Over-expression of mitochondrial ferritin affects the JAK2/STAT5 pathway in K562 cells and causes mitochondrial iron accumulation

Paolo Santambrogio,[1] Benedetta Gaia Erba,[1] Alessandro Campanella,[2,3] Anna Cozzi,[1] Vincenza Causarano,[4] Laura Cremonesi,[4] Anna Gallì,[5] Matteo Giovanni Della Porta,[5] Rosangela Invernizzi,[6] and Sonia Levi[1,3]*

[1]Proteomics of Iron Metabolism Unit, Division of Neuroscience, San Raffaele Scientific Institute, Milano, Italy; [2]IIT Network, Research Unit of Molecular Neuroscience; [3]Vita-Salute San Raffaele University; [4]Genomic Unit for the Diagnosis of Human Pathologies, Center for Genomics, Bioinformatics and Biostatistics, San Raffaele Scientific Institute; [5]Institute of Hematology and [6]Department of Internal Medicine, University of Pavia, IRCCS Policlinico San Matteo Foundation, Pavia, Italy

Citation: Santambrogio P, Erba BG, Campanella A, Cozzi A, Causarano V, Cremonesi L, Gallì A, Della Porta MG, Invernizzi R, and Levi S. Over-expression of mitochondrial ferritin affects the JAK2/STAT5 pathway in K562 cells and causes mitochondrial iron accumulation. Haematologica 2011;96(10):1424-1432 doi:10.3324/haematol.2011.042952

Online Supplementary Design and Methods

Cell cultures

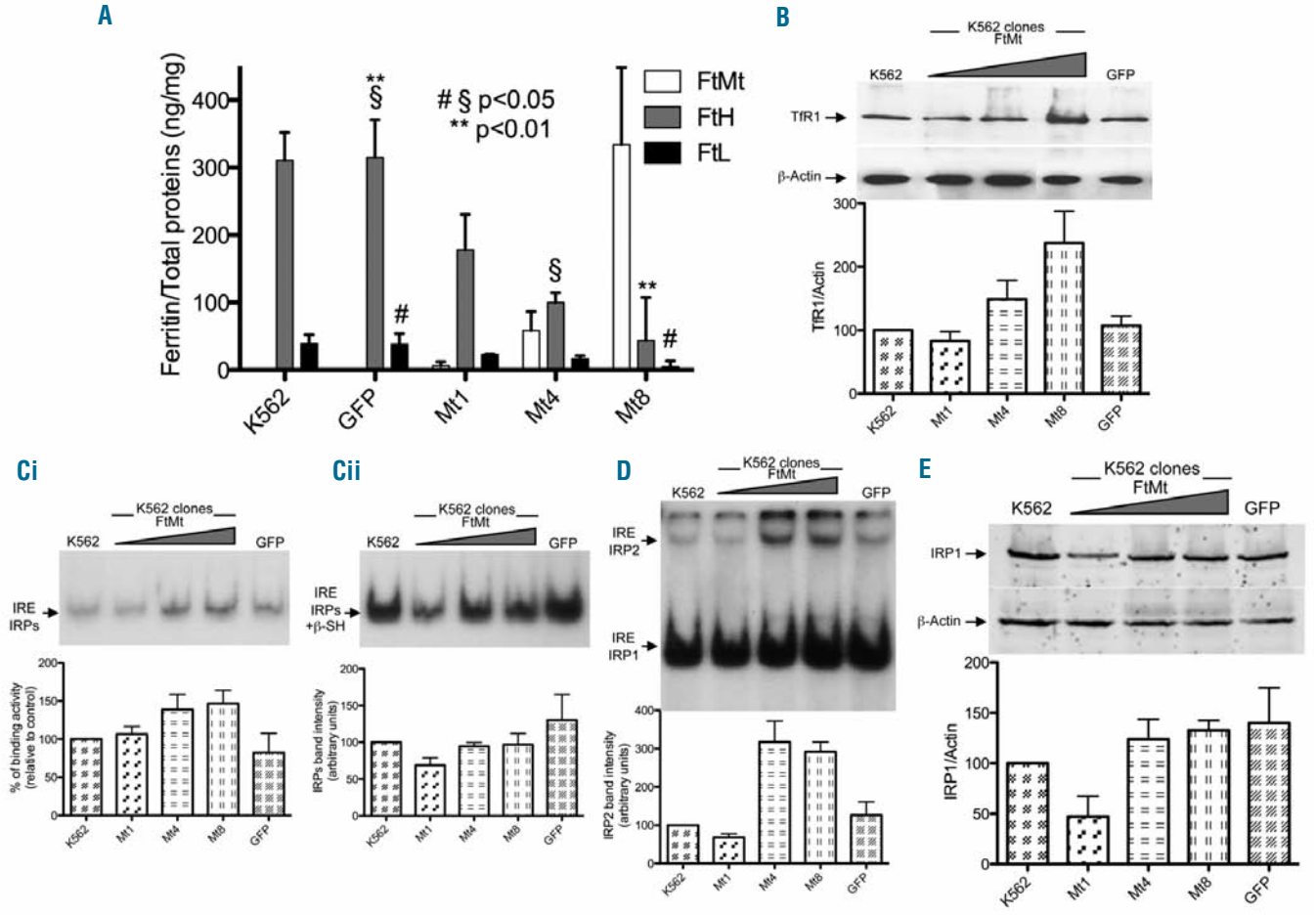
Human erythroleukemic (K562) and murine erythroleukemia (MEL) cell lines were grown in suspension in RPMI 1640 (Lonza, Treviglio, Italy) supplemented with 10% fetal bovine serum (Clontech, Saint Germain en Laye, France), 100 μg/mL streptomycin, 100 U/mL penicillin and 2 mM L-glutamine (Sigma, Milan, Italy). Cells were incubated with 0.1 mM desferrioxamine (DFO) for 18 h for western blot analysis or with 1 mM DFO for 48 h for real-time quantitative polymerase chain reaction and apoptosis analysis. $CD34^+$ bone marrow cells were separated by two cycles of positive selection using the MiniMacs system (Miltenyi Biotech, Bergish Gladbach, Germany), according to the manufacturer's instructions, on mononuclear cells isolated by density gradient centrifugation from the bone marrow of healthy donors. Informed consent was obtained from the subjects and the study followed the guidelines of the research ethical committee of the IRCCS Policlinico San Matteo in Pavia. FtMt-transduced and control $CD34^+$ cells (see below) were seeded onto plates at a density of 1×10^5 cells/mL in Iscove's medium (Sigma) supplemented with 1% penicillin and streptomycin, 1% L-glutamine, 15% BIT9500 serum substitute, 10 ng/mL interleukin-3, 10 ng/mL interleukin-6 and 25 ng/mL stem cell factor (Tebu-Bio, Magenta, Italy) and cultured for 21 days. Recombinant human erythropoietin (Santa Cruz Biotechnology, Heidelberg, Germany) was added to the medium from day 7 of culture.

Iron proteins and iron content analysis

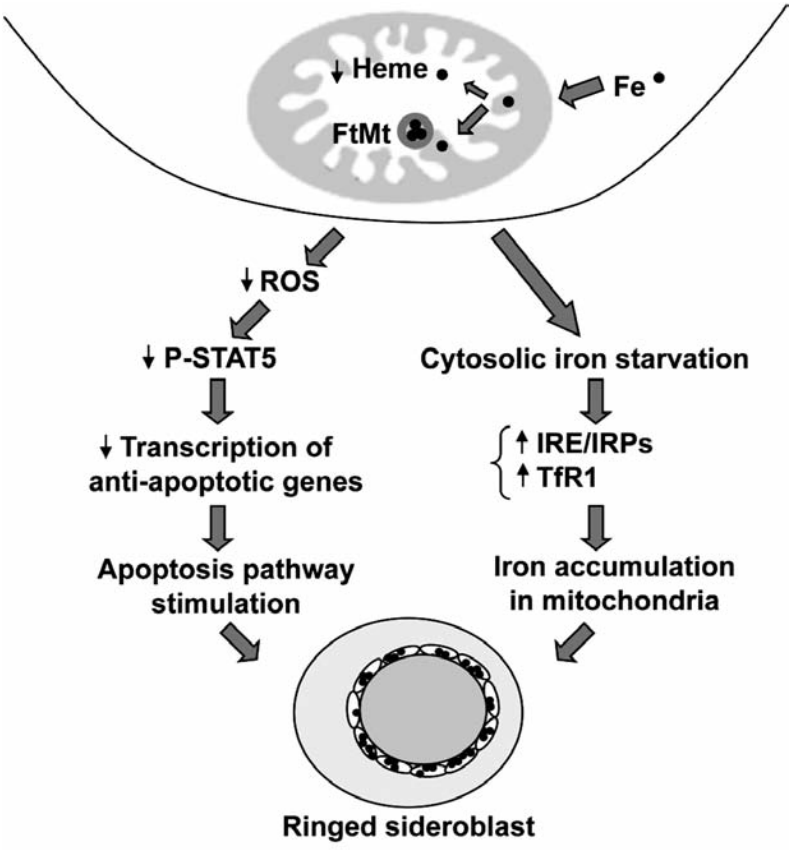
Ferritin quantification was performed by ELISA as described previously.[1,2] Electromobility shift assay was performed as described by Campanella *et al.*[3] Protein concentration was evaluated by the bicinchoninic acid method (Pierce, Pero, Italy). Cellular ^{55}Fe incorporation was determined in K562 cells (5×10^5) incubated for 18 h with 1 μM ^{55}Fe-ferric citrate (55Fe:citrate 1:2) (Perkin Elmer, Monza, Italy) and 10 μM ascorbic acid (Sigma) in RPMI with 0.5% fetal calf serum and 0.5% bovine serum albumin. Heme content was measured in control and transiently transfected MEL cells (1×10^6) grown for 3 days in RPMI containing 1.5% dimethylsulfoxide. Cells were washed with phosphate-buffered saline and dissolved in 0.5 mL of 98% formic acid and incubated for 15 min. Heme content was evaluated by analyzing the clear supernatant at 400 nm with an extinction coefficient of $1.56 \times 10^5 \times M^{-1} \times cm^{-1}$. Data were normalized to protein content determined by the BioRad Protein Assay (BioRad, Segrate, Italy).

References

1. Santambrogio P, Biasiotto G, Sanvito F, Olivieri S, Arosio P, Levi S. Mitochondrial ferritin expression in adult mouse tissues. J Histochem Cytochem. 2007;55(11):1129-37.
2. Cozzi A, Levi S, Bazzigaluppi E, Ruggeri G, Arosio P. Development of an immunoassay for all human isoferritins, and its application to serum ferritin evaluation. Clin Chim Acta. 1989;184(3):197-206.
3. Campanella A, Levi S, Cairo G, Biasiotto G, Arosio P. Blotting analysis of native IRP1: a novel approach to distinguish the different forms of IRP1 in cells and tissues. Biochemistry. 2004;43(1):195-204.



Online Supplementary Figure S1. Effect of FtMt expression on iron metabolism in K562 clones. (A) Mitochondrial (FtMt) and cytosolic heavy (FtH) and light (FtL) ferritin chains evaluated by ELISA using specific antibodies. Plots represent mean (±SD) of the amount of ferritin relative to total protein content in three independent experiments. Significant changes are marked. (B) Stable clones and control K562 soluble cell lysates (25 μg per lane) were separated by 10% SDS-PAGE, blotted onto nitrocellulose filters and probed with antibodies specific for TfR1 and β−actin. Plots show band intensities of TfR1 relative to β−actin representative of three independent experiments. Triangles indicate increasing amount of expressed protein. (C and D) Electromobility shift assay (EMSA) was performed on 2 μg of clones and control K562 soluble cell extracts using pSPT-Fer for IRE probe synthesis. (Ci) IRE binding activities and (Cii) total IRE binding activities obtained by addition of 2% β−mercapto-ethanol (β −SH). Plots show the percentage of IRP binding activity or band intensities of IRP representative of three independent experiments. In (D) IRE-IRP2 was separated from IRE-IRP1 by supershift with anti-IRP2 antibody. The IRE/IRP complex was separated by non-denaturing 6% PAGE. Plots show IRP2 band intensities representative of three independent experiments. (E) The same as in (B), except that the blot was probed with antibodies specific for IRP1 and β−actin. Plots show band intensities of IRP1 relative to β−actin representative of three independent experiments. Arrows indicate the position of protein bands. Triangles indicate increasing amount of expressed protein.



Online Supplementary Figure S2. Schematic representation of the proposed mechanism leading to a sideroblastic-like phenotype. The high expression of FtMt in erythroleukemia cells appears to decrease the amount of heme. Moreover its expression produces two parallel effects: (i) the FtMt-dependent decrease in ROS causes a reduction of P-Stat5, leading to diminished anti-apoptotic gene transcription and enhancement of apoptosis; (ii) the FtMt iron avidity promotes induction of cytosolic iron starvation, which stimulates the control of cellular iron importation (IRE/IRP machinery) and determines iron accumulation in mitochondria. When all these events occur in $CD34^+$ cells, they may promote the formation of sideroblasts.