Heme-regulated eIF2α kinase activated Atf4 signaling pathway in oxidative stress and erythropoiesis

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Heme-regulated eIF2α kinase (Hri) is necessary for balanced synthesis of heme and globin. In addition, Hri deficiency exacerbates the phenotypic severity of β-thalassemia intermedia in mice. Activation of Hri during heme deficiency and in β-thalassemia increases eIF2α phosphorylation and inhibits globin translation. Under endoplasmic reticulum stress and nutrient starvation, eIF2α phosphorylation also induces the Atf4 signaling pathway to mitigate stress. Although the function of Hri in regulating globin translation is well established, its role in Atf4 signaling in erythropoietic precursors is not known. Here, we report the role of the Hri-activated Atf4 signaling pathway in reducing oxidative stress and in promoting erythroid differentiation during erythropoiesis. One acute oxidative stress, Hri−/− erythroblasts suffered from increased levels of reactive oxygen species (ROS) and apoptosis. During chronic iron deficiency in vivo, Hri is necessary both to reduce oxidative stress and to promote erythroid differentiation. Hri−/− mice developed ineffective erythropoiesis during iron deficiency with inhibition of differentiation at the basophilic erythroblast stage. This inhibition is recapitulated during ex vivo differentiation of Hri−/− fetal liver erythroid progenitors. Importantly, the Hri-eIF2αP-Atf4 pathway was activated and required for erythroid differentiation. We further demonstrate the potential of modulating Hri-eIF2αP-Atf4 signaling with chemical compounds as pharmaceutical therapies for β-thalassemia. (Blood. 2012; 119(22):5276-5284)

Introduction

Heme-regulated eIF2α kinase (Hri) was the first discovered member of the family of eIF2α kinases, which control translational initiation under diverse stress conditions by phosphorylation of the α-subunit of eIF2 (eIF2αP).1,2 Hri is necessary to coordinate translation of globin mRNAs with the availability of heme for the production of large amounts of hemoglobin during erythroid maturation.3 In Hri deficiency, excessive globins synthesized during heme deficiency precipitate and form inclusions causing proteotoxicity.1,4 Beyond heme deficiency, Hri is also activated by oxidative stress and denatured proteins,5 both of which occur in thalassemia.6 Indeed, Hri is required to reduce the phenotypic severity of the Hbb−/− mouse model of β-thalassemia intermedia lacking both copies of β-globin major.4

Besides Hri, there are 3 additional mammalian eIF2α kinases: the double-stranded RNA-dependent eIF2α kinase (Pkr), the Gcn2 protein kinase, and the Pkr-like ER resident kinase (Perk).1 Each kinase elicits a major physiologic response in vivo: Pkr responds to viral infection; Gcn2 senses nutrient starvations; Perk is activated by endoplasmic reticulum (ER) stress; and Hri is regulated by heme. In addition to inhibition of global protein synthesis, the second function of eIF2α phosphorylation is to reprogram translation and transcription necessary for adaptation to stress7 as illustrated in Figure 7A. In mammalian cells, translation of activating transcriptional factor 4 (Atf4) is selectively increased by eIF2αP amid general inhibition of protein synthesis during ER stress and amino acid starvation via upstream open reading frames in the 5′-UTR (untranslated region).8,9 A major target gene of Atf4 is the transcription factor, C/EBP Homologous Protein-10 (Chop), which is up-regulated on stress.10 In ER stress, induction of Chop leads to expression of Gadd34, which recruits eIF2αP for dephosphorylation by Ppase1,11,12 This feedback mechanism of Gadd34 in regenerating active eIF2 is necessary for the recovery of protein synthesis during the late stage of the stress response.13,14

Although we demonstrated the necessity of Hri in inhibiting globin translation during iron/heme deficiency and in β-thalassemia, the role of Hri under these stress conditions is more than just inhibition of translation. Hri is also necessary to reduce ineffective erythropoiesis.3,4 To further understand the molecular mechanism by which Hri mitigates stress and reduces ineffective erythropoiesis, we investigated the eIF2αP-Atf4 signaling pathway in the stress response of the erythroid lineage.

In this article we demonstrate that Hri activates the Atf4 stress response pathway in nucleated erythroid precursors for adaptation to oxidative stress. Significantly, this Hri-dependent eIF2αP-Atf4 pathway is also operative and necessary for erythroid differentiation, especially under stress conditions. Our study reveals the function of Hri in translational activation of Atf4 mRNA and the subsequent regulation of gene expression necessary for stress adaptation. We also demonstrate the feasibility of targeting the Hri-eIF2αP-Atf4 pathway for the treatment of β-thalassemia with the small chemical salubrinal, which inhibits dephosphorylation of eIF2αP and promotes the Atf4 signaling pathway.15

The online version of this article contains a data supplement.
Methods

Animals

Mice were maintained at the Massachusetts Institute of Technology (MIT) animal facility, and all experiments were carried out using the protocols approved by the Division of Comparative Medicine at MIT. Mice were generated in our laboratory. The generations of Atf4\textsuperscript{+/+} and Hri\textsuperscript{+/+} mice were as previously described.\textsuperscript{16} All mice used for this study were of C57BL/6J genetic background. Mice were maintained in an iron-deficient diet (Harlan Teklad) on weaning for 2 months before analysis.

Fetal liver cell isolation and ex vivo differentiation

Ter119\textsuperscript{+} cells were isolated from E14.5 fetal liver (FL) and cultured for ex vivo differentiation as described.\textsuperscript{17} Morphologic examination of cells was performed with Sigma-Aldrich Mission small interfering RNAs (siRNAs) using the protocol described by Paradkar et al.\textsuperscript{20} Atf4 knockout siRNA sequences were shown in supplemental Table 2. Mission siRNA Universal Negative Control #1 (Sigma-Aldrich) was used as a negative control.

Salubral treatment and measurement of protein synthesis

Blood samples from adult Hri\textsuperscript{+/+}/Hbb\textsuperscript{+/+} mice, which contained approximately 30% reticulocytes, were recovered in complete DMEM medium as described. Cells were then treated with salubral as indicated. E14.5 FL cell were pretreated with 5μM of arsenite for 2 hours to enhance eIF2αP levels. Cells were washed with PBS to remove arsenite and then treated with various concentrations of salubral as indicated. Splenic Ter119\textsuperscript{+} cells from Hbb\textsuperscript{+/+} mice were isolated using magnetic-activated cell sorting MACS column purification (Miltenyi Biotech). Cells were then recovered for 30 minutes before salubral treatment at concentrations indicated for 3.5 hours. The rate of globin synthesis was determined by S\textsuperscript{35}-methionine/cysteine incorporation into globin proteins.\textsuperscript{3}

Data analysis

The statistical analyses among the various groups were performed by the 2-tailed Student t test with a P value of <.05 for statistical significance.

Results

Activation of Hri-eIF2αP-Atf4 pathway in erythroid precursors on arsenite-induced oxidative stress

We have shown earlier that Hri is the eIF2α kinase activated by treatment with sodium arsenite in erythroid precursors and that the activation of Hri by arsenite is prevented by pretreatment with the ROS scavenger N-acetylcysteine. To gain further insight into mechanistic details of ROS production and Hri activation, ROS levels in E14.5 FL cells, which are highly enriched in nucleated erythroid precursors, were measured after arsenite treatment. As shown in Figure 1A, ROS levels increased rapidly within 15 minutes and peaked at 30 minutes on arsenite treatment of FL cells. Concomitantly, activation of Hri also peaked at 30 minutes after arsenite treatment. Hri remained activated for at least 60 minutes as shown by the hyperphosphorylation of Hri and the increased levels of eIF2αP (Figure 1B).

Arsenite treatments also resulted in the Hri-dependent induction of the eIF2αP-Atf4 signaling pathway as shown by increased levels of eIF2αP, Atf4 and Chop proteins in Hri\textsuperscript{+/+} FL cells at 1 to 6 hours of exposure to arsenite (Figure 1C). There was no increase in eIF2αP, Atf4, or Chop protein levels in Hri\textsuperscript{−/−} cells on arsenite stress. Importantly, the Atf4 mRNA level did not change significantly on arsenite stress in either Hri\textsuperscript{+/+} or Hri\textsuperscript{−/−} cells (Figure 1D), consistent with the immediate translational regulation of Atf4 by increased eIF2αP.\textsuperscript{9} Both Chop and Gadd34 mRNA levels were increased in Hri\textsuperscript{+/+} cells on arsenite stress, consistent with their transcriptional induction by Atf4. In contrast to Gadd34, the constitutively expressed regulator of eIF2αP dephosphorylation Crep\textsuperscript{21} was not affected by arsenite stress as would be expected (Figure 1D). These results establish that activation of Hri by arsenite stress elicits the eIF2αP signaling pathway of up-regulating Atf4, Chop, and Gadd34 in primary erythroid precursors.

Increased oxidative stress in Hri\textsuperscript{−/−} erythroid precursors on arsenite stress

To investigate whether this up-regulation of the Hri-Atf4 signaling pathway might contribute to adaptation to oxidative stress, levels of ROS in Hri\textsuperscript{+/+} and Hri\textsuperscript{−/−} FL cells were measured at different time points. The statistical analyses among the various groups were performed by the 2-tailed Student t test with a P value of <.05 for statistical significance.

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Increased oxidative stress in Hri\textsuperscript{−/−} erythroid precursors on arsenite stress

To investigate whether this up-regulation of the Hri-Atf4 signaling pathway might contribute to adaptation to oxidative stress, levels of ROS in Hri\textsuperscript{+/+} and Hri\textsuperscript{−/−} FL cells were measured at different time
prominent and required higher concentrations compared with 
Hri<sup>−/−</sup> cells (Figure 2A). Furthermore, the level of Ho-1 mRNA was 
significantly increased on arsenite treatment in Hri<sup>+/+</sup> cells, 
but not in Hri<sup>−/−</sup> cells (Figure 2C).

We then examined whether the induction of Ho-1 by arsenite 
required Atf4, a downstream target of Hri signaling pathway 
(Figure 1C). As shown in Figure 2B, there is less Ho-1 protein in 
Atf4<sup>−/−</sup> FL cells compared with Atf4<sup>+/+</sup> or Atf4<sup>+/−</sup> cells at 
the baseline without arsenite treatment. On arsenite treatment, Ho-1 
protein expression was increased in FL cells from all the 3 genotypes. 
However, there was still less Ho-1 protein in Atf4<sup>−/−</sup> cells (Figure 2B). These results demonstrate that Ho-1 expression in primary 
erythroid precursors is dependent on both Hri and Atf4.

Besides Hri-, arsenite treatment also induced expression of other 
antioxidant genes (Figure 2C), such as glutathione S-transferase 
μ (GSTM), NAD(P)H quinone oxidoreductase 1 (Nqo1) and 
superoxide dismutase 2 (Sod2) in Hri<sup>+/+</sup>, but not in Hri<sup>−/−</sup> FL cells. 
Similarly, induction of Ho-1 and Nqo1 mRNA was observed in 
Atf4<sup>+/+</sup>, but not in Atf4<sup>−/−</sup> cells (Figure 2D). These results 
demonstrate that both Hri and Atf4 are necessary to induce some 
antioxidant genes to mitigate oxidative stress incurred by arsenite 
treatment. Consistent with decreased expression of Ho-1 and Nqo1, 
RBCs from Hri<sup>−/−</sup> and Atf4<sup>−/−</sup> mice were more sensitive to 
H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and exhibited increased ROS levels 
compared with RBCs from wild-type (WT) and Atf4<sup>+/+</sup> mice 
(Figure 2E; quantitative data of ROS levels are shown in supplemen-
tal Figure 3A). These results indicate that Hri-Atf4 signaling may

Figure 1. Induction of the Hri-eIlf2α-P-Atf4 signaling pathway and protection 
against oxidative stress in FL erythroblasts. (A) ROS levels. Hri<sup>+/+</sup> FL cells 
were treated with 25μM sodium arsenite. At times indicated, cellular ROS levels 
were measured by DCF fluorescence. (B) Hyperphosphorylation and activation of Hri. 
Hyperphosphorylation of Hri was examined by the slower migration in SDS-PAGE. 
eIlf2α was used as a loading control. (C) Induction of the Hri-dependent Atf4 signaling pathway components. Atf4, Chop, Gadd34, and 
Crep mRNA levels from FL cells treated with 5μM As for 3 hours were analyzed by 
qPCR. Expression of each of the mRNA in untreated Hri<sup>−/−</sup> cells was defined as 1.

Hri and Atf4 dependent expression of Ho-1 and antioxidant 

genes

To further investigate the mechanism by which Hri protects against 
oxidative stress, we examined the expression of heme oxygenase 1 (Ho-1), 
which catalyzes heme to biliverdin, CO, and Fe, and plays a pivotal role in 
reducing oxidative stress. We found that Ho-1 protein in Hri<sup>+/+</sup> 
erythroblasts was increased by treatment with arsenite from 0.1 to 3μM (Figure 2A). Interestingly, the 
increase of Ho-1 expression by arsenite in Hri<sup>−/−</sup> cells was less 

Figure 2. Hri and Atf4 dependent induction of antioxidant genes. (A) 
Hri-dependent Ho-1 protein expression. Hri<sup>+/+</sup> and Hri<sup>−/−</sup> FL cells were treated for 
5 hours with increasing concentrations of arsenite as indicated. (B) Atf4-dependent 
Ho-1 protein expression. Atf4<sup>+/−</sup>, +/−, and −/− FL cells were treated with 5μM arsenite for 3 hours. (C-D) Relative expression of mRNA of antioxidant genes. Hri<sup>+/+</sup>, 
Hri<sup>−/−</sup>, Atf4<sup>+/+</sup>, and Atf4<sup>−/−</sup> FL cells were treated with 5μM arsenite for 3 hours. 
mRNA levels of treated and untreated cells were analyzed by qPCR. Data are 
presented as mean ± SD (n = 3). P values denote the comparison between arsenite-
treated Hri<sup>+/+</sup> and Hri<sup>−/−</sup> samples; P < .001 for Atf4 and Gadd34; P < .005 for Chop. (E) ROS levels. Hri<sup>+/+</sup> and Hri<sup>−/−</sup> FL cells were treated with 5 or 25μM arsenite 
for 9 hours before ROS measurement.
also be required during erythroid development. Impairment of this pathway may generate RBCs that are more sensitive to oxidative insults.

In increased oxidative stress in Hri−/− erythroid cells during chronic iron deficiency in vivo

As shown above, Hri is required for reducing acute oxidative stress in vitro (Figure 1E). We investigated whether Hri also participated in reducing oxidative stress of erythroid cells in vivo during chronic iron deficiency. Levels of ROS were measured in RBCs from WT and Hri−/− (KO) mice maintained on either a normal diet or an iron-deficient diet. Representative histograms of ROS levels by FACS analysis are shown in Figure 2F. The quantitative data of ROS levels are shown in supplemental Figure 3B. It is important to note that iron deficiency alone (WT-Fe) did not significantly increase ROS levels in RBCs. However, during iron deficiency, the absence of Hri (KO-Fe) resulted in dramatically elevated ROS levels in RBCs compared with those of WT-Fe cells (Figure 2F). These results demonstrate that Hri is necessary to mitigate oxidative stress in erythroid cells under chronic stress of iron deficiency in vivo. These findings are also consistent with our gene profiling data indicating that expression of many redox genes, such as catalase, Sod2, Gsts, thioredoxin reductase 2, peroxiredoxins, ferritins, and Ho-1 is significantly lower in KO-Fe FL cells compared with WT-Fe cells.23 Together, these results indicate that both Hri-mediated inhibition of globin translation and up-regulation of the Atf4 signaling pathway contribute in mitigating oxidative stress during iron deficiency.

Inhibition of erythroid differentiation in Hri−/− mice during chronic iron deficiency in vivo

We have shown earlier that fetal definitive erythropoiesis in iron deficiency is inhibited at the basophilic erythroblast stage and that this inhibition is more severe in Hri deficiency.23 Here, we examined erythroid differentiation in adult mice during iron deficiency. We found that there was no statistically significant increase of CD71highTer119high erythroblasts in adult WT mice during iron deficiency (Figure 3A). However, there was a mild inhibition of erythroid differentiation at the basophilic stage along with mild splenomegaly in approximately 20% of WT-Fe mice. The more severe inhibition of erythroid differentiation during fetal definitive erythropoiesis in response to iron deficiency is probably attributable to the much greater need for iron to accommodate a higher rate of erythropoiesis during embryonic development.

Most importantly, there was a profound inhibition of erythroid differentiation with a substantial accumulation of CD71highTer119high erythroblasts concomitant with diminished CD71lowTer119low erythroid cells in the spleens of Hri−/− mice during iron deficiency (Figure 3A, P < .005). Furthermore, these CD71highTer119high erythroblasts exhibited basophilic morphology (Figure 3B). The inhibition of erythroid differentiation was also observed in the bone marrow of KO-Fe mice (supplemental Figure 4). Further analysis of erythroid differentiation using CD44 described by Chen et al24 showed a significant accumulation of CD44highFSChigh cells in the bone marrow and spleens of Hri−/− mice under iron deficient conditions (supplemental Figure 5). Thus, these results indicate that erythroid expansion in the spleen and bone marrow of KO-Fe mice is associated with severe inhibition of erythroid differentiation.

Apoptosis of CD71+ erythroblasts during iron deficiency in vivo

We have previously shown that the percentage of apoptotic cells in Ter119+ erythroid precursors from FL, spleen, and bone marrow of Hri−/− mice increased during iron deficiency.3,23 We investigated apoptosis more closely by examining the degree to which erythroid precursors at different stages of differentiation undergo apoptosis during iron deficiency. Representative density plots of the apoptosis of Ter119+ erythroid precursors are shown in Figure 3C, whereas the quantitative data analysis is shown in Figure 3D. We found that the percentage of AnV+ apoptotic cells in total splenic Ter119+ erythroid precursors (Figure 3C right top and bottom quadrants combined) significantly increased during iron deficiency (P < .05), and further increased on combined deficiencies of Hri and iron (P < .05). In addition, this increase in apoptosis occurred mostly in CD71+Ter119+ erythroblasts (top right quadrants of Figure 3C); there were few AnV+ cells among CD71−Ter119+ erythroblasts (bottom right quadrants of Figure 3C). These results
also confirm an expansion of splenic CD71+ erythroblasts among Ter119+ erythroid precursors from KO-Fe mice (top left and right quadrants of Figure 3C).

It is possible that the increase in percentage of AnV+ apoptotic cells in KO-Fe splenic Ter119+ erythroid precursors might simply be because of the expansion of CD71−Ter119+ erythroblasts in the spleens of KO-Fe mice. We therefore analyzed the percentage of AnV+ apoptotic cells in CD71−Ter119+ erythroblasts (Figure 3D). The percentage of apoptotic cells in CD71−Ter119+ erythroblasts was increased in WT-Fe spleens, but was not further increased in KO-Fe spleens (Figure 3D). Similar observations were made with bone marrow samples (data not shown).

Collectively, these results demonstrate that ineffective erythropoiesis of KO-Fe mice is primarily because of the profound inhibition of erythroid differentiation at the CD71−Ter119+ erythroblast stage. The increase of apoptotic cells in total Ter119+ erythroid precursors observed in KO-Fe mice is the consequence of the expansion and inhibition of erythroid differentiation of CD71−Ter119+ erythroblasts, which undergo apoptosis normally.25

**Hri is necessary for differentiation of erythroid progenitors ex vivo**

Ter119− cells in E14.5 FLs are primarily erythroid progenitors, which undergo proliferation and differentiation in ex vivo culture.17 We used this system to further investigate the role of Hri in erythroid differentiation. Differentiation of Hri−/− Ter119− erythroid progenitors progressed more slowly than that of Hri+/+ cells (Figure 4). Compared with Hri+/+ cells, Hri−/− cells had a greatly reduced population in the P3 basophilic erythroblast stage at 20 hours of culture (top panels, \( P < .001 \), supplemental Figure 6A). Whereas the percentage of cells in the P3 stage appeared similar at 40 hours, Hri−/− cells had higher CD71 and lower Ter119 expression, indicative of retarded erythroblast differentiation (middle panels). At 60 hours of differentiation (bottom panels), there was a significantly lower percentage of Hri−/− cells in the P4 polychromatophilic and orthochromatophilic erythroblast stage compared with Hri+/+ cells (\( P < .005 \), supplemental Figure 6A).

To further substantiate the inhibition of erythroid differentiation of Hri−/− erythroblasts, the formation of reticulocytes during ex vivo differentiation was measured. Reticulocytes, which are devoid of nuclei and are characterized as the Hoechst 33342lowTer119high population, are shown in magenta in Figure 4B. Reticulocyte formation was greatly reduced in the differentiation of Hri−/− Ter119− erythroid progenitors compared with Hri+/+ cells at 20, 40 and 60 hours (Figure 4B). This inhibition of differentiation was further corroborated by observations of cell morphologies and hemoglobinization (supplemental Figure 6B). Taken together, these results demonstrate that Hri is required for differentiation of erythroid progenitors ex vivo, similar to the requirement of Hri in erythroid differentiation of adult spleens in vivo during iron deficiency (Figure 3A). Thus, this ex vivo erythroid differentiation system recapitulates the stress erythropoiesis observed in vivo, and provides an excellent in vitro system to investigate the molecular mechanism by which Hri regulates erythroid differentiation.

![Figure 4](image-url) - Differentiation of Ter119− FL erythroblast progenitors ex vivo and the activation of the Hri signaling pathway. (A) Erythroid differentiation, and (B) reticulocyte production of Hri+/+ and −/− FL Ter119− erythroid progenitors at 20, 40, and 60 hours of ex vivo culture. (C) Activation of Hri and protein expression of its downstream targets. Vertical lines have been inserted to indicate a repositioned gel lane. The middle 3 lanes, which contains HbbP−/− samples, were removed of this Western blot because these results is not necessary for this figure. (D) qPCR analysis of the mRNA expression at 36 hours of ex vivo culture. Data are presented as relative expression normalized to elf2α control with mean ± SD (\( n = 3 \)); **\( P < .005 \); ***\( P < .01 \). Triplicate of ex vivo differentiation were carried out using Hri+/+ or −/− FL erythroid progenitors isolated from embryos of the same mother. This set of experiment was repeated 3 times with similar results.

**Activation of the Hri signaling pathway during erythroid differentiation**

We then examined the activation of the Hri-elf2αP-Atf4 signaling pathway during ex vivo differentiation of Hri+/+ and Hri−/− cells. Hri protein expression was increased at 36 and 48 hours. Furthermore, Hri was activated by hyperphosphorylation (Hri-P; Figure 4C). Most notably, there was Hri-dependent elf2α phosphorylation and induction of Atf4, Chop, and Ho-1 protein expression during erythroid differentiation of Hri+/+, but not Hri−/− Ter119− erythroid progenitors (Figure 4C). Furthermore, we found that at 36 hours of ex vivo differentiation, Hri−/− cells also had significantly lower levels of Atf4, Chop, Ho-1, Gstμ, and Sod2 mRNAs (Figure 4D). Together, these results demonstrate the activation of the Hri signaling pathway during erythroid differentiation ex vivo.
We found that the dominant-negative Hri mutant inhibits erythroid differentiation (Figure 5A). Importantly, S123 knockdown samples also had a reduced Atf4 mRNA level to 34.5% of the control siRNA combination of all 3 siRNAs (S123) worked most effectively and Atf4 mRNA, whereas S3 was less effective (Figure 5A). The requirement of Atf4 for erythroid differentiation was shown in Figure 5C and were scored morphologically (Figure 5D). These results demonstrated that knockdown of Atf4 expression in MEL cells resulted in inhibition of erythroid differentiation at the basophilic stage and in reduction of benzidine positive cells. Furthermore, S123-treated cells were less hemoglobinized as assessed by benzidine staining (Figure 5C-D), consistent with decreased β-globin major mRNA (Figure 5B). Together, these results support the requirement of Atf4 for erythroid differentiation.

It was reported that ablation of the Atf4 gene in mice results in growth retardation and transient fetal anemia, which is attributed to a proliferation deficit in erythroid burst-forming unit (BFU-E) and erythrocyte colony-forming unit (CFU-E) erythroid progenitors. We investigated here the proof of concept for Hri and its signaling pathway as possible potential novel pharmaceutical targets for treatment of β-thalassemia by reducing ineffective erythropoiesis.

We used salubrinal, a small chemical that selectively inhibits the dephosphorylation of eIF2α, to test its capability to enhance the Hri signaling pathway in β-thalassemic erythroid precursors by salubrinal. Increased oxidative stress and decreased erythroid differentiation exacerbate severity of β-thalassemia. We have shown earlier that Hri+/−/Hbb−/− embryos died of severe anemia at E18.5 and Hri+/−/Hbb−/− mice had more severe adult β-thalassemic phenotype. We investigated here the proof of concept for Hri and its signaling pathway as possible potential novel pharmaceutical targets for treatment of β-thalassemia by reducing ineffective erythropoiesis.

Modulation of Hri signaling pathway in β-thalassemic erythroid precursors by salubrinal

Figure 5. Inhibition of erythroid differentiation of MEL cells by knockdown of the Atf4 expression. (A) Knockdown of Atf4 expression in MEL cells by siRNAs. (B) Decreased expression of β-globin major mRNA in Atf4 knockdown cells. Data are presented as mean ± SD (n = 3, 3 separate transfections were performed). (C) Inhibition of erythroid differentiation in Atf4 knockdown cells. At 5 days after siRNA transfection, differentiating cells were stained with Giemsa and benzidine. (D) Percentages of cells at the basophilic erythroblast (Baso), polychromatophilic erythroblast (Poly), and orthochromatophilic erythroblast (Ortho) stages as well as benzidine-positive cells were determined by scoring the Giemsa-benzidine stained objects as describe for Figure 3B.

Requirement of Atf4 for erythroid differentiation

To determine whether Atf4 is necessary for erythroid differentiation, siRNA knockdown of Atf4 expression was performed in MEL cells. Three Atf4 siRNAs, S1, S2, and S3 were used individually and in combination. Both S1 and S2 siRNA significantly reduced Atf4 mRNA, whereas S3 was less effective (Figure 5A). The combination of all 3 siRNAs (S123) worked most effectively and reduced the Atf4 mRNA level to 34.5% of the control siRNA (Figure 5A). Importantly, S123 knockdown samples also had a lower mRNA level of β-globin major compared with the control siRNA (Figure 5B), indicating an inhibition of differentiation by knocking down Atf4 expression. Erythroid differentiation of MEL cells after treatment with control and S123 siRNAs was further examined by benzidine-Giemsa staining. Cells at different stages of differentiation were shown in Figure 5C and were scored morphologically (Figure 5D). These results demonstrated that knockdown of Atf4 expression in MEL cells resulted in inhibition of erythroid differentiation at the basophilic stage and in reduction of benzidine positive cells. Furthermore, S123-treated cells were less hemoglobinized as assessed by benzidine staining (Figure 5C-D), consistent with decreased β-globin major mRNA (Figure 5B). Together, these results support the requirement of Atf4 for erythroid differentiation.

We used salubrinal, a small chemical that selectively inhibits the dephosphorylation of eIF2α, to test its capability to enhance the Hri signaling pathway in β-thalassemic erythroid precursors. As shown in Figure 6A, salubrinal treatment of Hri+/−/Hbb−/− reticulocytes resulted in increased eIF2αP as well as in a significant decrease in the rate of globin protein synthesis (Figure 6B). It is important to note that phosphorylation of only 20%–30% of eIF2α is sufficient to completely inhibit protein synthesis. Furthermore, there was less 35S-globin in the insoluble pellet fractions of salubrinal-treated reticulocytes compared with the DMSO-treated control (Figure 6B). Salubrinal treatment also increased the levels of eIF2αP and Chop in Hri+/−/Hbb−/− FL cells (Figure 6C).

Similarly, salubrinal treatment of Hri+/−/Hbb−/− Ter119+ erythroid precursors increased eIF2αP and inhibited protein synthesis in a concentration-dependent manner (Figure 6D). It is to be noted that Atf4 protein was synthesized in Hri+/−/Hbb−/− Ter119+ erythroid precursors, consistent with activation of Hri in β-thalassemia. Importantly, at 10µM salubrinal, Atf4 mRNA translation (as determined by immunoprecipitation of newly synthesized 35S-Atf4 protein) was increased by 21%, whereas globin mRNA translation was decreased by 17%. At 25µM salubrinal, eIF2αP was further increased resulting in the shut-off of protein synthesis including Atf4. This is expected, as no eIF2 can be recycled when eIF2αP sequesters all eIF2B.

Together, these results demonstrate that salubrinal is effective in increasing eIF2αP and reducing denatured globins in β-thalassemic
reticulocytes. Furthermore, salubrinal also enhances Atf4 translation and its subsequent signaling pathway in β-thalassemic erythroid precursors. These observations provide the foundation for exploiting the Hri-eIF2αP signaling pathway for treatment of thalassemia.

Discussion

Regulation of ROS levels and oxidative stress is extremely important in erythropoiesis. Starting at the basophilic erythroblast stage, erythroid precursors synthesize large amounts of hemoglobin, which requires heme as a prosthetic group. Thus, iron uptake for heme biosynthesis also increases, potentially generating ROS through the iron-catalyzed Fenton reaction. To date, 2 nonredundant Foxo3 and Nrf2-mediated pathways to combat oxidative stress have been identified in erythroid cells. We show here for the first time that the Hri-eIF2αP-Atf4 pathway is also required in the erythroid lineage for the adaptation to oxidative stress.

As illustrated in Figure 7A, phosphorylation of eIF2α by activated Hri not only leads to inhibition of globin translation but also leads to the selective enhanced translation of Atf4 and subsequent expression of redox genes (ie, Ho-1, Gstα, and Nqo1) for adaptation to acute and chronic oxidative stress. During chronic iron deficiency of Hri−/− mice, both reduced antioxidant gene expression (Figure 2 and Liu et al23) and heme-free globin precipitates probably contribute to the increased ROS levels in Hri−/− erythroid cells. Although the exact mechanisms by which misfolded proteins in the cytosol generate oxidative stress have yet to be defined, there are 2 possibilities. Denatured heme-free globin aggregates may overwhelm and compromise capacities of molecular chaperons and proteosomal degradation. Both of these processes have been shown to mitigate ROS during ER stress. Reduction of improper disulfide bonds in misfolded proteins by glutathione (GSH) can deplete GSH level. In addition, degradation of misfolded proteins is necessary to prevent ROS accumulation during ER stress.

Furthermore, this Hri-eIF2αP-Atf4 pathway is also necessary for erythroid differentiation (Figure 7B). In nucleated erythroblasts, Hri not only inhibits globin translation, but also increases Atf4 translation to mitigate oxidative stress and to promote erythroid differentiation. At the enucleated reticulocyte stage, the role of Hri is only to regulate globin translation to prevent
Hri signaling in erythropoiesis

The ineffective erythropoiesis in β-thalassemia is thought to be because of increased proliferation and accelerated apoptosis of erythroid precursors. However, a recent study by Libani et al demonstrates that decreased differentiation exacerbates the ineffective erythropoiesis in β-thalassemia. We show that the ineffective erythropoiesis occurring in Hri−/− mice during iron deficiency is primarily because of the profound inhibition of erythroid differentiation at the basophilic erythroblast stage (Figure 3). Inhibition of erythroid differentiation is also observed in several other mouse models of stress erythropoiesis, such as in Rb deficiency, and in Stat5a/5b deficiency.

Although Hri−/− FL cells displayed a mild defect in erythroid differentiation in vivo, Ter119+ Hri−/− FL erythroid progenitors showed a significant inhibition of erythroid differentiation at the basophilic erythroblast stage when cultured and differentiated ex vivo (Figure 4), recapitulating the inhibition of erythroid differentiation in vivo during iron deficiency. We demonstrate that the Hri-dependent eIF2αP-Atf4 pathway is activated during ex vivo differentiation of Ter119+ erythroid precursors (Figure 4), and during erythroid differentiation of MEL cells. Furthermore, knockdown of Atf4 in MEL cells resulted in inhibition of erythroid differentiation (Figure 5). Thus, the Hri-Atf4 signaling pathway may be necessary for inducing transcription of genes required for erythropoiesis starting at the early erythroblast stage (Figure 7B).

Targeting the Hri-eIF2αP-Atf4 pathway for treatment of thalassemia

Accumulation of unpaired α-globin, oxidative stress, and ineffective erythropoiesis are well documented in β-thalassemia. We have shown earlier that Hri deficiency exacerbates the pathologic severity of Hbb−/− β-thalassemic mice. We demonstrate here the feasibility of augmenting the eIF2αP signaling pathway with salubrinal in erythroid precursors from β-thalassemic mice (Figure 6). Elevated eIF2αP achieved by salubrinal treatment resulted in inhibition of globin translation and reduction of insoluble globin protein. Furthermore, salubrinal treatment also enhances Atf4 signaling in erythroid precursors, which may be important in reducing the oxidative stress encountered in this disease.

In summary, the Hri-eIF2αP-Atf4 signaling pathway is necessary not only for mitigating oxidative stress, but also necessary for erythroid differentiation. This study reveals the novel function of Hri in erythroid differentiation beyond its established function in coordinating globin translation with heme availability.

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Heme-regulated eIF2α kinase activated Atf4 signaling pathway in oxidative stress and erythropoiesis

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