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### ► To cite this version:

Maryam Nikpour, Andrea Pellagatti, Anquan Liu, Mohsen Karimi, Luca Malcovati, et al.. Gene expression profiling of erythroblasts from refractory anaemia with ring sideroblasts (RARS) and effects of G-CSF. *British Journal of Haematology*, 2010, 149 (6), pp.844. 10.1111/j.1365-2141.2010.08174.x . hal-00552589

**HAL Id: hal-00552589**

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Submitted on 6 Jan 2011

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**Gene expression profiling of erythroblasts from refractory anaemia with ring sideroblasts (RARS) and effects of G-CSF**

Journal:	<i>British Journal of Haematology</i>
Manuscript ID:	BJH-2009-01784.R1
Manuscript Type:	Ordinary Papers
Date Submitted by the Author:	05-Feb-2010
Complete List of Authors:	Nikpour, Maryam; Karolinska Institute, Department of Medicine, Division of Hematology Pellagatti, Andrea; LRF Molecular Haematology Unit, NDCLS Liu, Anquan; Karolinska Institute, Department of Medicine, Division of Hematology Karimi, Mohsen; Karolinska Institutet, Department of Medicine, Division of Hematology Malcovati, Luca; University of Pavia, Division of Hematology Gogvadze, Vladimir; Karolinska Institutet, The Institute of Environmental Medicine (IMM) Forsblom, Ann-Mari; Karolinska Institute, Department of Medicine, Division of Hematology Wainscoat, Jim; Oxford university, NDCLS Cazzola, Mario; University of Pavia, Division of Hematology Zhivotovsky, Boris; Karolinska Institute, The Institute of Environmental Medicine (IMM) Grandien, Alf; Karolinska Institute, Department of Medicine Boulwood, Jackie; University of Oxford, LRF Molecular & Cytogenetic Haem. Unit Hellström-Lindberg, Eva; Karolinska Institute, Department of Medicine, Division of Hematology
Key Words:	MDS, ANAEMIA, IRON METABOLISM, ERYTHROID CELL DIFFERENTIATION, ARRAYS, APOPTOSIS

# Gene expression profiling of erythroblasts from refractory anaemia with ring sideroblasts (RARS) and effects of G-CSF

**Authors:** Maryam Nikpour [1], Andrea Pellagatti [2], Anquan Liu [1], Mohsen Karimi [1], Luca Malcovati [4], Vladimir Gogvadze [3], Ann-Mari Forsblom [1], James S. Wainscoat [2], Mario Cazzola [4], Boris Zhivotovsky [3], Alf Grandien [1], Jacqueline Boulton [2], Eva Hellström-Lindberg [1]

**Affiliation:** [1] Center of Experimental Haematology, Department of Medicine (Huddinge), Karolinska Institute, Stockholm, Sweden [2] LRF Molecular Haematology Unit, NDCLS, John Radcliffe Hospital, Oxford, UK [3] Institute of Environmental Medicine, Division of Toxicology, Karolinska Institute, Stockholm, Sweden [4] Department of Haematology Oncology, University of Pavia & Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy

## Corresponding Author

Eva Hellström-Lindberg

**Postal address:** Department of Medicine, Division of Haematology, Karolinska University Hospital- Huddinge, SE-141 86 Stockholm, Sweden

**E-mail address:** [Eva.Hellstrom-Lindberg@ki.se](mailto:Eva.Hellstrom-Lindberg@ki.se)

**Running title:** Biology of sideroblastic anaemia and mechanisms of G-CSF

**Disclaimers:** none

## Summary

RARS is characterized by anaemia, erythroid apoptosis, cytochrome *c* release and mitochondrial ferritin accumulation. Granulocyte-CSF inhibits the first three of these features *in vitro* and *in vivo*. To dissect the molecular mechanisms underlying the RARS phenotype and anti-apoptotic effects of G-CSF, erythroblasts generated from normal (NBM) and RARS marrow CD34<sup>+</sup> cells were cultured  $\pm$ G-CSF and subject to gene expression analysis (GEP).

Several erythropoiesis-associated genes deregulated in RARS CD34<sup>+</sup> cells showed normal expression in erythroblasts, underscoring the importance of differentiation-specific GEP. RARS erythroblasts showed a marked deregulation of several pathways including apoptosis, DNA damage repair, mitochondrial function, and the JAK/Stat pathway. *ABCB7*, transporting iron from mitochondria to cytosol and associated with inherited ring sideroblast formation was severely suppressed and expression decreased with differentiation, while increasing in NBM cultures. The same pattern was observed for the mitochondrial integrity gene *MFN2*. Other down-regulated key genes included *STAT5b*, *HSP5A*, *FANCC*, and the negative apoptosis regulator *MAP3K7*. Methylation status of key down-regulated genes was normal. The mitochondrial pathway including *MFN2* was significantly modified by G-CSF, and several heat shock protein genes were up-regulated, as evidence of anti-apoptotic protection of erythropoiesis. By contrast, G-CSF had no effect on iron-transport or erythropoiesis-associated genes.

**Key words:** Refractory anaemia with ring side oblasts, Gene expression profiling, Pathway analyses, *ABCB7*, *MFN2*

## Introduction

Myelodysplastic syndromes (MDS) are clonal haematologic disorders characterized by defects in the haemopoietic stem cell compartment resulting in failure of one or more of the cell lineages. The WHO subtype refractory anaemia with ring sideroblasts (RARS) presents with isolated anaemia, hyperplastic ineffective erythropoiesis, hypochromic erythrocytes, and presence of mitochondrial accumulation of mitochondrial ferritin in erythroblasts. According to WHO classification RARS, and refractory cytopenia with multilineage dysplasia and ring sideroblasts (RCMD-RS) are defined by the presence of more than 15% ringed sideroblasts and less than 5% myeloblasts in bone marrow with an isolated erythroid versus a multilineage dysplasia respectively (Jaffe ES 2001, Swerdlow SH 2008). The risk of transformation to acute myeloid leukaemia from RARS is very low, and from (RCMD-RS) around 9% (Germing, *et al* 2000).

We have previously shown that mitochondria in RARS erythroblasts constitutively release cytochrome *c* from the mitochondrial intermembrane space (Cazzola, *et al* 2003, Tehranchi, *et al* 2003, Tehranchi, *et al* 2005). The molecular basis for the abnormal iron accumulation, defect mitochondrial function and ineffective heme biosynthesis in RARS remains unknown. We recently identified the *ABCB7* gene as a candidate gene for sideroblast formation in RARS because of its low expression levels in CD34<sup>+</sup> RARS cells, and by the analogy of RARS with the hereditary syndrome X-linked sideroblastic anaemia with ataxia (XLSA-A), in which *ABCB7* is mutated (Boultonwood, *et al* 2008). However, no mutations have been detected in acquired RARS (Boultonwood, *et al* 2008, Steensma, *et al* 2007).

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5 Chronic transfusion need of RARS (Bennett, *et al* 1982, Greenberg, *et al* 1997) may  
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7 respond to treatment with Erythropoietin alone or in combination with Granulocyte  
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9 Colony- stimulating factor (G-CSF). The synergistic effect of the addition of G-CSF  
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11 more pronounced in RARS than in other subgroups of low-risk MDS (Hellstrom-  
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13 Lindberg, *et al* 1998, Jadersten, *et al* 2005). In a series of studies we showed that G-CSF  
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15 strongly inhibited apoptosis in differentiating RARS erythroblasts *in vitro* as well as *in*  
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17 *vivo*, through a marked inhibition of mitochondrial cytochrome *c* release and subsequent  
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19 decrease in caspase-9 and caspase-3 activity (Schmidt-Mende, *et al* 2001, Tehranchi, *et*  
20  
21 *al* 2003, Tehranchi, *et al* 2005). By contrast, G-CSF did not affect the accumulation of  
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23 mitochondrial ferritin at any measured time point of differentiation (Tehranchi, *et al*  
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25 2005).

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30 There are several reports on gene expression profiling of purified CD34<sup>+</sup> fractions  
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32 obtained from the bone marrow of patients with MDS (Chen, *et al* 2004, Hofmann, *et al*  
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34 2002, Pellagatti, *et al* 2006, Sternberg, *et al* 2005). We recently demonstrated that the  
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36 expression profile of MDS CD34<sup>+</sup> cells, an in particular RARS cells, show similarities to  
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38 IFN- $\gamma$ -induced gene expression in CD34<sup>+</sup> cells from healthy individuals. Moreover,  
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40 altered expressions of heme biosynthesis and mitochondrial genes were reported  
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42 (Pellagatti, *et al* 2006). The CD34 compartment of MDS bone marrow is, however,  
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44 heterogeneous and may reflect a different mix of progenitors depending on marrow blast  
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46 percentage and degree of erythroid hypo vs. hyperplasia. To specifically address the  
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48 abnormalities in differentiating erythroblasts we performed gene expression profiling on  
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50 cultured RARS and normal erythroid progenitors at an intermediate maturation level,  
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52 with a specific focus on genes involved in erythropoiesis, apoptosis, iron  
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metabolism/transport, and mitochondrial function. We also analyzed the effects of G-CSF on gene expression to further understand its anti-apoptotic role in RARS erythropoiesis.

For Peer Review

## Materials and Methods

### Patients

The diagnostic procedure was performed according to the WHO 2008 classification (Swerdlow SH 2008) and routines put forth by the Nordic MDS Group (Jadersten, *et al* 2005). Informed consent was obtained from patients and controls, and the study followed the guidelines of the ethical committee for research at Karolinska Institute and the Declaration of Helsinki. Seven patients with either RARS (n=3) or refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS) (n=4) with a median age of 75 years were included. Four were transfusion dependent and 3 had a stable anaemia at the time of sampling. There were no major differences in clinical presentation between the two WHO subgroups. Five patients had a normal karyotype, one 46XY,del(13q), and one had 46,XY[23]/47,XY,+8[2]. Bone marrow (NBM) samples from 6 healthy individuals (4 females and 2 males) with a median age of 54 years were used as controls. In addition, a larger cohort of RARS and RCMD-RS (n=19) and normal controls (n=10) were assessed for methylation status of identified candidate genes.

### CD34<sup>+</sup> cell separation and erythroblast cultivation

Bone marrow mononuclear cells (MNC) were isolated by using Lymphoprep (Axis-Shield, Oslo, Norway) density gradient within one hour from aspiration, and CD34<sup>+</sup> cells were positively selected using a MACS magnetic labelling system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' protocols. The purity of CD34<sup>+</sup> cells isolated with this system was assessed at regular intervals and shown to be



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3 above 95% (Tehranchi, *et al* 2003). Following separation, CD34<sup>+</sup> cells were cultured (0.1  
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6 x 10<sup>6</sup> /ml) for 14 days in Iscove's medium supplemented with 15% BIT9500 serum  
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9 substitute (containing bovine serum albumin, bovine pancreatic insulin, and  
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12 iron/saturated human transferrin, and recombinant human interleukin (rh-IL)-3 (10ng/ml),  
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14 rh-IL-6 (10ng/ml), rh-stem cell factor (rh-SCF; 25 ng/ml), 1% penicillin and  
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17 streptomycin and 1% L-glutamine. Epo (2 IU/ml) was added to the medium at day 7, and  
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20 fresh medium supplemented as above (plus Epo) was added at day 9 and 11. We used  
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23 cells obtained at day 7 of culture, in order to compare gene expression profiling with  
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26 previous extensive cell biological studies on this cell cohort. Phenotype and erythroid  
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29 maturation of cells was, as previously validated and reported, analyzed at day 4, 7, and 14  
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32 using CD36 and glycoporphin A (GpA) antibodies. At day 4, 42% of cells were CD34<sup>+</sup>  
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34 CD36<sup>-</sup>, while 24% were CD34<sup>+</sup> CD36<sup>+</sup> and 12% were positive only for CD36 (median  
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37 values). At day 7, 22% were CD34<sup>+</sup> CD36<sup>-</sup>, 14% were CD34<sup>+</sup> CD36<sup>+</sup>, 26% CD36<sup>+</sup> and  
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40 5% CD36<sup>+</sup> GpA<sup>+</sup>. At day 14, the median percentages of CD36<sup>+</sup> and GpA<sup>+</sup> cells were 92%  
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42  
43 and 77% respectively (Tehranchi, *et al* 2003, Tehranchi, *et al* 2005).  
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45  
46 At day 7, an aliquot of cells were treated with granulocyte colony-stimulating factor (G-  
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49 CSF) (100ng/ml) (Neupogen; Amgen, Twelve Oaks, CA) for 4 hours, e.g. the same  
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52 concentration and exposure time that were used in previous experiments (Tehranchi, *et al*  
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55 2003, Tehranchi, *et al* 2005).  
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### Total RNA extraction

Total RNA from cultured erythroid progenitors at day 7 (untreated and G-CSF-treated cells from RARS patients and healthy controls) was extracted using TRIZOL (Invitrogen, Paisley, United Kingdom) following the protocol supplied by the manufacturer. RNA yields were determined spectrophotometrically at 260 nm and RNA integrity assayed using Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

### HUMARA analysis

Clonality of cultured erythroblasts was evaluated through the study of the X-Chromosome Inactivation Pattern (XCIP) by both DNA methylation and differential allelic expression analysis of the *HUMARA* (human androgen receptor) gene after 7 days of culture. DNA methylation status analysis was performed as previously described (Tonon, *et al* 1998). The expression clonality assay was based on a nested primer RT-PCR as reported by Busque L. (Busque, *et al* 1994). Amplified bands were subjected to density analysis by Molecular Imager FX software (Bio-Rad laboratories, Hercules, CA).

### Affymetrix experiments

Extracted total RNA (50 ng for each sample) was amplified and labelled with the 2-Cycle cDNA Synthesis and the 2-Cycle Target Labelling and Control Reagent packages (Affymetrix, Santa Clara, CA) following the manufacturer's recommendations. Labelled fragmented cRNA (10 µg) was hybridized to oligonucleotide probes on an Affymetrix Human Genome U133 Plus 2.0 GeneChip, containing 54,675 probe sets representing approximately 39,000 human genes. Hybridization occurred for 16 hours at 45°C with

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4 constant rotation at 60 rpm. Washing was carried out in accordance with the  
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7 manufacturer's instruction on the GeneChip Fluidics Station 450, and the arrays were  
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10 scanned using a GeneChip Scanner 3000 (Affymetrix).

### 14 **Data analysis**

15  
16 Scanned GeneChip images were processed using GeneChip Operating Software (GCOS).  
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18 Data analysis was performed by GeneSpring 7.3 (Agilent Technologies). Quality control  
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20 was performed within the GCOS software after scaling the signal intensities of all arrays  
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22 to a target of 100. Scale factors, background levels, percentage of present calls,  
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24  $3'/5'GAPDH$  ratio and intensities of spike hybridization controls were within the  
25  
26 acceptable range for all samples. Affymetrix CEL files were uploaded and pre-processed  
27  
28 using Robust MultiChip Analysis (RMA) (Irizarry, *et al* 2003). Differentially expressed  
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30 genes (t-test,  $p < 0.05$ , Benjamini-Hochberg multiple testing correction) between  
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32 conditions were identified using GeneSpring and used for pathway analysis using  
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34 Ingenuity 5.0. Hierarchical clustering was performed with GeneSpring software using  
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36 Pearson correlation.  
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### 45 **Quantitative real-time RT-PCR**

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47 For confirmation of microarray results, real-time quantitative polymerase chain reaction  
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49 (QRT-PCR) was performed for selected genes. We selected the 5 most deregulated probe  
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51 sets in patient compared to normal erythroblasts (sorted according to p-value) including  
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53 *NSMCE4A*, *UQCC*, *ABCB7*, *ITFG1* and *DNAJA2* as well as 5 genes with potential role in  
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55 RARS pathogenesis or potential mechanism of G-CSF effect including *MAP3K7*, *MFN2*,  
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5 *HSPA1B*, *FANCC*, and *FOXO3A*.  
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7 The expression level of the housekeeping gene GAPDH was used to normalize for  
8 differences in input cDNA. RT-PCR was carried out using TaqMan gene expression pre-  
9 synthesized reagents and master mix (Applied Biosystems, Warrington, UK) in 7500  
10 Real-Time PCR system (Applied Biosystems). The expression ratio was calculated using  
11 the  $\Delta\Delta CT$  method (Livak and Schmittgen 2001).  
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### 20 21 **Western Blotting**

22 Cells were lysed with lysis buffer (Complete Lysis-M, Roche). Total protein  
23 concentration was determined using the BCA Protein Assay Kit (Uptima). Twenty five  
24 micrograms of total protein was separated on a 7.5-10 % acrylamide gel and transferred  
25 to nitrocellulose membrane. Membranes were probed with antibodies that recognize  
26 HSPA1B (Abcam), MFN2 (Abcam) and GAPDH (Abcam). After incubation with  
27 appropriate secondary antibodies, specific proteins were detected using ECL reagents  
28 (Amersham Bioscience).  
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### 43 **DNA extraction and Bisulfite modification**

44 Genomic DNA was extracted from myeloid cell fractions from 19 patients and 10 healthy  
45 individuals using Gene Elute mammalian genomic extraction kit (Sigma, Sweden)  
46 according to the manufacturer's instructions. Bisulfite modification of genomic DNA was  
47 carried out using EZ DNA methylation Gold™ kit (ZYMO research) following standard  
48 protocol. Epi-Tect<sup>®</sup> PCR control DNA set (Qiagen) was used as methylated and  
49 unmethylated DNA controls for all PCR reactions.  
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### MCA-Meth

MCA-Meth (Melting curve analysis-Methylation assay) was performed as described by Lorente (Lorente, *et al* 2008). Briefly, 20ng of bisulfite modified DNA was amplified in a total volume of 20µl PCR mix, containing 10µl of 2X SYBR Green master mix (Applied biosystems, CA) and 5pmol of forward and reverse primers. Primers for ABCB7, MFN2, FANCC, and FOXO3 were designed using Methprimer software (Li and Dahiya 2002). The cycling program was 95°C for 10Min, following by 40 cycles of 30 Sec at 94°C, 30 sec at corresponding annealing temperatures (Supplementary table I) and 30 sec at 72°C. Melting curve analysis was performed from 60°C to 95°C. Both amplification reaction and melting curve analysis were carried out using an ABI 7500 FAST real time PCR system (Applied Biosystems, CA)

## Results

### RARS erythroblasts are clonal

We investigated the clonality of cultured day 7 erythroblasts from five female patients with RARS through X-chromosome inactivation pattern, using both DNA methylation- and allelic expression-based approaches. At day 7, all samples showed a skewed XCIP (corrected allelic ratios: 4.28, 5.62, 8.19 and complete inactivation of one allele in two cases), indicative of a clonal erythroid progenitor population. Three of these were also analyzed at start of culture (CD34<sup>+</sup> cells), and all showed skewed XCIP (corrected allelic ratios: 18.41 and complete inactivation of one allele in two cases) (data not shown).

### Differentially expressed genes in RARS and normal erythroblasts

6228 probe sets were significantly different between day 7 RARS and normal erythroblasts. From these, 3960 probe sets were up- and 2268 probe sets were down-regulated. Table I shows the 10 most up- and down-regulated probe sets, sorted according to p-value, in RARS compared to normal erythroblasts (category A). Table I also lists deregulated genes specifically involved in iron metabolism, haematopoiesis and apoptosis (category B), even if they did not fall within the top 10 genes, as well as marks the genes significantly altered by G-CSF. Genes included in both categories or affected by G-CSF were of particular interest. In addition the 50 most deregulated probe sets, sorted only according to p-value, are presented in supplementary Table II. The most deregulated gene in RARS compared to normal erythroblasts was NSMCE4A, which was down-regulated. NSMCE4A mutant yeast displays genome instability and hypersensitivity to DNA

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3 damage (Hu, *et al* 2005). PCR confirmed deregulation of 9 of the 10 selected genes  
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6 (selected data shown in Fig 1-3).  
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### 10 11 12 **Deregulation of genes involved in cellular iron metabolism and mitochondrial** 13 **function increases during differentiation** 14 15

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17 We previously showed that *ABCB7* expression, mutated in XLSA/A and involved in  
18 maturation of cytosolic Fe/S proteins, is inversely correlated to the percentage of ring  
19 sideroblasts in MDS marrow smears (Boultonwood, *et al* 2008). Here we show that *ABCB7*  
20 is suppressed throughout erythroid maturation in RARS, and that the suppression is more  
21 pronounced in mature erythroblasts, in contrast to the increasing expression over time in  
22 normal erythroblast cultures (Fig 1c).  
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32 Another key down-regulated mitochondrial gene was mitofusin 2 (*MFN2*), involved in  
33 mitochondrial membrane integrity (Sugioka, *et al* 2004). A corresponding decrease of the  
34 protein was shown (Fig 2c). *MFN2* expression during culture followed the pattern of  
35 *ABCB7*, and increased 2-3-fold during normal erythroid differentiation, while decreasing  
36 in the RARS cultures (Fig 2b).  
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44 In addition, Sideroflexin 1 (*Sfxn1*), mutated in flexed-tail (f/f) mice with siderocytic  
45 anaemia, and PGRMC1, a heme binding protein, were also down-regulated in RARS  
46 erythroblasts.  
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### 50 51 52 **Erythropoiesis-related genes are not over-expressed in RARS erythroblasts** 53

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55 Next, we compared expression profiling from a recent study using RARS CD34<sup>+</sup> cells  
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(Pellagatti, *et al* 2006) with the GEP results obtained from intermediate RARS erythroblasts in the present study. The expression level of all heme biosynthesis pathway enzymes was normal in RARS erythroblasts, except aminolevulinic acid synthase 2 (*ALAS2*), the first enzyme in the heme biosynthetic pathway (Table I, category B). RARS erythroblasts showed a moderate over-expression of *ALAS2*, however at a much lower magnitude than RARS CD34<sup>+</sup> cells (1.37-fold vs. 12.77-fold). Interestingly, also other genes involved in the heme pathway and erythropoiesis including *ALAD*, *FECH*, *GATA1*, and *EPO-R*, were up-regulated in CD34<sup>+</sup> cells but showed normal expression levels in RARS erythroblasts. This indicates that part of the over-expression observed in CD34<sup>+</sup> cells may be due to an enrichment of early erythroid cells in that fraction. Down-regulated genes in the erythropoiesis group encompassed *FOXO3A*, a transcription factor regulating oxidative stress in erythropoiesis and *FANCC*, a key mutated gene in Fanconi anaemia.

### **Genes involved in cell survival and apoptosis**

The expression of pro and anti-apoptotic genes in RARS erythroblasts constituted a complex pattern. *MAP3K7*, an important negative regulator of apoptosis (Tang, *et al* 2008) was one of the most down-regulated genes in RARS erythroblasts. By contrast, two members of the heat shock protein 70 family, *HSPA1B* and *HSPA9B* genes, showed moderate over-expression, which may indicate their involvement in protection of erythroid cells survival in RARS. *HSPA1B* is an inducible HSP70 which regulates haematopoiesis and protects GATA-1 from caspase-3 cleavage, while, *HSPA9B* is an anti-apoptotic protein and a mediator of erythropoietin signalling (Ohtsuka, *et al* 2007,



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Ribeil, *et al* 2007).

A key finding of the paper by Pellagatti (Pellagatti, *et al* 2006) was up-regulation of several interferon induced genes which may be responsible for enhancement of apoptosis in erythroid progenitors, hence in ineffective erythropoiesis in RARS. Deregulation of several interferon induced genes including *IFR2*, *IFR6*, *IRF2BP2*, *IFRG15*, *IFNA17*, *ISG20L1*, and *ISG20L2* were observed also in RARS erythroblasts.

### **Pathway analysis of RARS compared to normal erythroblasts**

Functional classification revealed 25 significantly deregulated pathways in RARS compared to normal erythroblasts ( $P < 0.01$ ), the top 10 being listed in Table II, A. RARS erythroblasts showed a marked deregulation of several important pathways for haematopoiesis and cell cycle control, including integrin, PI3K/AKT and VEGF signaling, protein ubiquitination, apoptosis, DNA damage checkpoint regulation, mitochondrial function, and the JAK/Stat pathway.

The JAK/Stat signalling pathway mediates cellular responses to growth factors, however, erythropoietin (EpoR) and granulocyte colony-stimulating factor (G-CSFR) receptors activate distinct but overlapping sets of signalling molecules within the pathway. While Stat5 activation is essential for signalling through the Epo-R, it is not necessary for the terminal erythroid differentiation induced by G-CSF (Milot, *et al* 2001). Interestingly, *Stat5b* was dramatically down regulated in RARS erythroblasts, which could explain why RARS patients benefit from the addition of G-CSF to EPO treatment. The deregulation of protein ubiquitination pathway, which targets abnormal or short-lived proteins for degradation, might reflect an abnormal protein synthesis in RARS.

### Potential mechanisms behind the anti-apoptotic effects of G-CSF in RARS

1153 probe-sets were significantly different ( $P < 0.05$ ) between G-CSF treated and untreated RARS erythroblasts. The 10 most up and down-regulated genes, sorted according to p-value are shown in supplementary Table II. The influence of G-CSF treatment on probe sets categorized as A or B is also shown in Table I.

Only 163 probe-sets were differentially expressed in treated and untreated normal erythroblasts, which is in line with the very moderate effect of G-CSF on normal erythroblasts shown by us in previous cell biological studies (Tehranchi, *et al* 2003, Tehranchi, *et al* 2005). G-CSF had no effect on the expression of *ABCB7*, or erythropoiesis-associated genes. The slight over-expression of *ALAS2* was normalized by G-CSF. Importantly, several genes, which in RARS were altered in a direction of enhanced apoptosis, were reverted back to the normal range by G-CSF. The reduced *MFN2* expression was reverted to normal range (confirmed by RT-PCR and western blotting) (Fig. 2). However, the expression level of *MAP3K7*, a negative regulator of apoptosis and down-regulated in RARS erythroblasts, was not altered by G-CSF. The expression level of *HSPA9B*, which was slightly up-regulated in RARS, further increased after G-CSF treatment. *HSPA9B* inhibits apoptosis via the inactivation of P53 (Kaul, *et al* 2000) and its loss in zebra fish recapitulates the ineffective haematopoiesis of the myelodysplastic syndrome including anaemia and dysplasia (Craven, *et al* 2005). G-CSF also significantly enhanced the expression and protein level of *HSPA1B* in RARS (Fig 3).

### Pathway analysis of RARS erythroblasts after G-CSF treatment

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3 Pathway analysis revealed 17 significantly deregulated pathways in G-CSF treated and  
4 un-treated RARS erythroblasts ( $P < 0.05$ ), listed in Table II, B. Interestingly, the  
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6 “mitochondrial dysfunction” pathway, one of the main deregulated pathways in RARS  
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8 erythroblasts, was significantly modulated after G-CSF treatment ( $p = 0.04$ ). Caspase-9,  
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10 which is increased in RARS was down-regulated by G-CSF (Tehranchi, *et al* 2003).  
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12 G-CSF also reverted the endoplasmic reticulum stress (ERS) pathway, which is triggered  
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14 by the accumulation of unfolded proteins in the endoplasmic reticulum (ER), towards the  
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16 normal pattern. For instance, HSPA5, an ER associated member of HSP70 family and  
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18 shown to inhibit cytochrome *c* release and apoptosis, was up-regulated. Moreover, G-  
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20 CSF over-expressed several genes in “NRF2-mediated Oxidative Stress Response  
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22 pathway” involved in protein folding including DNAJB9, DNAJB11, DNAJB6, DNAJC7  
23  
24 and HERPUD1 (Table II, B). As a reflection of the minor cellular effects of G-CSF on  
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26 normal erythroblasts, only two pathways were deregulated in normal erythroblasts after  
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28 G-CSF treatment (Table II, C)  
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### 39 **Methylation analysis**

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41 In order to investigate whether down-regulation of key genes was caused by  
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43 hypermethylation, we studied methylation status by MCA-Methylation assay (Lorente, *et*  
44  
45 *al* 2008). The *MFN2*, *FANCC* and *FOXO3A* genes were not methylated in the RARS  
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47 samples. Regarding *ABCB7*, there was 50% methylation in females (both RARS and  
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49 control), which is in line with the fact that one allele of *ABCB7* gene is inactivated  
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51 because of its location on chromosome X (Fig 1d). Hence, DNA methylation was not a  
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53 significant cause of low mRNA expression. Methylation status of *MFN2* in RARS  
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5 samples is shown in Figure 2e.  
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## 8 9 **Discussion**

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11 Gene expression profiling of MDS CD34<sup>+</sup> cells showed that RARS patients constitute a  
12 relatively homogenous group with IFN- $\gamma$ -induced gene expression, and altered expression  
13 of heme biosynthesis and mitochondrial genes (Pellagatti, *et al* 2006). However, the  
14 CD34<sup>+</sup> compartment may reflect different cell mixes in different MDS subtypes as MDS  
15 progenitors may retain the CD34 antigen longer than normal progenitors (Kanter-  
16 Lewensohn, *et al* 1996). Considering the typical morphology of RARS, it is likely that  
17 CD34<sup>+</sup> mRNA from this subtype mirror a higher proportion of erythroblasts compared to  
18 e.g. 5q- syndrome CD34<sup>+</sup> cells. To address this problem, we used cultured erythroblasts  
19 for the microarray analyses using a well-validated culture method (Tehranchi, *et al* 2003,  
20 Tehranchi, *et al* 2005). Interestingly, several genes involved in erythropoiesis and  
21 significantly over-expressed in RARS CD34<sup>+</sup> cells showed normal or nearly normal  
22 expression in erythroblasts. By contrast, the suppressed expression of *ABCB7* and *MFN2*  
23 genes in RARS erythroblasts was reinforced during erythroid differentiation.  
24 The severely reduced *ABCB7* expression in mature erythroblasts deserves more  
25 consideration (Boulwood, *et al* 2008). *ABCB7* is required for maturation of cytosolic Fe-  
26 S proteins such as iron regulatory protein 1, which regulates protein levels of the major  
27 iron homeostasis genes (Cairo and Recalcati 2007). Hence, disruption of Fe-S cluster  
28 biogenesis pathways can result in maladaptive changes in iron metabolism. *ABCB7*  
29 silencing in HeLa cells causes an iron-deficient phenotype with mitochondrial iron  
30 overload (Cavadini, *et al* 2007). In normal erythroblast cultures *ABCB7* expression  
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4 increased in parallel with hemoglobinisation, but intriguingly, it decreased gradually from  
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6 day 0 to day 14 in RARS, supporting a critical role of *ABCB7* in aberrant iron  
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8 accumulation also in acquired RARS.  
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12 The single most down-regulated gene in RARS was *NSMCE4A*, which is involved in  
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14 DNA damage repair and hence may be associated with DNA instability in RARS (Hu, *et*  
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16 *al* 2005, Novotna, *et al* 2008). Moreover, *FANCC*, a key mutated gene in Fanconi  
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18 anaemia and involved in DNA damage repair and apoptosis inhibition in haematopoietic  
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20 cells exposed to IFN- $\gamma$  (Pang, *et al* 2001), was also markedly suppressed, which may  
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22 explain the similarities of MDS CD34<sup>+</sup> and IFN- $\gamma$ -induced normal CD34<sup>+</sup> cells  
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24 (Pellagatti, *et al* 2006). Finally, apoptosis of RARS erythroblasts was paralleled by  
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26 *MAP3K7* down regulation. *MAP3K7* deletion leads to a massive haematopoietic cells  
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28 apoptosis in mice (Tang, *et al* 2008).  
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36 Several pathways showed deregulation in RARS compared to normal erythroblasts,  
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38 including mitochondrial function, apoptosis, and JAK/Stat signaling, all key mediators of  
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40 functional erythropoiesis. Deregulation of JAK/Stat signaling pathway and in particular  
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42 Stat5 down regulation, is in line with the impaired response to erythropoietin (Epo) in  
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44 RARS patients (Hoefsloot, *et al* 1997). However, RARS erythroblasts are able to undergo  
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46 terminal differentiation induced by G-CSF because of the dispensable role of Stat5b in G-  
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48 CSF-R signaling (Milot, *et al* 2001). This may be a relevant explanation for the  
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50 particularly good synergistic *in vivo* and *in vitro* effects of G-CSF and EPO on the  
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52 anemia and erythroid apoptosis in RARS (Jadersten, *et al* 2005, Tehranchi, *et al* 2003,  
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55 Tehranchi, *et al* 2005).  
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3 G-CSF has virtually no effect on normal erythroblasts and haemoglobin of normal  
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5 individuals, which was reflected by its very moderate effects on normal gene expression.  
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8 To further address the anti-apoptotic role of G-CSF in RARS cells we assessed genes  
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10 reverted back to the normal expression range by G-CSF. Interestingly, the expression of  
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12 several anti-apoptotic genes, such as *Bcl-2* and *Bcl-xL*, was normal in RARS and  
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14 unaffected by G-CSF (Tehranchi, *et al* 2003) and neither did G-CSF affect *MAP3K7*.  
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18 Instead, G-CSF restored *MFN2* gene and protein expression and had also a significant  
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20 effect on the whole mitochondria pathway in RARS cells. *MFN2* participates in the  
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22 mitochondrial pathway and its silencing leads to enhanced apoptosis in HeLa cells  
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24 (Sugioka, *et al* 2004) and reduced mitochondrial membrane potential (Bach, *et al* 2003).  
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28 The reduced *MFN2* expression in RARS erythroblasts may therefore link to cytochrome *c*  
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30 release in these cells and G-CSF-induced re-expression of *MFN2* to the inhibitory effect  
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32 of the growth factor on cytochrome *c* release and erythroid apoptosis (Tehranchi, *et al*  
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34 2003). Furthermore, G-CSF up-regulated *HSPA1B*, a member of *HSP70s*, which was  
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36 recently shown to protect *GATA-1* from caspase-3-mediated proteolysis during  
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38 differentiation (Ribeil, *et al* 2007). This may constitute another potential mechanism for  
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40 G-CSF, which inhibits caspase-3 activation in RARS cells (Schmidt-Mende, *et al* 2001).  
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45 *HSPA9B*, another member of *HSP70s*, had a similar response pattern to G-CSF. *HSPA9B*  
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47 inhibits P53 function (Wadhwa, *et al* 1998) and is also involved in EPO signalling  
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49 (Ohtsuka, *et al* 2007).  
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52 The endoplasmic reticulum stress (ERS) pathway is activated in response to an  
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54 accumulation of unfolded proteins in the endoplasmic reticulum (ER) and protects cells  
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56 against different types of stress (Zhang and Kaufman 2006). Interestingly, the ERS  
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3 pathway was modified by G-CSF, which might be a survival mechanism in particular  
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5 since several genes involved in protein folding including DNAJB9, DNAJB11, DNAJB6,  
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7 DNAJC7, and HERPUD1 were up-regulated by G-CSF. In addition, HSPA5, an ER  
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9 associated member of the HSP70 family, was up-regulated by G-CSF. HSPA5 over-  
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11 expression is associated with inhibition of cytochrome *c* release and apoptosis (Shu, *et al*  
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13 2008).

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17 In contrast to its anti-apoptotic effects, G-CSF does not seem to affect sideroblast  
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19 formation or mitochondrial iron accumulation, as shown by the *ABCB7* results and  
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21 unaffected accumulation of mitochondrial ferritin (Tehranchi, *et al* 2005). Interestingly,  
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23 RARS erythrocytes from patients responding to growth factors develop a more abnormal  
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25 phenotype with increased cell size and hypochromatic cytosol, which supports this  
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27 interpretation (Ljung, *et al* 2004). We therefore suggest that G-CSF promotes  
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29 erythroblast survival until the erythrocyte stage, in spite of impaired cellular function.  
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33 Our data shows the strength of assessing gene expression during differentiation. Through  
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35 this technique and by assessing the G-CSF effects, we have identified a number of  
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37 candidate genes involved in mitochondrial iron accumulation and cytochrome *c* release  
38  
39 for further investigation. The stable clinical course of WHO RARS may suggest its  
40  
41 monogenetic – or oligogenetic nature. In the absence of *ABCB7* mutations or  
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43 hypermethylation, we hypothesize that upstream mechanisms with impact on cellular iron  
44  
45 transport may be involved in RARS pathogenesis.  
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## 51 **Acknowledgments**

52  
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54 This work was supported by grants from the Swedish Cancer Society (contract 08 0601),  
55  
56 and through a strategic grant for Centrum For Experimental Haematology (EHL and  
57  
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1  
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4 AG), the Medical Research Council (contract 70293801), the Cancer Society in  
5  
6  
7 Stockholm (EHL), Leukaemia Research Fund of the UK (JB, AP, JW), and Fondazione  
8  
9 IRCCS Policlinico San Matteo, Pavia, (M.C).  
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For Peer Review



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## Tables

**Table I.** List of up-regulated (a) and down-regulated (b) probe sets in RARS

erythroblasts compared to normal erythroblasts. Genes were categorized by A: the 10 most up- and down regulated probe sets sorted according to p-value, B: potential role in RARS pathogenesis or potential mechanism of G-CSF effect. Genes in category B are shown in rows with grey shadow. In column “Effects of G-CSF”, ↑ symbol indicates gene up-regulation; ↓ symbol shows gene down-regulation and – indicates no change by G-CSF.

**Table II.** A. The 10 most deregulated pathways in day 7 RARS erythroblasts compared to normal erythroblasts. B. The 10 most deregulated pathways in day 7 RARS erythroblasts after G-CSF treatment. C. The only 2 deregulated pathways in day 7 normal erythroblasts after G-CSF treatment.

**Supplementary Table I.** List of the oligonucleotides used for methylation analysis.

**Supplementary Table II.** List of the 50 most deregulated probe sets in RARS erythroblasts compared to normal erythroblasts, sorted according to p-value. In column “Effects of G-CSF”, ↑ symbol indicates gene up-regulation; ↓ symbol shows gene down-regulation and – indicates no change by G-CSF.

**Supplementary Table III.** The 10 most significantly up-/down-regulated probe sets in G-CSF-treated RARS erythroblasts versus untreated RARS erythroblast

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## Figure legends

**Figure 1.** *ABCB7* expression level in intermediate (day 7) erythroblasts by **a.** microarray analysis and **b.** RT-PCR (7 RARS and 6 normal samples); **c.** *ABCB7* expression level in differentiating RARS and normal erythroblasts, with *ABCB7* expression normalized to that of day 0 healthy controls (results shown as mean  $\pm$  SD) (3 RARS and normal samples); **d.** Analysis of *ABCB7* promoter methylation by MCA-Meth. Melting temperature of the amplified fragment (274 bp) was determined as described in materials and methods. Results from 19 RARS patients and 10 healthy donors plus un-methylated, 50% methylated and 100% methylated controls are presented. Female samples from RARS as well as NBM showed both unmethylated and methylated peaks.

**Figure 2. a.** *MFN2* expression level by microarray analysis in intermediate (day 7) RARS and normal erythroblasts  $\pm$  G-CSF (7 RARS and 6 normal samples); **b.** *MFN2* expression level in differentiating RARS and normal erythroblasts, with *MFN2* expression normalized to that of day 0 healthy controls (results shown as mean  $\pm$  SD) (3 RARS and normal samples); **c.** *MFN2* protein level in representative RARS and normal cultures, with and without G-CSF treatment (2 RARS and normal samples); **d.** *MFN2* expression level by RT-PCR in day 7 RARS erythroblasts  $\pm$  G-CSF (7 RARS and 6 normal samples). **e.** Analysis of *MFN2* promoter methylation by MCA-Meth. Melting temperature of the amplified fragment (183 bp) was determined by 7500F real time PCR system. Results from 19 RARS patients and 10 healthy donors are presented. Methylation was not observed in either group. Two methylated peaks are 50%- and 100 % methylated DNA controls.

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6 **Figure 3.** *HSPA1B* expression level by **a.** microarray and **b.** RT-PCR in intermediate  
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8 (day 7) RARS and normal erythroblasts  $\pm$  G-CSF (7 RARS and 6 normal samples); **c.**  
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10 *HSPA1B* protein level in two representative RARS cultures, with and without G-CSF  
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**Table I. List of up-regulated (a) and down-regulated (b) probe sets in RARS erythroblasts compared to normal erythroblasts**

Gene	Affymetrix ID	Map	Function	p-value	Average ratio RARS/NBM $\pm$ SD	Effect of G-CSF	Category
<b>a. Up-regulated probe sets</b>							
UQCC	229672_at	20q11.22	Ubiquinol-cytochrome c reductase complex chaperone	0.0001	7.00 $\pm$ 1.80	-	A
DNAJA2	226994_at	16q11.1-q11.2	Co-chaperone of Hsp70s in protein folding and mitochondrial protein	0.0003	2.19 $\pm$ 0.23	↓	A
LMNA	214213_x_at	1q21.2-q21.3	Nuclear stability, Gene expression	0.0003	1.26 $\pm$ 0.05	-	A
SEC22L1	214257_s_at	1q21.2-q21.3	ER-Golgi protein trafficking	0.0003	2.08 $\pm$ 0.18	-	A
LOC440983	227338_at	3q25.1	Unknown	0.0003	2.06 $\pm$ 0.13	-	A
C2orf67	231252_at	2q34	Unknown	0.0003	3.61 $\pm$ 0.57	-	A
MED19	226293_at	11q12.1	Mediator complex subunit RNA polymerase II transcription mediator	0.0003	1.52 $\pm$ 0.08	-	A
-	231576_at	12p12.1	Unknown	0.0003	1.94 $\pm$ 0.27	↓	A
TBC1D8	241017_at	2q11.2	GTPase activator activity	0.0003	14.95 $\pm$ 7.06	-	A
DDX27	215693_x_at	20q13.13	Helicase activity, ATP binding	0.0003	1.72 $\pm$ 0.15	-	A
HSPA1B	202581_at	6p21.3	Regulation of erythropoiesis	0.0366	1.62 $\pm$ 0.27	↑	B
HSPA9B	200690_at	5q31.1	Anti-apoptosis, Mediator of erythropoietin signaling	0.0035	1.67 $\pm$ 0.21	↑	B
ALAS2	238813_at	Xp11.21	Heme biosynthetic pathway	0.017	1.37 $\pm$ 0.20	↓	B
<b>b. Down-regulated probe sets</b>							
NSMCE4A	211376_s_at	10q26.13	Essential for cell cycle progression and DNA repair	0.00002	0.18 $\pm$ 0.02	-	A, B

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
ABCB7	209620_s_at	Xq12-q13	Maturation of Fe/S enzymes, mutated in XLSA/A	0.0001	0.31 ± 0.05	-	A , B																																									
ITFG1	221449_s_at	16q12.1	Protein binding	0.0001	0.29 ± 0.05	-	A																																									
SPAG4L	237188_x_at	20q11.21	Spermatogenesis	0.0003	0.66 ± 0.04	-	A																																									
ATXN10	208833_s_at	22q13.31	Involved in autosomal dominant cerebellar ataxias	0.0003	0.45 ± 0.04	-	A																																									
YWHAB	208743_s_at	20q13.1	Potential role in linking mitogenic signaling and the cell cycle	0.0003	0.43 ± 0.04	-	A																																									
UQCC	229672_at	20q11.22	Ubiquinol-cytochrome c reductase complex chaperone	0.0001	0.38 ± 0.18	-	A																																									
RTF1	212301_at	15q15.1	Paf1/RNA polymerase II complex	0.0003	0.41 ± 0.06	-	A																																									
ZNF587	243121_x_at	19q13.42	Unknown	0.0004	0.52 ± 0.05	-	A																																									
TM2D1	211703_s_at	1p31.3	Induction of apoptosis by extracellular signals	0.0005	0.50 ± 0.06	-	A																																									
MAP3K7	206854_s_at	6q16.1-q16.3	Negative regulation of apoptosis	0.0005	0.57 ± 0.07	-	B																																									
SFXN1	232055_at	5	Mutated in mice with siderocytic anemia	0.0256	0.61 ± 0.18	-	B																																									
MFN2	201155_s_at	1p36.22	Mitochondrial membrane stability	0.0192	0.52 ± 0.16	↑ 6 of 7 patients	B																																									
FANCC	205189_s_at	9q22.3	Genomic stability, mutated gene in fanconi anemia	0.0023	0.53 ± 0.08	-	B																																									
FOXO3A	204132_s_at	6q21	Regulation of oxidative stress in erythropoiesis	0.0126	0.19 ± 0.07	-	B																																									
STAT5B	212549_at	17q11.2	Signal transducer activity	0.0136	0.65 ± 0.15	-	B																																									

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Table II. Pathway Analysis

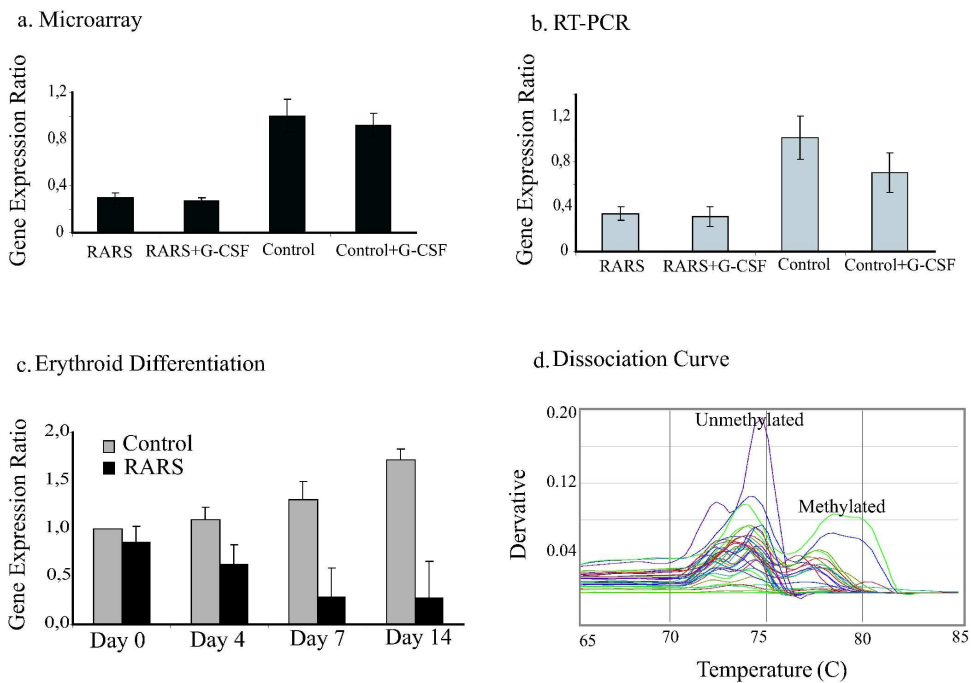
Pathways	P-value	Selection of the involved Genes
<b>A. The 10 most deregulated pathways in day 7 RARS erythroblasts compared to normal erythroblasts</b>		
Integrin Signaling	5.37E-06	69 genes including MAP2K4, RAP2B, RAC2, RAF1, MAP3K11, ARHGAP26, MAPK1, PIK3R1, HRAS, TLN1
PI3K/AKT Signaling	1.78E-05	43 genes including GAB2, RAF1, PIK3CA, JAK1, YWHAH, MAPK1, PPP2CA, PIK3R1, ILK, HRAS
B Cell Receptor Signaling	4.27E-05	52 genes including MAP2K4, RAF1, FCGR2C, GAB2, RAC2, MAP3K11, MAPK1, NFATC3, PIK3R1, HRAS
Actin Cytoskeleton Signaling	4.90E-05	69 genes including RAC2, RAF1, PFN1, MAPK1, DIAPH3, PIK3R1, HRAS, ARHGEF1, LIMK2, MYH11
Protein Ubiquitination Pathway	6.31E-05	64 genes including UBE2H, USP45, PSMA3, UBE2A, PSMA7, UBE3B, UBR2, UBE2D2, USP20, UBE2V2
JAK/Stat Signaling	1.66E-04	26 genes including RAF1, SOCS1, SOCS3, PIK3CA, PIAS2, JAK1, MAPK1, PIK3R1, HRAS, MAP2K2, STAT5B, SOS1
VEGF Signaling	1.70E-04	33 genes including RAF1, EIF2S2, PIK3CA, PTK2B, MAPK1, PIK3R1, EIF1, HRAS, EIF2S1, EIF2B2
Insulin Receptor Signaling	2.57E-04	44 genes including PRKACB, PPP1CC, SOCS3, FYN, RAF1, PIK3CA, JAK1, MAPK1, PIK3R1, LIPE
Apoptosis Signaling	5.25E-04	32 genes including MAP2K4, RAF1, MAPK1, HRAS, MAP4K4, DFFA, BCL2, ACIN1, CASP6, IKBKB
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	7.24E-04	18 genes including CDKN2A, TP53, PRKDC, UBB, YWHAH, YWHAB, WEE1, PTPMT1, CUL1, YWHAZ
<b>B. The 10 most deregulated pathways in day 7 RARS erythroblasts after G-CSF treatment (p&lt;0.05)</b>		
Biosynthesis of Steroids	2.69E-07	SQLE, FDFT1, DHCR7, EBP, IDI1, MVK, PDSS2, LSS, HMGCR, SC5DL
Hypoxia Signaling	2.69E-03	VEGFA, UBB, NFKBIA, UBE2D3, HIF1A, UBE2L6, UBE2E1, PTEN, ARNT
Endoplasmic Reticulum Stress Pathway	6.76E-03	CASP9, ERN1, MAP3K5, HSPA5
Toll-like Receptor Signaling	1.70E-02	ECSIT, TLR4, NFKBIA, CD14, EIF2AK2, IRAK2
IL-10 Signaling	1.74E-02	IL1R2, NFKBIA, BLVRA, IL10RA, BLVRB, CD14, FCGR2B
Propanoate Metabolism	1.74E-02	ACSL3, ACAT2, ACACB, DHCR24, ABAT, ACSS1, ALDH6A1

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LXR/RXR Activation	2.63E-02	IL1R2, TLR4, CD14, ABCG1, HMGCR, MMP9, ABCA1
Acute Phase Response Signaling	3.47E-02	ECSIT, TCF4, RRAS, NOLC1, SOCS2, SERPINF1, MAP3K5, TCF3, NFKBIA, RIPK1, SOS1, SERPINE1, IL1RAP
Inositol Phosphate Metabolism	3.47E-02	PAK1, INPP4B, SGK1, PDIA3, CSNK1A1, CDK6, LIMK2, IHPK2, EIF2AK2, PI4KA, PTEN
Pyruvate Metabolism	3.55E-02	ACSL3, AKR1A1, ACAT2, ACACB, DLAT, ACSS1, ACOT9
<b>C. The only 2 deregulated pathways in day 7 normal erythroblasts after G-CSF treatment (p&lt;0.05)</b>		
SAPK/JNK Signaling	2.51E-02	FADD, RIPK1, ZAK
Galactose Metabolism	4.17E-02	GLA, GALT

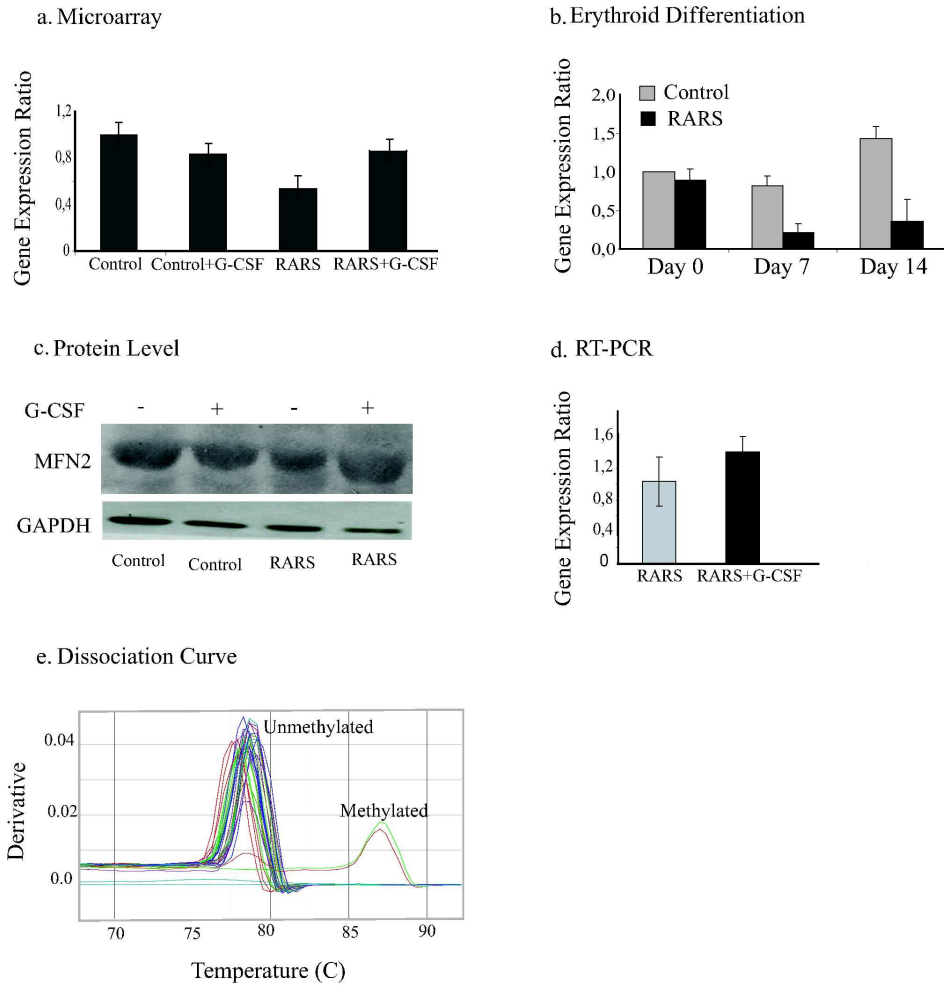
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Eva Hellström-Lindberg, Figure 1, Top



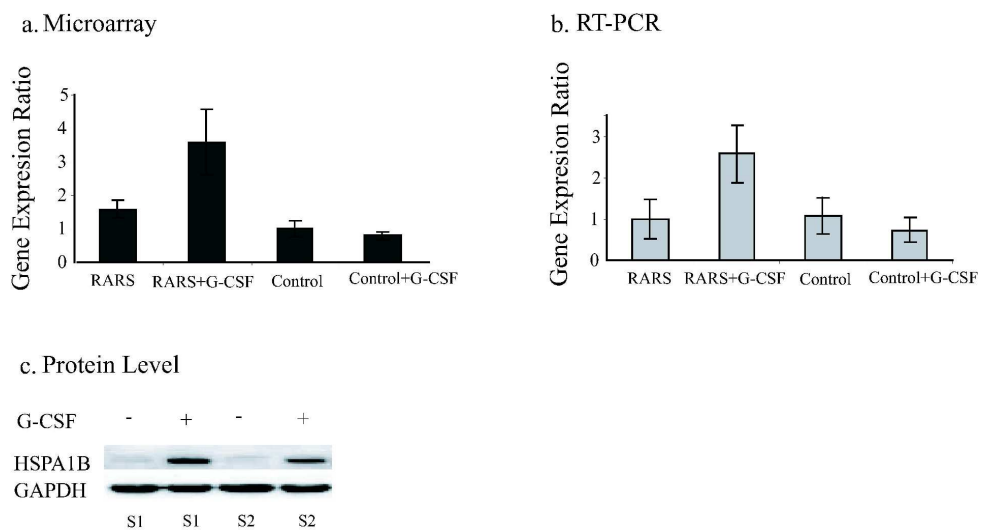
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Eva Hellström-Lindberg, Figure 2, down



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Eva Hellström-Lindberg, Figure 3, Top



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Supplementary Table I. List of the oligonucleotides used for methylation analysis

Gene	Sequence	Product size	Annealing
ABCB7-F	5'-AATAAGAAGAGGAGAGATAATTAAGG-3'	274 bp	56°C
ABCB7-R	5'-TCAATAACATCTCACCTAATAAACTC-3'		
MFN2-F	5'-TTATAGTTTTTATGATGTAGTGGGAG-3'	183 bp	52°C
MFN2-R	5'-AAACTAATAAACCCCTAAACCCAACC-3'		
FANCC-F	5'-GGGTTTATTTTYGTTAGAGTTTTGG-3'	128 bp	56°C
FANCC-R	5'-ATCAACAATACATTCTAAAACCTAACT128-3'		
FOXO3A-F	5'-GTGTGTTTATAATTTTGTGTTGTTG-3'	234 bp	52°C
FOXO3A-R	5'-CCTACCTCRCTTCCTCCCTTCA-3'		

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Supplementary Table II. List of 50 most deregulated probe sets in RARS erythroblasts compared to normal erythroblasts

No.	Gene	Affymetrix ID	Map	Function	p-value	UP/ Down	Effect of G-CSF
1	NSMCE4A	211376_s_at	10q26.13	Essential for cell cycle progression and DNA repair	2.00E-05	Down	-
2	UQCC	229672_at	20q11.22	Ubiquinol-cytochrome c reductase complex chaperone	0.0001	Up	-
3	NSMCE4A	219067_s_at	10q26.13	Essential for cell cycle progression and DNA repair	0.0001	Down	-
4	ABCB7	209620_s_at	Xq12-q13	Maturation of Fe/S enzymes, mutated in XLSA/A	0.0001	Down	-
5	ITFG1	221449_s_at	16q12.1	Protein binding	0.0001	Down	-
6	DNAJA2	226994_at	16q11.1-q11.2	Co-chaperone of Hsp70s in protein folding, mitochondrial protein import	0.0003	Up	↓
7	LMNA	214213_x_at	1q21.2-q21.3	Nuclear stability, gene expression	0.0003	Up	-
8	SEC22B	214257_s_at	1q21.2-q21.3	ER-Golgi protein trafficking	0.0003	Up	-
9	LOC440983	227338_at	3q25.1	Unknown	0.0003	Up	-
10	SPAG4L	237188_x_at	20q11.21	Spermatogenesis	0.0003	Down	-
11	C2orf67	231252_at	2q34	Unknown	0.0003	Up	-
12	MED19	226293_at	11q12.1	RNA polymerase II transcription mediator activity	0.0003	Up	-
13	-	231576_at	12p12.1	Unknown	0.0003	Up	↓
14	ATXN10	208833_s_at	22q13.31	Involved in autosomal dominant cerebellar ataxias	0.0003	Down	-
15	TBC1D8, RPL31	241017_at	2q11.2	GTPase activator activity, ribosomal protein L31	0.0003	Up	-
16	YWHAB	208743_s_at	20q13.1	Transcription corepressor activity	0.0003	Down	-
17	UQCC	217935_s_at	20q11.22	Ubiquinol-cytochrome c reductase complex chaperone	0.0003	Down	-
18	DDX27	215693_x_at	20q13.13	ATP-dependent helicase activity	0.0003	Up	-
19	RTF1	212301_at	15q15.1	Paf1/RNA polymerase II complex component	0.0003	Down	-
20	BAT2L2	214052_x_at	1q23.3	Protein C-terminus binding	0.0004	Up	-
21	ZNF845	243121_x_at	19q13.42	Unknown	0.0004	Down	-
22	HIATL1	233964_at	9q22.32	Protein binding	0.0005	Up	-

23	TM2D1	211703_s_at	1p31.3	Induction of apoptosis by extracellular signals	0.0005	Down	-
24	AGK	218568_at	7q34	ATP binding, Transferase activity	0.0005	Up	-
25	TRNAU1AP	228997_at	1p35.3	Protein binding, Nucleotide binding	0.0005	Up	-
26	DDX27	219108_x_at	20q13.13	ATP-dependent helicase activity	0.0005	Up	-
27	EIF2C2	229841_at	8q24	short-interfering-RNA-mediated gene signalling	0.0005	Up	-
28	ZNF587	60794_f_at	19q13.43	DNA binding	0.0005	Up	-
29	MAP3K7	206854_s_at	6q16.1-q16.3	Apoptosis and response to environmental stresses	0.0005	Down	-
30	FBXW8	237317_at	12q24.22	Phosphorylation-dependent ubiquitination	0.0005	Up	-
31	-	241310_at	13q21.1	Unknown	0.0005	Up	-
32	PPP2R5A	202187_s_at	1q32.2-q32.3	Protein phosphatase type 2A regulator activity	0.0006	Down	-
33	KIAA0406	212898_at	20q11.23	Unknown	0.0006	Down	-
34	SYNJ2	216180_s_at	6q25.3	Intracellular distribution of mitochondria	0.0006	Up	-
35	UTP14A	221514_at	Xq25	Ribosome biogenesis and 18S rRNA synthesis	0.0007	Up	-
36	-	209535_s_at	15q25.3	signal transducer activity, Protein binding	0.0007	Up	-
37	RBMS1	209868_s_at	2q24.2	DNA replication, gene transcription	0.0007	Down	-
38	COX5B	213736_at	2cen-q13	Cytochrome-c oxidase activity	0.0007	Up	-
39	SFRS3	232392_at	6p21	Protein binding, Nucleotide binding	0.0008	Up	-
40	MAP3K12	230921_s_at	12q13	Protein binding, ATP binding	0.0008	Up	-
41	ANKRD45	216496_s_at	1q25.1	Unknown	0.0009	Up	-
42	RBMS1	207266_x_at	2q24.2	DNA replication, gene transcription	0.001	Down	-
43	MRPS30	222275_at	5q11	mitochondrial ribosomal protein	0.001	Up	-
44	C22orf39	227086_at	22q11.21	Unknown	0.001	Up	-
45	UQCC	244795_at	20q11.22	Ubiquinol-cytochrome c reductase complex chaperone	0.001	Up	-
46	C6orf48	222968_at	6p21.3	Unknown	0.001	Up	-
47	ME2	210153_s_at	18q21	Oxidoreductase activity	0.0011	Down	-
48	RBMS1	203748_x_at	2q24.2	DNA replication, gene transcription	0.0011	Down	-
49	RARS2	225264_at	6q16.1	Arginyl-tRNA synthetase, Translation	0.0011	Up	-

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50	RAP1GDS1	217457_s_at	4q23-q25	GTPase activator activity	0.0011	Down	-
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**Suppl. Table II.** The 10 most significant up-/down-regulated genes in G-CSF-treated RARS erythroblasts versus untreated RARS erythroblast

Gene	Affymetrix ID	Map	Function	Average ratio RARS/normal	Average ratio RARS+G /RARS
<b>a. The 10 most up-regulated probe sets after G-CSF treatment</b>					
PSAT1	223062_s_at	9q21.2	Phosphoserine transaminase activity	1.68	2.35
FRMD3	229893_at	9q21.32	Cytoskeletal protein binding, essential for maintaining erythrocyte shape	0.86	1.82
TXNRD1	201266_at	12q23-q24.1	Electron carrier activity, Oxidoreductase activity	1.31	1.28
HMGCR	202540_s_at	5q13.3-q14	Cholesterol biosynthesis, Oxidoreductase activity	0.91	1.88
FDFT1	208647_at	8p23.1-p22	Cholesterol biosynthesis, Oxidoreductase activity	1.14	1.20
LRP8	205282_at	1p34	Apolipoprotein E receptor activity, Calcium ion binding	1.29	1.60
ANXA3	209369_at	4q13-q22	Phospholipase A2 inhibitor activity, Calcium ion binding	0.86	1.77
SLC38A1	224579_at	12q13.11	Sodium ion binding, Glutamine transporter	0.91	1.52
SLC1A4	235875_at	2p15-p13	Chloride channel activity	1	1.39
NME1	201577_at	17q21.3	Nucleotide binding	0.80	1.19
<b>b. The 10 most down-regulated probe sets after G-CSF treatment</b>					
SBF2	226169_at	11p15.4	Phosphatase activity	1.31	0.69
NSMCE2	226536_at	8q24.13	Prevention of DNA damage-induced apoptosis	1.27	0.77
MANBA	203778_at	4q22-q25	Catalytic activity	0.88	0.66

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ZNF292	236435_at	6q15	Zinc ion binding,	0.53	0.80
ABCG1	204567_s_at	21q22.3	Cholesterol binding, phospholipid binding	1.62	0.13
MAN1A1	237849_at	6q22	Mannosidase activity	1.27	0.43
MYLIP	220319_s_at	6p23-p22.3	Cytoskeletal protein binding, Ubiquitin-protein ligase activity	0.99	0.34
GALNT7	222587_s_at	4q31.1	Transferase activity, Calcium ion binding	1.14	0.81
TNKS	202561_at	8p23.1	Transferase activity	1.49	0.76
FAM175A	226521_s_at	4q21.21-q21.23	DNA repair	1.72	0.81

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