ml of membrane suspension was diluted to 1 ml with 2M HCl and treated with 40 ml of 5 mM 2,4-dinitrophenylhydrazine (2,4-DNPH) (final concentration 0.2 mM) in 2M HCl for one hour at room temperature to allow denaturation and complete reaction of carbonyl groups and their conversion into the 2,4-dinitrophenylhydrazone derivatives. Absorbance at 370 nm was then measured subtracting a blank, which contained 0.2 mM 2,4-DNPH alone.

FOXO- PRDX2 alignment analysis. The sequence alignment between the nucleotide sequence of the promoter region of PRDX2 and the FOXO-DNA binding motif was performed using the web available program ClustalW.

Statistical analysis. Data were analyzed using either *t*-test or the 2-way analysis of variance (ANOVA) algorithm for repeated measures between control and β thalassemic cultures or mice with or without resveratrol at the different time point studied. A difference with a *P* value less than 0.05 was considered significant.

Table 1S. Details of primers for the specific Q-RT PCR of the analyzed genes in sorted CFU-E

Gene Symbol	Forward Primer Sequence (5'→ 3')	Reverse Primer Sequence (5'→ 3')
FOXO3 (**FOXO3A**)	GCCGGCTGGAAGAACTCC	CTTGCCAGTTCCCTCATTCTG
FOXO1 (**FOXO1A**)	CCCAACCAAAGCTTCCCAC	GTGTAACCTGCTCACTAACCCTCA
HBG1 (**HBGA**)	GACTTCCTTGGGAGATGCCAC	AAACGGTCACCAGCACATTTCC
HBG2	GACTTCCTTGGGAGATGCCAT	AAACGGTCACCAGCACATTTCC
CAT (catalase)	CCAAATACTCCAAGGCAAAGGT	CTCCAGCAACAGTGGAGAACC
PRDX2	ACAAAGGGAAGTACGTGGTCCTC	GCTGAACGCGATGATCTCG
GAPDH	CCACATCGCTCAGACACCAT	AGTTAAAAGCAGCCCTGGTGAC

Table 2S. Details of primers for the specific Q-RT PCR of the analyzed genes on sorted mouse basophilic erythroblasts and polychromatic erythroblasts.

Gene Symbol	Forward Primer Sequence (5'→ 3')	Reverse Primer Sequence (5'→ 3')
Foxo3 (FOXO3A)	CAGGCTGAAGGATCACTGAGG	TCTGTAGGTCTTCCGTCAGTTTGAG
Prdx2	CGCCTAGTCCAGGCCTTTC	GATGGTGTCACTGCCGGG
Cat (catalase)	GGAGGAGAGGAAACGCCTGT	TTGACCGCTTTCTTCTGAATGA
Gapdh	GTAGACAAAATGGTGAAGGTCGG	GCCACTGCAAATGGCAGC

SUPPLEMENTAL FIGURES

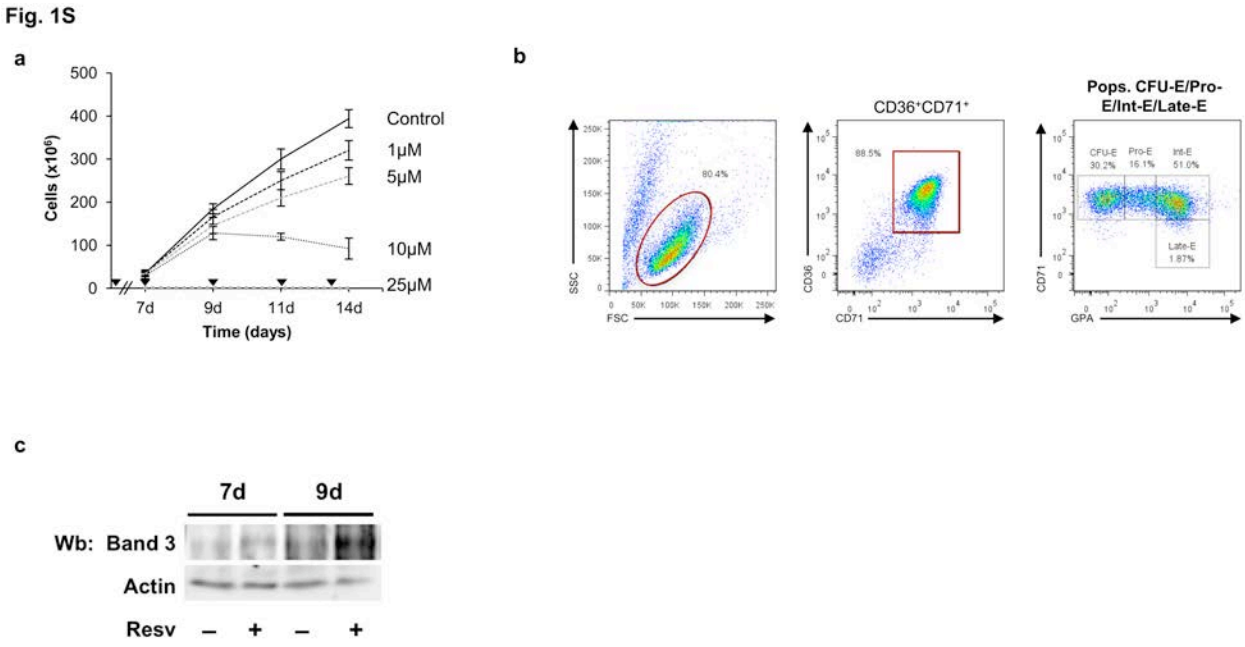

Fig. 1S. (a) Dose response effects of resveratrol (no resveratrol: control; 1, 5, 10, 25 μM) on cell growth of human erythroid precursors derived from $CD34^+$ cells isolated from peripheral blood of normal subjects (n=5). Data are presented as means ± SD. The arrows indicate when resveratrol was added to the medium of the cultures. **(b)** Flow cytometric strategy of human erythroid precursors derived from $CD34^+$ cells analyzed using CD36, CD71 and GPA as surface markers as previously reported by Merryweather *et al*. (22). **(c).** Immunoblot analysis of band 3 from sorted $CD71^+$, $CD36^+$ erythroblasts with and without resveratrol treatment. One experiment similar to the other 4 is shown. Actin was used as a loading control protein.

Fig. 2S

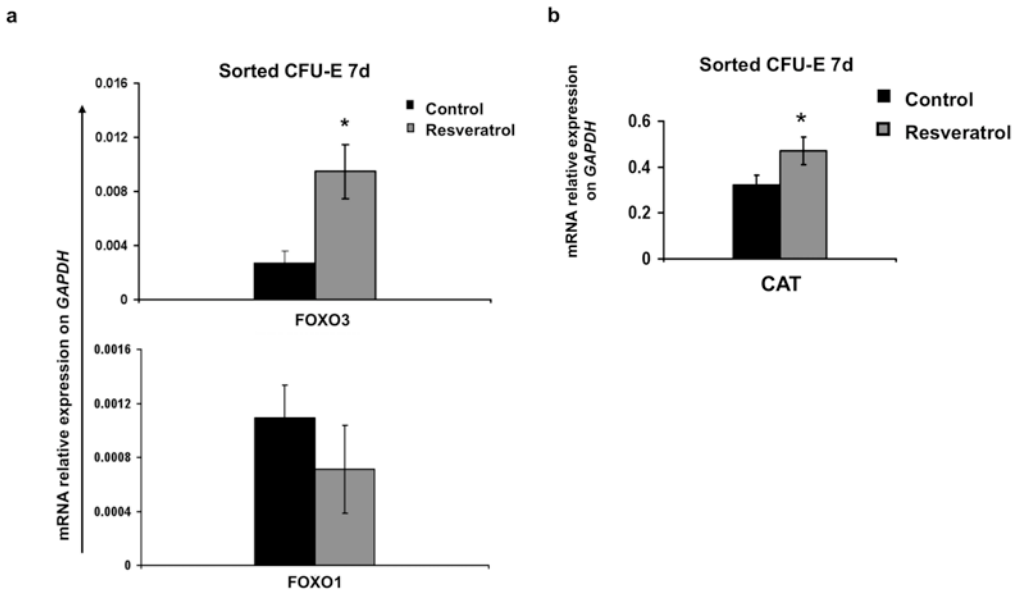

Fig. 2S. (a) RT-PCR expression of FOXO3 and FOXO1 on sorted CFU-E cells at day 7 of culture with and without resveratrol. Sorted CFU-E cells from 4 different cell cultures were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; **P* <0.01 compared to untreated cells; *n*=4). **(b)** RT-PCR expression of catalase (CAT) on sorted CFU-E cells at day 7 of culture with and without resveratrol. Sorted CFU-E cells from 4 different cell cultures were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; **P* <0.01 compared to untreated cells; *n*=4).

Fig. 3S

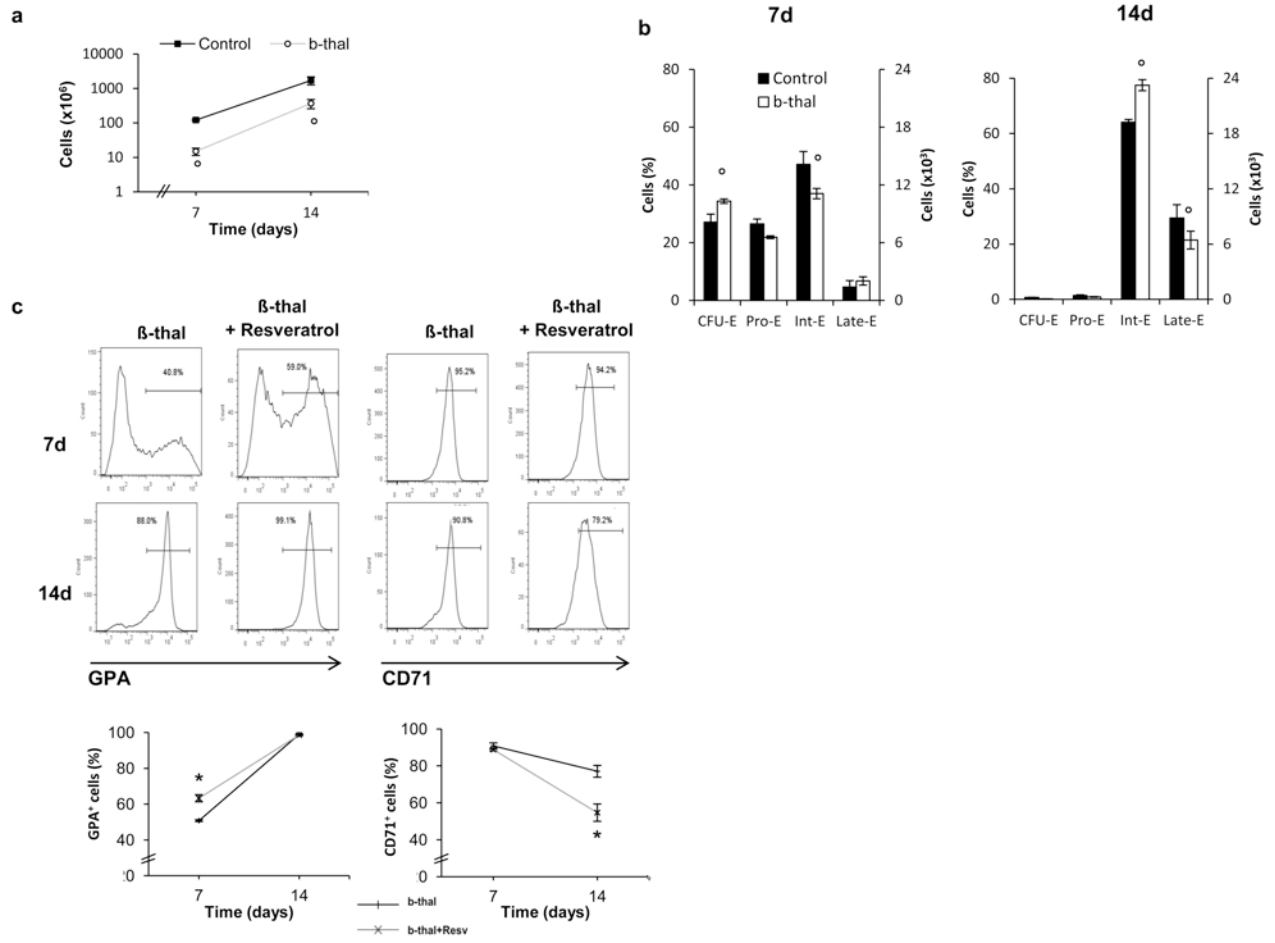

Fig. 3S. (a) Cell proliferation of normal (control) and β-thalassemic (b-thal) erythroid precursors derived by *in vitro* liquid culture of CD34$^+$ cells isolated from peripheral blood of healthy and β-thalassemic (b-thal) intermedia subjects (*n*=10). Data are presented as means± SD, *n*=10; ° $P<0.05$ compared to control cells. **(b)** Cyto-fluorimetric analysis of maturation pattern of normal (Control) and β-thalassemic erythroid precursors at different time of cell culture 7, and 14 days (d) using the following surface markers: CD36, glycophorin-A and CD71 (see also on line Methods). This cyto-fluorimetric strategy allows the identification of the following homogenous cell populations: pro-erythroblasts (Pro-E), basophilic erythroblasts corresponding to intermediate erythroblasts (Int-E) and polychromatic and orthochromatic erythroblasts as late erythroblasts (Late- E). Data are presented percentage and absolute cell counts and shown as means± SD (*n*=10); °$P< 0.05$ compared to control cells. **(c)** Kinetic of GPA appearance and reduction of CD71 in resveratrol (Resv) treated β-thalassemic cells compared to untreated cells at 7 and 14 days of culture. Data are presented as means ± SD (*n*=10); *$P< 0.05$ compared to untreated cells.

Fig. 4S

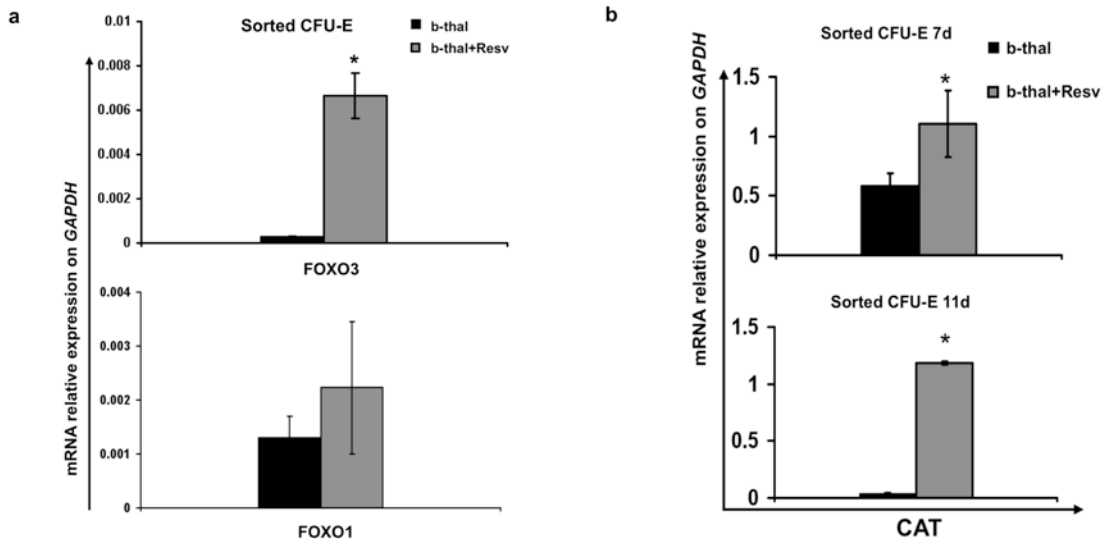

Fig. 4S. (a) RT-PCR expression of FOXO3 and FOXO1 on sorted β-thalassemic (b-thal) CFU-E cells at day 7 of culture with and without resveratrol (Resv). Sorted β-thalassemic CFU-E cells from 6 different cell cultures were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD, n=4; *P <0.05 compared to untreated cells). **(b)** RT-PCR expression of catalase (CAT) on sorted β-thalassemic CFU-E cells at day 7 and 11 of culture with and without resveratrol (Resv). Sorted CFU-E cells from 4 different cell cultures were analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD; n=4; *P <0.01 compared to untreated cells).

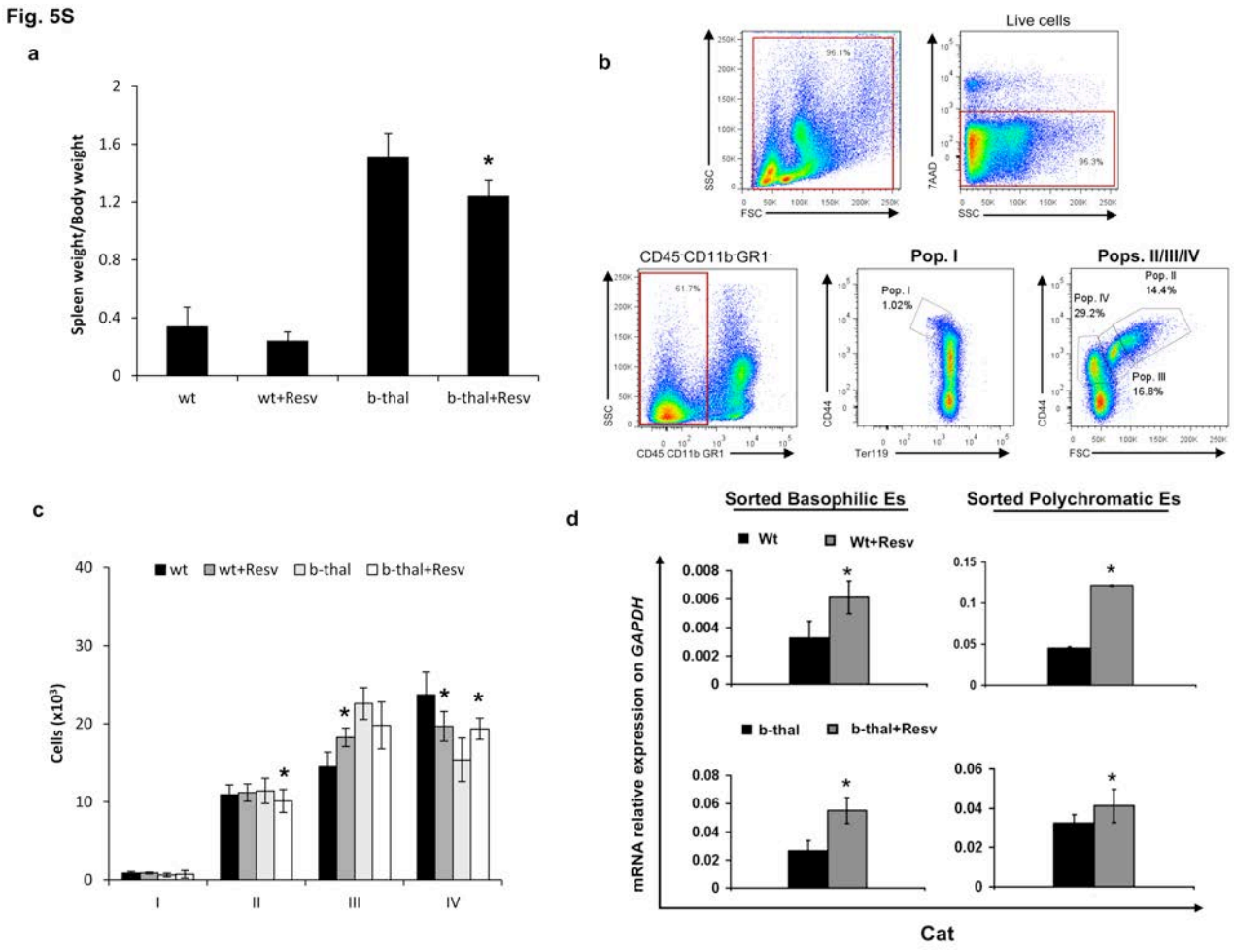

Fig. 5S. (a) Spleen weight/ mouse weight ratio in wild-type (wt) and β-thalassemic (b-thal) mice with and without resveratrol supplementation. Data are presented as means ± SD (n=12); *P< 0.05 compared to untreated mice. **(b)** Flow cytometric strategy of mouse erythroid precursors using CD44, TER119 strategy as previously reported (31, 41). **(c)** Cyto-fluorimetric analysis of maturation pattern of wild-type (wt) and β-thalassemic (b-thal) erythroid precursors from bone marrow of mice with or without resveratrol supplementation using the following surface markers: CD44 and TER119 (see also supplementary Materials and Methods). This cyto-fluorimetric strategy allows the identification of the following homogenous cell populations: population I corresponding to pro-erythroblasts, population II corresponding to basophilic erythroblasts, population III corresponding to polychromatic erythroblasts and population IV corresponding to orthochromatic erythroblasts. Data are presented as absolute cell counts and shown as means ± SD (*n*=10); *P< 0.05 compared to untreated mice. **(d)** RT-PCR expression of catalase (Cat) on sorted basophilic and polychromatic erythroblasts from wild-type (WT) and β-thalassemic (b-thal) mice with or without resveratrol (Resv) supplementation. Sorted cells from 6 mice from each group were

analyzed. Experiments were performed in triplicate. Error bars represent the standard deviations (mean ± SD, n=6; *P <0.01 compared to untreated cells).