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Haem-regulated eIF2 α kinase is necessary for adaptive gene expression in erythroid precursors under the stress of iron deficiency

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Summary

Haem-regulated eIF2 α kinase (HRI) is essential for the regulation of globin gene translation and the survival of erythroid precursors in iron/haem deficiency. This study found that in iron deficiency, fetal definitive erythropoiesis is inhibited at the basophilic erythroblast stage with increased proliferation and elevated apoptosis. This hallmark of ineffective erythropoiesis is more severe in HRI deficiency. Microarray gene profiling analysis showed that HRI was required for adaptive gene expression in erythroid precursors during chronic iron deficiency. The number of genes with expression affected more than twofold increased, from 213 in iron deficiency and 73 in HRI deficiency, to 3135 in combined iron and HRI deficiencies. Many of these genes are regulated by Gata1 and Fog1. We demonstrate for the first time that Gata1 expression in developing erythroid precursors is decreased in iron deficiency, and is decreased further in combined iron and HRI deficiencies. Additionally, Fog1 expression is decreased in combined deficiencies, but not in iron or HRI deficiency alone. Our results indicate that HRI confers adaptive gene expression in developing erythroblasts during iron deficiency through maintaining Gata1/Fog1 expression.

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Author contribution

SJL and JJC designed the research, analyzed the data and wrote the paper; SJL, APH, RNS and WTZ performed experiments; SB and SJL performed the genechip data analysis; SB and RCF participated in writing the paper.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

Data deposition

Microarray data are available at NCBI GEO data base accession no. GSE6808.

Supplementary material

The following supplementary material is available for this article online:

Table S1. Differentially expressed erythroid and iron/haem homeostasis genes in FL cells during iron and HRI deficiencies.

The material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2141.2008.07293.x>

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Keywords

iron and haem; erythroid differentiation; eIF2 α kinase; Gata1 and Fog1; gene expression

Haem-regulated eIF2 α kinase (HRI, also known as eIF2 α kinase 1, gene symbol *Eif2ak1*) is highly expressed in the erythroid lineage. The kinase activity of HRI is regulated by haem through its two haem-binding domains located in the N-terminus and the kinase insertion (Chen, 2007). In haem deficiency, haem dissociates from the binding site in the kinase insertion and HRI is activated by autophosphorylation. Subsequently, the α -subunit of eukaryotic translation initiation factor 2 (eIF2) is phosphorylated and the recycling of eIF2 for another round of protein synthesis is inhibited. Thus, HRI normally insures that no globin is synthesized in excess of what can be assembled into haemoglobin for the amount of haem available (Chen, 2007).

Iron-deficiency anaemia is one of the most prevalent human diseases (Stoltzfus, 2003), which has long been known to impair physical and mental function. We have demonstrated that HRI is an important physiological regulator of protein synthesis and cell survival of the erythroid lineage during iron deficiency. In the absence of HRI and under iron deficiency, free globins precipitate within the red blood cells (RBCs) and its precursors. Further, in iron deficiency *Eif2ak1*^{-/-} erythroid precursors have increased apoptosis and *Eif2ak1*^{-/-} mice exhibit ineffective erythropoiesis with splenomegaly (Han *et al*, 2001). We have shown recently that HRI also reduces the phenotypic severity of β -thalassaemia in mice (Han *et al*, 2005). While anaemia *per se* is well tolerated in mice and humans, ineffective erythropoiesis is the cause of major complications in β -thalassaemia (Rund & Rachmilewitz, 2005) because of increased iron absorption (Andrews & Schmidt, 2007) and iron overload in multiple organs.

Gata1 is a key transcriptional regulator of erythropoiesis (Crispino, 2005; Ferreira *et al*, 2005). Gata1 works in concert with its cofactor, Friend of GATA1 (Fog1, also known as zinc finger protein, multitype 1, gene symbol *Zfpml1*) (Tsang *et al*, 1997). Both Gata1 and Fog1 are expressed mainly in haematopoietic cells, and are essential for the production of RBCs and megakaryocytes (Crispino, 2005; Ferreira *et al*, 2005). *Gata1*^{-/-} and *Zfpml1*^{-/-} embryos die from severe anaemia between E10.5 and E11.5 of gestation (Fujiwara *et al*, 1996; Tsang *et al*, 1998). Although there are primitive erythroid cells in the *Gata1*^{-/-} embryonic blood, these cells are arrested in their maturation at the proerythroblast stage. A similar block in erythroid maturation was also observed in *Zfpml1*^{-/-} embryos (Tsang *et al*, 1998). In addition, *Gata1*^{-/-} embryonic stem cells fail to yield mature definitive erythroid cells (Pevny *et al*, 1991; Weiss *et al*, 1994). Recently, the study of the conditional knockout of *Gata1* in adult mice revealed that Gata1 is essential for both steady-state and stress erythropoiesis (Gutierrez *et al*, 2008).

This study investigated the mechanisms involved in the ineffective erythropoiesis of chronic iron deficiency and the role of HRI in this process. We report here for the first time the inhibition of erythroid differentiation at basophilic erythroblast stage in iron deficiency. In addition, HRI was found to be necessary for adaptive gene expression in erythroid precursors in iron deficiency. *Gata1* expression in developing fetal liver (FL) erythroblasts

was shown to be decreased in iron deficiency and further decreased in combination with HRI deficiency. Moreover, *Zfpml* expression is decreased in combined iron and HRI deficiencies. Decreased *Gata1* and *Zfpml* expression is most likely to be responsible for a substantial change of gene expression in *Eif2ak1*^{-/-} erythroblasts in iron deficiency.

Materials and methods

Mouse breeding and sample collection

Mice production and experimentation were approved by the Committee on Animal Care at MIT. *Eif2ak1*^{-/-} mice, induction of iron deficiency and staining of blood smears were as described previously (Han *et al*, 2001). Blood samples and FLs were collected from E14.5 embryos.

Erythroid differentiation, cell cycle and apoptosis analyses

Erythroid differentiation of FL cells was examined by fluorescent-activated cell sorting (FACS) analyses using phycoerythrin (PE)-conjugated anti-Ter119 and fluorescein isothiocyanate (FITC)-conjugated anti-CD71 antibodies (BD Biosciences, San Jose, CA, USA) according to Zhang *et al* (2003). Sorting of FL cells at different stages of differentiation was performed using a BD FACS ARIA machine. Cell cycle status was examined by FACS analysis using propidium iodide (Chan *et al*, 2006). Apoptosis was analyzed using FITC-conjugated Annexin V as described previously (Han *et al*, 2001).

Western blot and quantitative reverse transcription polymerase chain reaction analyses

Protein extracts were prepared as described previously (Han *et al*, 2005) except with the supplement of 0.1% sodium dodecyl sulphate (SDS) and sonication. Western blot analyses of protein extracts (20 µg) were performed using antibodies against eIF2α, phosphorylated eIF2α (BioSource, Camarillo, CA, USA), *Gata1* and *Fog1* (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Total RNA isolation and quantitative reverse transcription polymerase chain reaction (RT-PCR) were performed as described (Liu *et al*, 2007). Primers for quantitative RT-PCR (qRT-PCR) were: *Gata1* forward primer 5'-GGCAAGACG-GCACTCTACC-3', reverse primer 5'-CAAGAACGTGTTGTTGCTCTTC-3'; *Zfpml* forward primer 5'-CTGAAGAAG-CCGCCAACTCA-3', and reverse primer 5'-AAGGCGCACATATAGCAGTCC-3', *eIF2a* primers were as described (Liu *et al*, 2007).

Gene profiling and microarray analysis

Affymetrix mouse expression array 430 A and B chips were used for gene profiling. Total RNAs from E14.5 FLs of iron-sufficient and deficient embryos of wild type (Wt) and *Eif2ak1*^{-/-} (Ko) (Wt+Fe, Wt-Fe, Ko+Fe and Ko-Fe) were used to prepare cRNA probes according to the Affymetrix protocol. Three replicates of gene chip microarray were performed for each of the four conditions. Raw data were normalized by quantile-based robust multichip average analysis (Jain *et al*, 2003). A local pooled error test (Jain *et al*, 2003) was used to identify genes that were expressed with statistical significance among the four groups. *P*-values were adjusted to reflect the false discovery rate (FDR) using the Benjamini-Hochberg method (Benjamini *et al*, 2001). Differentially expressed genes were defined by changes of gene expression at either twofold greater or less (with a FDR <0.1) in

Wt-Fe, Ko+Fe or Ko-Fe when compared to Wt+Fe. Functional classification of differentially expressed genes and prediction of protein interactions were performed using the Ingenuity Knowledge Base Pathway Analysis Solution software (Ingenuity Systems, Inc. Redwood City, CA, USA). Gene expression data is available at NCBI GEO database, accession no. GSE6808.

Statistical analysis

Two-tailed Student's *t*-test was used to analyze the statistical significance of FACS, cell cycle and qRT-PCR data; a *P*-value of <0.05 determined statistical significance.

Results

Impairment of erythroid differentiation in iron and HRI deficiencies

During embryonic development, FL is the main erythropoietic organ for definitive erythropoiesis. Erythroid precursors are the predominant cells in the E14.5 FL, which is widely used as a model for studying definitive erythropoiesis. To evaluate the effects of deficiencies of iron and HRI on erythroid differentiation, FACS analyses of E14.5 FL cells from iron sufficient and deficient *Eif2ak1*^{+/+} and *Eif2ak1*^{-/-} embryos (Wt+Fe, Wt-Fe, Ko+Fe and Ko-Fe) were carried out. As shown in Fig 1A, FL cells were divided into five populations based on their expression of CD71 and Ter119. P1, the CD71^{low} TER119^{low} population, contained mainly primitive progenitors. P2, the CD71^{high}TER119^{low} population, was mostly proerythroblasts. P3, the CD71^{high}TER119^{high} population, was mainly composed of basophilic erythroblasts. P4, the CD71^{med}TER119^{high} population, was predominantly chromatophilic and orthochromatophilic erythroblasts and P5, the CD71^{low}TER119^{high}, comprised late orthochromatophilic erythroblasts and reticulocytes. Most of Wt+Fe FL cells were present at the P4 stage (48.32%, Fig 1 and Table I). Under iron deficiency, however, most FL cells from both Wt-Fe (57.37%) and Ko-Fe (55.65%) were present at the less differentiated P3 stage. These results showed that erythroid differentiation of Wt-Fe and Ko-Fe FL cells was inhibited at the basophilic erythroblast stage. In addition, Ko+Fe FL cells also had a significant increase in percentage of cells in P3 when compared to Wt+Fe (31.72% vs. 20.52%, *P*= 0.019), albeit to a lesser degree than in iron deficiency.

The blood smears of E14.5 embryos were examined for the effects of HRI and iron deficiencies on embryonic red blood cell (RBC) development. The anaemia was more severe in Ko-Fe embryos with many globin inclusions in definitive embryonic RBCs and reticulocytes. No inclusion was observed in Wt+Fe, Ko+Fe or Wt-Fe embryonic blood cells (Fig 1B). These results underscore the role of HRI in inhibiting globin translation during embryonic red cell development in iron deficiency as seen in the adult mice (Han *et al*, 2001). Interestingly, the primitive nucleated RBCs were not affected in Ko-Fe embryos; there were no visible globin inclusions or morphological changes (Fig 1B). These results suggested that there was sufficient iron for primitive erythropoiesis under the iron-deficient conditions implemented.

Increased proliferation and accelerated apoptosis in Ko-Fe erythroid cells

To investigate the mechanism of impaired erythropoiesis in HRI and iron deficiencies, cell cycle and apoptosis analyses were performed. Cell cycle status was not affected by HRI deficiency alone (Fig 1C). However, the percentage of cells G0/G1 phase decreased significantly in Wt-Fe (54.17%, $P=0.001$) and was further decreased in Ko-Fe (47.91%, $P<0.001$) FL cells compared to that in Wt+Fe (60.36%). Concomitantly, there was increase in the percentage of cells in S phase in Wt-Fe (45.56%, $P=0.001$) and in Ko-Fe (51.34%, $P<0.001$) as compared to Wt+Fe (39.62%) (Fig 1C). These results showed the increased cell cycling in Wt-Fe and Ko-Fe FL cells. There was also an increase in the percentage of Annexin V⁺ apoptotic cells in the Wt-Fe FL cells (32.51%, $P=0.001$), and further increase in Ko-Fe FL cells (53.06%, $P<0.001$) compared with that in Wt+Fe cells (12.23%) (Fig 1C). The increase in apoptosis during iron deficiency was accompanied with a significantly decreased expression of antiapoptotic *Akt1* (3.25-fold for Wt-Fe and 10.27-fold for Ko-Fe) and *Bcl2l1* (2.11-fold for Wt-Fe and 4.99-fold for Ko-Fe) as seen in microarray analyses (data available at NCBI GEO database, no. GSE6808). Together, these results demonstrate that FLs develop ineffective erythropoiesis of increased proliferation and enhanced apoptosis during chronic iron deficiency, and that this ineffective erythropoiesis is more severe in HRI deficiency.

Alternation of gene expression in iron and HRI deficiencies

To gain insights into the protective role of HRI in the ineffective erythropoiesis of iron deficiency, gene profiling was carried out using RNAs from E14.5 FLs of Wt+Fe, Wt-Fe, Ko+Fe and Ko-Fe embryos as probes. The number of differentially expressed genes in Ko+Fe (73) was much smaller than that in Wt-Fe or Ko-Fe, consistent with the rather mild phenotypes in Ko+Fe embryos (Fig 1) and mice (Han *et al*, 2001). Expression of 213 genes was significantly altered in Wt-Fe. Strikingly, the number of differentially expressed genes increased 15-fold to 3135 in Ko-Fe (data available at NCBI GEO database, no. GSE6808). It is important to note that there was no significant difference between Wt-Fe and Ko-Fe FL cells in cell populations along erythroid differentiation based on CD71 and Ter119 expression (Fig 1 and Table I). Therefore, the difference in gene expression between Wt-Fe and Ko-Fe FL is intrinsic to HRI deficiency in these cells.

Many of differentially expressed genes in Ko-Fe FL were involved in cell growth and survival, protein synthesis and cellular assembly and organization, as would be expected from the increased proliferation and enhanced apoptosis in Ko-Fe erythroid precursors described above (Fig 1). Importantly, 5% of the differentially expressed genes are involved in hematological diseases, such as *Akt1*, *Bcl2l1*, *Casp8*, *Epor*, *Jak2*, *Myc*, *Pten*, *Sod2* etc. Expression of 6 and 34 known erythroid genes were altered significantly in Wt-Fe and Ko-Fe FLs, respectively. Many genes that are upregulated during erythroid differentiation, such as *Zfpml*, *Abcb10*, *Epor*, *Jak2*, *Eklf*, *Gypa*, *Epb 4-1*, *4-2* and *Stom*, *Trim10* and *Tspan33*, were downregulated three- to fourfold in Ko-Fe, and were known to be regulated by Gata1/Fog1 (Table SI). These results strongly support the hypothesis that, in iron deficiency, HRI is necessary to sustain terminal erythroid differentiation by maintaining the expression of many erythroid genes required for differentiation. Furthermore, these results also suggest an impairment of Gata1- and Fog1-induced erythropoiesis in Ko-Fe FL.

Diminished *Gata1* and *Zfpm1* expression in Ko-Fe erythroblasts

Ingenuity Pathway Analysis Solution software was used to help identifying the potential network of these altered gene expression. Decreased *Gata1* and *Zfpm1* expression was predicted to be linked to the changes of gene expression observed in Wt-Fe and Ko-Fe FL cells. In addition, our manual analysis of gene profiling results also indicated that *Gata1*/*Fog1* activity might be compromised, particularly in Ko-Fe FLs, as many of the affected genes were regulated by *Gata1*/*Fog1*. We therefore examined the expression of *Gata1* and *Zfpm1*. Although the microarray analysis did not reveal significant changes of *Gata1* mRNA, qRT-PCR analysis showed a significant decrease of *Gata1* mRNA in Wt-Fe and Ko-Fe FL cells (Fig 2A, $P < 0.05$). The difference in the expression changes of *Gata1* between genechip and qRT-PCR data is probably because the genechip data does not fully reflect the gene changes, especially for those genes with only one probe-set, such as *Gata1*. Consistent with microarray data, *Zfpm1* mRNA expression was significantly decreased only in Ko-Fe (Fig 2B, $P < 0.05$). In addition, both *Gata1* and *Fog1* proteins were expressed significantly lower in Ko-Fe as compared to Wt-Fe (Fig 2C, $P < 0.05$ $n = 3$).

To determine whether the decreased expression of *Gata1* and *Zfpm1* in iron and HRI deficiencies occurred at specific stages of erythroid differentiation, FL cells were sorted into three populations, Ter119^{low} (P1 + P2), Ter119^{high}CD71^{high} (P3) and Ter119^{high}CD71^{med} (P4). Expression of *Gata1* and *Fog1* in these three populations was shown in Fig 3. *Gata1* mRNA was decreased in all three populations during iron deficiency. In addition, *Gata1* mRNA was further decreased upon combined iron and HRI deficiencies in all three populations. Similarly, *Gata1* protein was also decreased in iron deficiency in all three populations and further decreased upon HRI deficiency. However, *Zfpm1* mRNA and protein were significantly decreased in all three populations only in Ko-Fe (Fig 3). We also found that *Gata1* and *Zfpm1* expression remained high during normal FL erythroblast maturation (Fig 3A). This result is consistent with reports that *Gata1* expression is necessary throughout erythroid maturation (Zheng *et al*, 2006; Marinkovic *et al*, 2007). Together, these results demonstrated that the decrease of *Gata1* and *Zfpm1* expression in combined iron and HRI deficiencies occurred throughout erythroid differentiation.

Upregulation of *Eif2ak1* during erythroid differentiation

To further understand the relationship of HRI and *Gata1*/*Zfpm1* expression, we examined the expression of *Eif2ak1* during erythroid differentiation in the three populations of FL cells described above. *Eif2ak1* expression, both mRNA and protein, were increased during erythroid differentiation with higher expression in Ter119^{high} cells (Fig 4). This higher expression of *Eif2ak1* in late erythroblasts, in which globin proteins are actively made in large quantities, is consistent with its known function in regulating globin translation. The contribution of HRI to eIF2 α phosphorylation among the family of eIF2 α kinases was also increased during erythroid maturation, from 35% in Ter119⁻ cells (P1 + P2) to 67.2% Ter119^{high}CD71^{high} cells (P3) and to 93.9% in Ter119^{high}CD71^{med} cells (P4) (Fig 4B, comparing *Eif2ak1*^{+/+} to *Eif2ak1*^{-/-} cells). It is important to note that the temporal expression of *Gata1* and *Zfpm1* (Fig 3A) is similar to that of *Eif2ak1* (Fig 3), consistent with the possibility of the regulation of *Gata1* and *Zfpm1* expression by HRI activated in iron deficiency.

Comparison of gene profiling of Ko-Fe with G1E-Er4 cells

To evaluate the significance of reduced expression of *Gata1/Zfpml* on gene expression in FL cells, the gene profiling data of Ko-Fe erythroid precursors were compared with that of G1E-Er4 cells (Welch *et al*, 2004). G1E-Er4 cells were derived from *Gata1*^{-/-} ES cells and expressed Gata1-ER fusion protein, which is activated upon induction of oestradiol. G1E-Er4 cells proliferate as immature erythroblasts, but undergo terminal differentiation upon restoration of Gata1 function (Weiss *et al*, 1997). At 30 h after Gata1 activation, most of G1E-Er4 cells resembled basophilic erythroblasts expressing haemoglobin (Weiss *et al*, 1997). Given that *Gata1* expression was decreased in Ko-Fe cells, we would expect that a number of differentially expressed genes were in common but regulated in opposite directions between Ko-Fe and Gata1-activated G1E-Er4 cells. The number of these genes increased from 15 at 3 h to 120 at 30 h after the induction of G1E-Er4 cells by oestradiol, with a total of 133 genes throughout the 30 h of Gata1 activation. The majority of these genes (79.7%) were upregulated by Gata1 in G1E-Er4 cells and downregulated in Ko-Fe. Some genes of interest relating to erythroid maturation are summarized in Table II.

Consistent with our results (Fig 4), *Eif2ak1* expression was increased during differentiation of G1E-Er4 cells upon activation of *Gata1* starting at 7 h [Table II and (Welch *et al*, 2004)]. This result provides further support that Gata1 activity was diminished in Ko-Fe FL cells and suggests that HRI is required to maintain *Gata1* expression and function in these cells during chronic iron deficiency.

Discussion

We have previously shown that HRI is important in protecting erythroid precursors during iron/haem deficiency and in β -thalassaemia (Han *et al*, 2001, 2005). HRI exerts this protective function in part by inhibiting protein synthesis to reduce globin aggregates. However, it is also established that activation of eIF2 α kinases upon stress also increases translation of specific mRNAs to alter gene expression for the adaptation to stress (Holcik & Sonenberg, 2005; Chen, 2007). We report here the alternation of gene expression in developing FL erythroblasts upon iron and HRI deficiencies. We demonstrate that in chronic iron deficiency HRI is also required for adaptive gene expression in the cellular processes of proliferation, apoptosis, erythroid differentiation and iron/haem homeostasis.

This study demonstrated for the first time that definitive erythropoiesis is inhibited at the basophilic erythroblast stage during iron deficiency in both *Eif2ak1*^{+/+} and *Eif2ak1*^{-/-} FLs. However, *Eif2ak1*^{-/-} embryos exhibit more severe ineffective erythropoiesis and anaemia with reticulocytosis and pronounced globin inclusions in the reticulocytes of embryonic blood. These phenotypic changes in fetal definitive erythropoiesis induced by iron deficiency correlated very well with the gene profiling data of E14.5 FL cells under these conditions. The extent of differentially expressed genes in iron deficiency was increased 15-fold upon HRI deficiency. These results underline the significant role of HRI in erythroid development during iron deficiency. Although Ko+Fe FL cells displayed a mild defect in erythroid differentiation *in vivo*, these cells showed a significant block of maturation at the basophilic erythroblast stage when cultured *in vitro* where conditions may not be as optimal as *in vivo* (R.N.V.S. Suragani and J.-J. Chen unpublished observations). We have also

reported earlier that the survival of *Eif2ak1*^{-/-} mice is severely compromised upon phenylhydrazine induced acute hemolytic anaemia as compared to Wt mice (Han *et al*, 2001). Together, these results underscore a functional role of HRI in stress erythropoiesis and are consistent with the fact that HRI and other eIF2 α kinases are stress-activated kinases (Holcik & Sonenberg, 2005; Chen, 2007).

Our microarray data also demonstrated changes of expression of many of Gata1/Fog1 target genes in Ko-Fe FL. We show for the first time that *Gata1* expression is decreased in developing erythroblasts during iron deficiency. Interestingly, *Zfpm1* expression is reduced only under combined iron and HRI deficiencies. Consistent with the compromised Gata1/Fog1 functional activity in Ko-Fe erythroblasts, we demonstrated that more than 100 genes were downregulated more than twofold in Ko-Fe FL cells, but were upregulated in G1E-ER4 cells by Gata1 activation.

Both Gata1 and Fog1 are essential for the proliferation, survival and differentiation of the erythroid lineage (Cantor & Orkin, 2005). Previous studies of knockdown mutations of *Gata1*, *Gata1*^{low} and *Gata1-05/X*, have revealed that erythroid maturation is dependent on the level of Gata1 (McDevitt *et al*, 1997; Takahashi *et al*, 1998). Moreover, expression of *Gata1* at late-stage maturation is essential for the terminal differentiation of primitive and definitive erythroblasts (Zheng *et al*, 2006). It should be noted that the level of Gata1 in Ko-Fe (26%) FL is similar to that of *Gata1*^{low}. Therefore, the attenuation of phenotypic severity and *Gata1* expression by HRI in iron deficiency reported here provides further support for such a dose-dependent effect of Gata1 on erythroid maturation. In addition, a reduced level of Gata1 is observed in *Lyn*^{-/-} J2E-NR cells, which fail to differentiate upon exposure to erythropoietin (Tilbrook *et al*, 1997). Like *Gata1*^{low} and Ko-Fe mice, *Lyn*^{-/-} mice (Tilbrook *et al*, 1997) also display an impairment of erythropoiesis with severe splenomegaly. Thus, reduced Gata1 activity in erythroid precursors may be a general mechanism responsible for ineffective erythropoiesis. Physical interaction of Gata1 and Fog1 is necessary for erythroid maturation (Cantor & Orkin, 2005; Crispino, 2005). Mutations of *Gata1* that disrupt its binding to Fog1 have been documented in several families with X-linked congenital anaemia and thrombocytopenia (Cantor & Orkin, 2005; Crispino, 2005). Thus, decreased *Zfpm1* expression in HRI deficiency also contributes to ineffective erythropoiesis in iron deficiency.

Cleavage of Gata1 by caspase 3 has been implicated as a negative regulatory mechanism of erythropoiesis (De Maria *et al*, 1999). Recently, hsp70 has been shown to regulate erythropoiesis by preventing Gata1 cleavage by caspase 3 (Ribeil *et al*, 2007). The present study found that, in iron deficiency, decreased Gata1 protein expression correlated well with decreased mRNA expression. These results suggest that regulation of Gata1 in FL erythroblasts during chronic iron deficiency probably occurs at the RNA level rather than at the protein stability. While the mechanism of decreased *Gata1* and *Zfpm1* expression in HRI deficiency remains to be further investigated, it is possible that activation of HRI in iron deficiency may inhibit the translation of some unknown short-lived proteins that are necessary to maintain *Gata1* mRNA level. Such a translational repression has recently been documented for Mcl-1 upon stress activation of eIF2 α kinases (Fritsch *et al*, 2007). Little is known about the regulation of *Gata1* mRNA besides auto-regulation (Vyas *et al*, 1999; Nishimura *et al*, 2000; Yu *et al*, 2002a). It was reported that the BMP/Smad pathway could

direct the onset of *Gata1* expression during erythroid differentiation of the embryonic body (Adelman *et al*, 2002). We recently found that the serum level of BMP2 was decreased threefold in Ko-Fe, but not in Wt-Fe mice (S.J. Liu and J.-J. Chen, unpublished observations). It is possible that the effect of HRI on *Gata1* expression in iron deficiency may be indirect through BMPs. These possibilities remains to be investigated further.

The rapid induction of *Eif2ak1* expression upon activation of Gata1 in G1E-ER cells (Table II) and the temporal of *Eif2ak1* and *Gata1* expression during normal erythroid differentiation (Fig 3) also raised the possibility of the potential regulation of *Eif2ak1* expression by Gata1, which would confer the high level expression of *Eif2ak1* in the erythroid lineage (Chen, 2007). Mutation of *Gata1* with either reduced interaction with Fog1 (V205G) (Crispino *et al*, 1999) or decreased DNA binding (R216N) (Yu *et al*, 2002b) has been shown to result in reduced expression of *Eif2ak1* in G1E-ER cells, consistent with the potential regulation of *Eif2ak1* expression by Gata1.

In summary, our study demonstrates the impairment of erythroid differentiation and *Gata1* expression in iron deficiency. Furthermore, HRI is required to maintain the Gata1/Fog1 function during stress erythropoiesis in the setting of iron deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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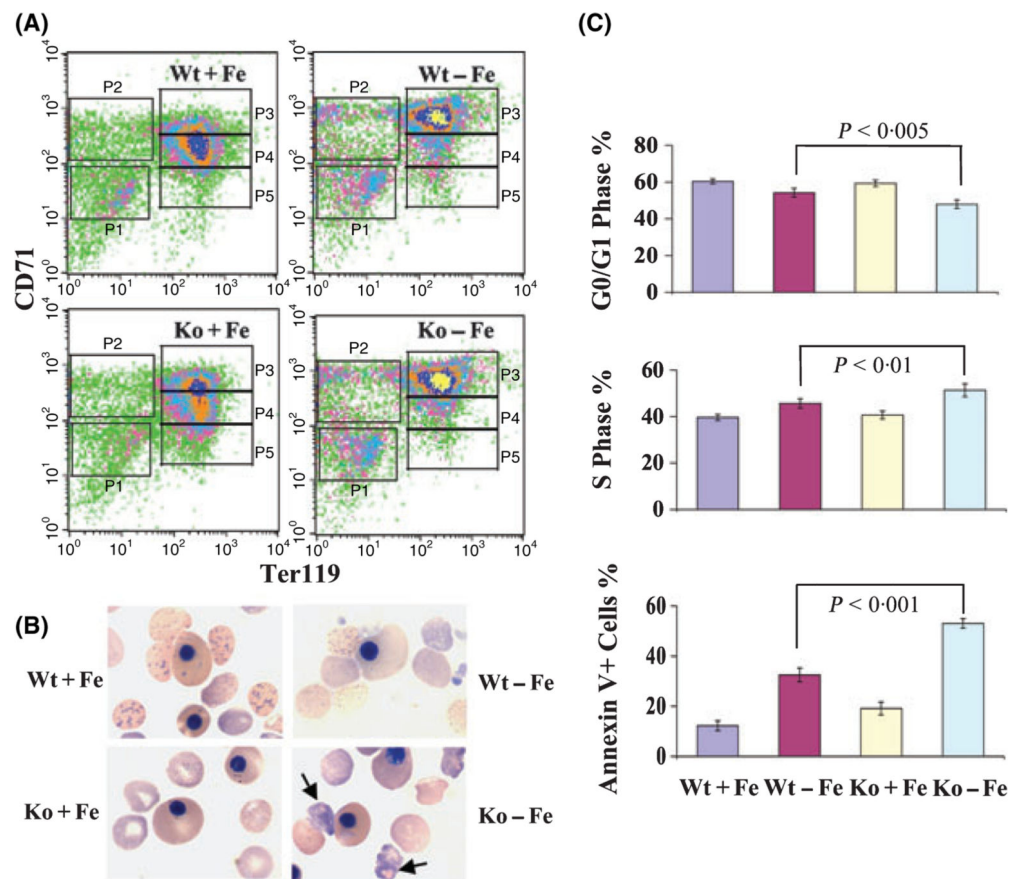


Fig 1. Erythroid differentiation of FL in iron deficiency and HRI deficiency. (A) FACS analysis of the erythroid differentiation of E14.5 FL cells. (B) Blood smears of E14.5 embryos. Arrows indicate globin inclusions in Ko-Fe reticulocytes. (C) Cell cycle and apoptosis in E14.5 FL cells. Results are presented as mean \pm SD ($n = 6$).

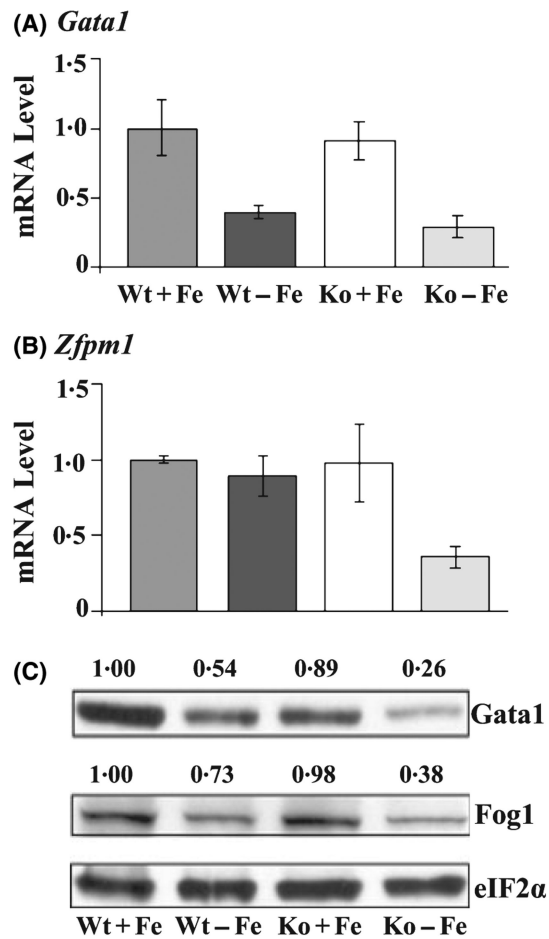


Fig 2. Expression of *Gata1* and *Zfpml* in FL under iron and HRI deficiencies. (A) Expression of *Gata1* mRNA. (B) Expression of *Zfpml* mRNA. (C) Expression of *Gata1* and *Zfpml* proteins. The results of mRNA expression are presented in mean \pm SD ($n = 4$). The intensities of the autoradiograms in the Western blots were quantified by Alpha EaseFC software. Expression of proteins was normalized with eIF2 α . Expression of proteins in Wt +Fe is defined as 1. The normalized values are shown above the autoradiograms.

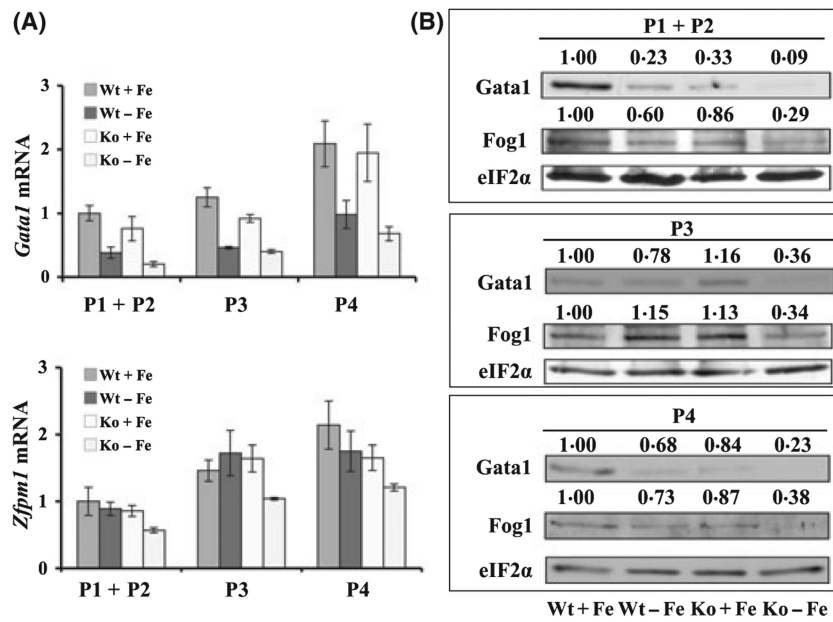


Fig 3. Expression of *Gata1* and *Zfpml1* in sorted cell populations of FL under iron and HRI deficiencies. (A) Expression of *Gata1* and *Zfpml1* mRNAs in three sorted populations. Results are presented in mean \pm SD ($n = 4$). (B) Expression of *GATA1* and *Zfpml1* proteins in sorted cells. Quantification of Western blots was as described in the legend of Fig 2.

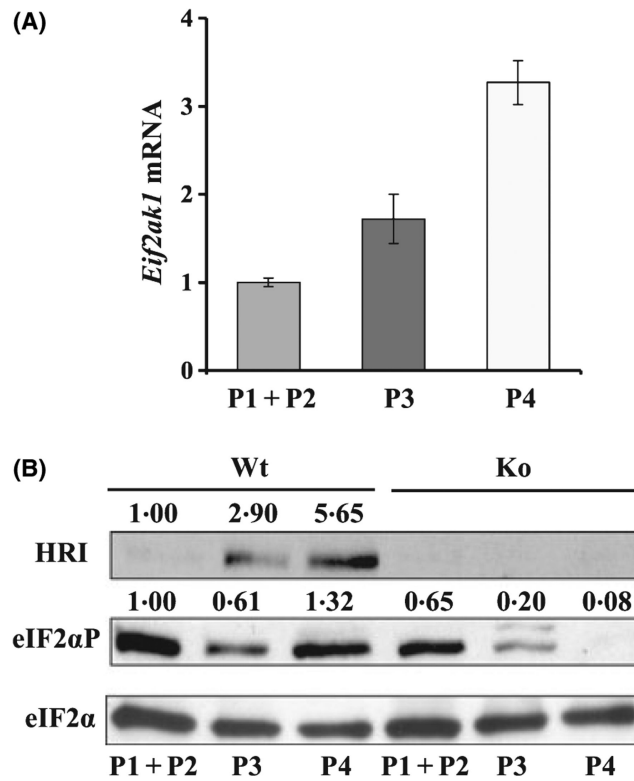


Fig 4. Upregulation of *Eif2ak1* during erythroid differentiation. (A) Expression of *Eif2ak1* mRNA in three sorted populations. (B) Western blot analyses of HRI, eIF2αP, and eIF2α. Expression of proteins in P1 + P2 of Wt is defined as 1. Quantification of Western blots was as described in the legend of Fig 2.

Table I

Distribution of Wt and Ko fetal liver cells at different stages of erythroid differentiation under iron-sufficient or iron-deficient conditions.

	P1 (%)	P2 (%)	P3 (%)	P4 (%)	P5 (%)
Wt+Fe	8.95 ± 2.37	5.47 ± 1.98	20.52 ± 6.74	48.32 ± 7.52	6.50 ± 2.63
Wt-Fe	10.47 ± 1.05	8.53 ± 2.24 *	57.37 ± 3.20 *	9.96 ± 3.32 *	2.67 ± 0.73 *
Ko+Fe	10.27 ± 2.09	4.60 ± 1.60	31.72 ± 5.19 *	36.80 ± 6.15 *	6.37 ± 1.31
Ko-Fe	11.46 ± 1.21	12.28 ± 2.80 *†	55.65 ± 6.23 *	6.61 ± 1.08 *†	1.79 ± 0.92 *†

Results are presented in mean ± SD ($n = 5-6$).

* $P < 0.05$, compared with Wt+Fe.

† $P < 0.05$, compared with Wt-Fe.

Table II

Comparison of gene expression between G1ER and Ko-Fe FL cells.

Gene symbol	Gene name	3h	7h	14 h	21 h	30 h	Ko-Fe
Erythroid genes							
<i>Add1</i>	Adducin 1	↑	↑↑	↑↑↑	↑↑↑	↑↑↑↑	↓↓↓
<i>Eif2ak1</i>	Haem-regulated eIF2α kinase	–	↑	↑↑	↑↑↑	↑↑↑	N/A
<i>Gypa</i>	Glycophorin A	–	↑	↑↑↑	↑↑↑	↑↑↑	↓↓
<i>Stom (Epb7-2)</i>	Erythrocyte protein band 7-2	–	↑	↑	↑	↑	↓
<i>Zfpml</i>	Zinc finger protein, multitype 1	–	↑	↑	↑	↑↑	↓
<i>Klfl1</i>	Kruppel-like factor 1 (erythroid)	–	–	↑	↑	↑	↓
<i>Pkfr</i>	Pyruvate kinase liver and red blood cell	–	–	↑	↑	–	↓
<i>Bcl2l1</i>	Bcl2-like (Bcl2l1) 1	–	–	–	↑	↑↑	↓↓
<i>Aqp1</i>	Aquaporin 1	–	–	–	–	↑	↓↓
<i>Bpgm</i>	2,3-bisphosphoglycerate mutase	–	–	–	–	↑	↓↓
Iron/haem genes							
<i>Trfr2</i>	Transferrin receptor 2	–	↓	↓	↓↓	↓↓	↑
<i>Urod</i>	Uroporphyrinogen decarboxylase	–	–	↑	↑	↑	↓
<i>Fech</i>	Ferrochelatase	–	–	–	–	↑	↓↓
<i>B2m</i>	β-2 microglobulin	–	–	–	–	↑	↓↓↓
Proliferation, apoptosis and survival-related genes							
<i>Pim1</i>	Proviral integration site 1	↓	–	↑	↑	↑	↓↓
<i>Btg2</i>	B-cell translocation gene 2, anti-proliferative	–	↑	↑↑	↑↑	↑↑↑	↓↓↓
<i>Cdkn2c</i>	Cyclin-dependent kinase inhibitor 2C	–	–	↑	↑	↑	↓
<i>Cdkn1a</i>	Cyclin-dependent kinase inhibitor 1A	–	–	–	–	↑	↓
<i>Ptdss2</i>	Phosphatidylserine synthase 2	↑	↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↓↓
Antioxidant genes							
<i>Selenbp1</i>	Selenium binding protein 1	–	↑	↑	↑	↑↑	↓
<i>Ddt</i>	D-dopachrome tautomerase	–	–	↑	↑	↑↑↑↑	↓
<i>Txnrd2</i>	Thioredoxin reductase 2	–	–	↑	↑↑	↑	↓↓
Others							
<i>Tcf4</i>	Transcription factor 4	↓↓↓	↓↓	–	–	↓	↑↑
<i>Slc31a1</i>	Solute carrier family 31, member 1	–	–	↑	↑	↑↑	↓

Hr 3–30: time course of G1ER cells upon induction of *Gata1* in hours.

Fold changes: ↓↓↓↓, –32 to –16; ↓↓↓, –16 to –8; ↓↓, –8 to –4; ↓, –4 to –2; –, –2 to 2; ↑, 2 to 4; ↑↑, 4–8; ↑↑↑, 8–16; ↑↑↑↑, 16–32; N/A, not applicable.