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Research Article

Hematology

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Heme-regulated eIF2 α kinase modifies the phenotypic severity of murine models of erythropoietic protoporphyria and β -thalassemia

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Heme-regulated eIF2 α kinase (HRI) controls protein synthesis by phosphorylating the α -subunit of eukaryotic translational initiation factor 2 (eIF2 α). In heme deficiency, HRI is essential for translational regulation of α - and β -globins and for the survival of erythroid progenitors. HRI is also activated by a number of cytoplasmic stresses other than heme deficiency, including oxidative stress and heat shock. However, to date, HRI has not been implicated in the pathogenesis of any known human disease or mouse phenotype. Here we report the essential role of HRI in 2 mouse models of human rbc disorders, namely erythropoietic protoporphyria (EPP) and β -thalassemia. In both cases, lack of HRI adversely modifies the phenotype: HRI deficiency exacerbates EPP and renders β -thalassemia embryonically lethal. This study establishes the protective function of HRI in inherited rbc diseases in mice and suggests that HRI may be a significant modifier of many rbc disorders in humans. Our findings also demonstrate that translational regulation could play a critical role in the clinical manifestation of rbc diseases.

Introduction

Protein synthesis in intact reticulocytes and reticulocyte lysates is dependent on the availability of heme. Heme serves as the prosthetic group of hemoglobin, the predominant protein in rbc and late erythroid precursors. Under conditions of heme deficiency, or iron deficiency, protein synthesis is inhibited at the level of translation initiation due to the activation of the heme-regulated eIF2 α kinase (HRI). HRI is a heme-regulated protein kinase that phosphorylates the α -subunit of eukaryotic translational initiation factor 2 (eIF2 α) (reviewed in ref. 1). Phosphorylation of eIF2 impairs the recycling of eIF2 for the purpose of translational initiation and results in the cessation of protein synthesis (reviewed in ref. 2). The molecular mechanism of this phosphorylation-mediated inhibition has been extensively studied (reviewed in refs. 3, 4). In brief, the recycling of eIF2 involves the exchange of bound ganosine 5'-diphosphate (GDP) for ganosine 5'-triphosphate (GTP) and requires eIF2B, which is rate limiting and is present at 15% to 25% of the amount of eIF2 (5). Phosphorylated eIF2(α P)-GDP binds much more tightly to the regulatory subcomplex of eIF2B than eIF2-GDP does and prevents the GDP/GTP exchange activity of eIF2B (6). Thus, once the amount of phosphorylated eIF2 exceeds the amount of eIF2B, protein synthesis is shut off.

Phosphorylation of eIF2 α occurs under various stress conditions other than heme deficiency and is carried out by the family

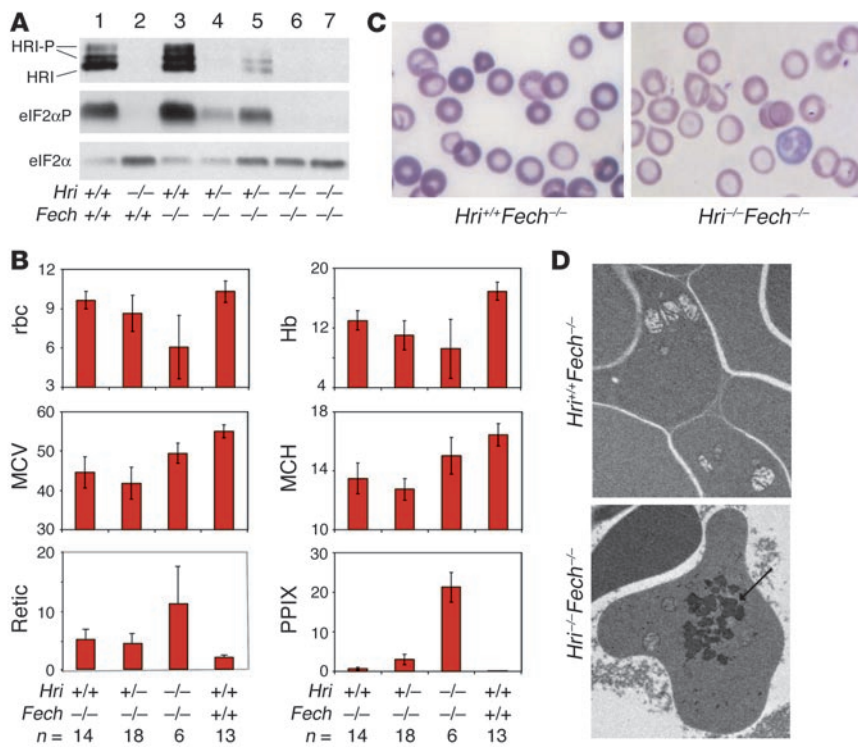
of eIF2 α kinases. In addition to HRI, 3 other eIF2 α kinases are known: the double-stranded RNA-dependent eIF2 α kinase (PKR), the general control nonderepressible 2 (GCN2) protein kinase, and the ER resident kinase, PKR-like ER kinase (PERK). The 4 eIF2 α kinases share extensive homology in their kinase catalytic domains (7–13) and phosphorylate eIF2 α at the same serine residue (Ser51) (12–15). While they share a common mode of action, each elicits a different physiological response as a consequence of distinctive tissue distributions and the signals to which they respond. PKR is ubiquitously expressed, induced by interferon, and regulated by double stranded RNA (dsRNA) through 2 N-terminal dsRNA-binding domains (reviewed in ref. 16). GCN2 is highly expressed in the liver and brain (17, 18) and is activated under conditions of amino acid starvation through the C-terminal domain, which contains a histidyl transfer RNA (His-tRNA) synthase-like sequence (reviewed in refs. 19). PERK is highly expressed in secretory tissues, particularly the pancreas, and is activated by ER stress. PERK contains a lumenal domain which is similar to the sensor domain of the ER-stress kinase, Ire1 (reviewed in refs. 13, 20). HRI is expressed predominantly in immature erythroid cells (21) and is regulated by heme via the 2 heme-binding domains located in the N-terminus and the kinase insertion (reviewed in refs. 1, 22, and 23).

We recently demonstrated that there is a significant increase in protein synthesis in *Hri*^{-/-} reticulocytes compared to *Hri*^{+/+} reticulocytes (24), which is accompanied by an increase in larger-sized polysomes. Furthermore, protein synthesis in *Hri*^{-/-} reticulocytes is not increased by the addition of hemin as is seen in *Hri*^{+/+} reticulocytes. Since there is no difference in globin mRNA levels between *Hri*^{+/+} and *Hri*^{-/-} reticulocytes, these results provide in vivo evidence for a role of HRI in the regulation of translational initiation. We have also demonstrated that HRI is essential for translational regulation of α - and β -globins and the survival of erythroid pro-

Nonstandard abbreviations used: ACN, acetonitrile; eIF2 α , the α -subunit of eukaryotic translational initiation factor 2; EPP, erythropoietic protoporphyria; Feh, ferrochelatase; GCN2, general control nonderepressible 2; HRI, heme-regulated eIF2 α kinase; MCV, mean cell volume; PERK, PKR-like ER kinase; PKR, double-stranded RNA-dependent eIF2 α kinase; PPIX, protoporphyrin IX; TFA, trifluoroacetic acid.

Conflict of interest: The authors have declared that no conflict of interest exists.

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**Figure 1**

HRI alters the hematological parameters of *Fech^{m1Pas/m1Pas}* mice. (A) Activation of HRI in the reticulocytes of *Fech*-deficient mice. Activation of HRI in the blood samples of 2-week-old mice from the same litter was analyzed by SDS-PAGE and Western blot analysis using anti-HRI antibody (top panel). The in vivo phosphorylation of eIF2 α (eIF2 α P) and the total eIF2 α in the blood samples of various mice were determined by Western blot analysis using antibodies specifically against phosphorylated eIF2 α (middle panel) and eIF2 α (lower panel). (B) The complete blood analyses. Data are presented as mean \pm SD. Retic, reticulocyte. Units of the hematological parameters: rbc, $\times 10^{12}/l$; Hb, g/dl; MCV, fl; MCH, pg; retic, %; PPIX, arbitrary units. (C) Examination of the blood smears by light microscopes. Original magnification, $\times 1,000$. (D) Examination of the blood smears by electron microscopes. Original magnification, $\times 22,000$. Arrow indicates intraerythroid inclusions. The blood samples from 12- to 16-week-old mice were used for these analyses.

genitors in iron deficiency; HRI is responsible for the physiological adaptation that produces hypochromic, microcytic erythrocytes in iron deficiency (24).

In addition to heme deficiency, HRI is activated by arsenite-induced oxidative stress, osmotic shock, and heat shock. HRI is the only eIF2 α kinase activated by arsenite and is the major eIF2 α kinase responsive to heat shock in erythroid cells (25). Consequently, it appears that HRI may protect erythroid cells against stress in general and may play a role in response to intrinsic rbc disorders.

In this report, we demonstrate the importance of HRI in 2 murine models of human inherited rbc disorders: erythropoietic protoporphyria (EPP) and β -thalassemia. *Hri*^{-/-} mice were interbred with *Fech^{m1Pas/m1Pas}* mice, which develop EPP due to a recessive loss of function mutation in ferrochelatase (*Fech*) (26), as well as a murine model of β -thalassemia intermedia, *Hbb^{mUNC}*, in which the murine β -major globin gene has been deleted (27). The findings demonstrate that in the absence of HRI, homozygous *Fech^{m1Pas}* mice are clinically and biochemically much more severely affected while β -thalassemic mice lacking HRI are embryonically lethal.

Results

Activation of HRI in murine EPP. We have previously shown that HRI is activated in iron deficiency to balance heme and globin synthesis (24). Although the regulation of HRI by heme is well documented in vitro, the in vivo evidence is still lacking. To address the question of whether it is heme or iron deficiency per se that regulates HRI in vivo as well as to determine the importance of HRI in the pathophysiology of heme-deficient disorders, *Hri*^{-/-} mice were interbred with *Fech^{m1Pas/m1Pas}* mice, which have a recessive severe loss of function mutation in *Fech*, the last enzyme of heme biosynthesis (26). Since *Fech* inserts iron into protoporphyrin IX (PPIX) to form heme, *Fech^{m1Pas/m1Pas}* mice are functionally heme deficient and accumulate PPIX, which is similar to what occurs in human EPP (26).

The activation of HRI in blood samples of *Fech^{m1Pas/m1Pas}* mice was examined by mobility shift in SDS-PAGE. We have shown previously that activation of HRI is accompanied by multiple auto-phosphorylation (28, 29), which can be assessed by Western blot analysis (25), as the activated hyperphosphorylated HRI (HRI-P) has a slower electrophoretic mobility than does unphosphorylated HRI. HRI in wild-type reticulocytes was present as 3 discrete bands, reflecting the baseline level of phosphorylation, with the majority of HRI protein present in the unphosphorylated state (Figure 1A, lane 1). However, in *Fech^{m1Pas/m1Pas}* reticulocytes (Figure 1A, lane 3), the majority of HRI was hyperphosphorylated. As expected, there is quantitatively less HRI in *Hri*^{+/-} reticulocytes (Figure 1A, lanes 4 and 5) while no HRI was detected in *Hri*^{-/-} reticulocytes (Figure 1A, lanes 2, 6, and 7). The activation of HRI was further validated by the increase in the in vivo phosphorylation of eIF2 α , the substrate for HRI. In particular, there was no increase in eIF2 α P in *Hri*^{-/-} *Fech^{m1Pas/m1Pas}* reticulocytes, indicating that HRI is the only eIF2 α kinase activated in heme-deficient *Fech^{m1Pas/m1Pas}* reticulocytes. Furthermore, these findings substantiate the in vitro evidence that HRI is regulated directly by heme and not by iron.

HRI is required for the inhibition of globin synthesis and microcytic hypochromic anemia in murine EPP. A total of 159 mice from 14 litters of *Hri*^{+/-} *Fech^{m1Pas/m1Pas}* double-heterozygote matings were analyzed. Mice of each of the expected 9 genotypes were born alive at near the expected Mendelian ratio (Table 1). The double-knockout mice, *Hri*^{-/-} *Fech^{m1Pas/m1Pas}*, were born alive at a frequency of 4.4%, somewhat less than the expected 6.25% (1/16). However, a similarly slight decrease in frequency was also observed for each of the mice with single homozygous mutations alone.

To determine the effect of HRI on the hematology of *Fech^{m1Pas}* mice, complete blood analyses (CBCs) of the 9 genotypes resulting from the double heterozygote crosses were performed. In the presence of HRI (*Hri*^{+/+} or *Hri*^{+/-}), *Fech^{m1Pas/m1Pas}* mice had a micro-



Table 1
F2 progeny of *Hri*^{-/-} and *Fech*^{m1Pas} mice crosses

Genotype	n	%	Expected %
<i>Hri</i> ^{+/+} <i>Fech</i> ^{+/+}	12	7.50	6.25
<i>Hri</i> ^{+/+} <i>Fech</i> ^{+/-}	28	17.70	12.50
<i>Hri</i> ^{+/+} <i>Fech</i> ^{-/-}	6	3.80	6.25
<i>Hri</i> ^{+/-} <i>Fech</i> ^{+/+}	18	11.30	12.50
<i>Hri</i> ^{+/-} <i>Fech</i> ^{+/-}	47	29.60	25.00
<i>Hri</i> ^{+/-} <i>Fech</i> ^{-/-}	16	10.00	12.50
<i>Hri</i> ^{-/-} <i>Fech</i> ^{+/+}	7	4.40	6.25
<i>Hri</i> ^{-/-} <i>Fech</i> ^{+/-}	18	11.30	12.50
<i>Hri</i> ^{-/-} <i>Fech</i> ^{-/-}	7	4.40	6.25

cytic hypochromic anemia with decreased hemoglobin, mean cell volume (MCV), and mean cell hemoglobin (MCH) (Figure 1B). *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} mice were significantly more anemic, with a 30% decrease in hemoglobin (*P* < 0.05). Furthermore, the MCV and MCH were not significantly different from that in wild type; the decreased hemoglobin was due to an overall decrease in the number of normochromic, normocytic rbc (Figure 1B; *P* < 0.01), similar to the unusual effect of HRI deficiency on the normally microcytic anemia of iron deficiency (24).

Examination of erythrocytes of *Fech*^{m1Pas/m1Pas} mice by light and electron microscopy revealed the presence of inclusion bodies in reticulocytes of both *Hri*^{+/-}*Fech*^{m1Pas/m1Pas} (data not shown) and *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} animals (Figure 1, C and D), with the number of inclusions noticeably increased in the latter group. The amounts of inclusions were quantitated biochemically by measuring aggregated globins in 100,000-g pellets as described previously (24). There were 1.34 fold and 2.7 fold more inclusions, respectively, in *Hri*^{+/-}*Fech*^{m1Pas/m1Pas} and *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} than in *Hri*^{+/+}*Fech*^{m1Pas/m1Pas} blood samples (data not shown). The composition of the globin chains in the inclusions of HRI-deficient *Fech*^{m1Pas/m1Pas} blood cells were analyzed by HPLC. As shown in Figure 2, both α and β chains were present in the pellet fraction. This is consistent with the global inhibition of protein synthesis by HRI in heme deficiency. In the absence of HRI, both α and β chains are synthesized in *Fech*^{m1Pas/m1Pas} rbc precursors. Thus, 2 copies of HRI are necessary to inhibit fully the accumulation of excess heme-free α - and β -globins in heme-deficient *Fech*^{m1Pas/m1Pas} mice. Similar inclusions containing both α and β chains were observed in the reticulocytes of *Hri*^{-/-} mice in iron deficiency (data not shown and ref. 24). The morphologically similar rbc abnormality elicited by the absence of HRI in both iron and heme deficiency further underscores the importance of HRI in inhibiting protein synthesis to avoid accumulation of excess heme-free α and β globins in heme-deficiency states.

HRI deficiency results in dramatic increase in PPIX and more severe manifestation of liver pathology and skin photosensitivity. Although *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} mice were viable, they were smaller in size than littermates of 8 other genotypes and appeared severely jaundiced. Most significantly, PPIX levels were dramatically increased, by 30-fold, in the rbc and reticulocytes of *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} mice (Figures 1B and 3A) compared to those in *Hri*^{+/+}*Fech*^{m1Pas/m1Pas} controls. Furthermore, *Fech*^{m1Pas/m1Pas} animals lacking one copy of HRI (*Hri*^{+/-}*Fech*^{m1Pas/m1Pas}) also had significantly increased PPIX (4.5 fold; *P* < 0.001). In contrast, HRI deficiency did not substantially alter protoporphyrin levels in *Fech*^{m1Pas/+} or *Fech*^{+/+} mice (data not shown).

Compared to heme, PPIX is a very poor inhibitor of HRI (30). Consequently, excessive PPIX biosynthesis in *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} mice is probably due to a failure of translational inhibition by HRI in the context of heme deficiency. Thus, sustained protein synthesis contributes to the excessive protoporphyrin synthesis in *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} animals, further supporting the role of HRI in globally regulating rbc protein expression.

While the erythron is the major source of excess protoporphyrin in EPP, the primary clinical complications are photosensitivity and hepatocellular toxicity (reviewed in ref. 31). At baseline, *Fech*^{m1Pas/m1Pas} mice have hepatomegaly and hepatic porphyrin deposits (26). These abnormalities were more severe in animals lacking HRI. In *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} mice, the liver accounted for 20–25% of body weight compared with 13% in *Hri*^{+/+}*Fech*^{m1Pas/m1Pas} mice (*P* = 0.01) and was histologically marked by more extensive deposits of PPIX (Figure 3B). A more modest enhancement of the severity in liver pathology was seen in *Hri*^{+/-}*Fech*^{m1Pas/m1Pas} animals. This observation is consistent with the results shown in Figure 3A indicating that both copies of HRI are necessary to reduce excess synthesis of PPIX and its accumulation in the liver.

Similarly to human patients with EPP, *Fech*^{m1Pas/m1Pas} animals exhibit photosensitivity (26, 32), which we examined in the presence and absence of HRI. Animals were exposed to a fixed UV dose and continuously monitored for toxicity. Representative animals of each genotype are shown in Figure 3C. The *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} mice were extremely jaundiced, as can be seen by the yellow color in their ears, and were uniformly dead 6 hours after exposure to light. In addition, *Fech*^{m1Pas/m1Pas} mice lacking 1 copy of HRI developed grossly more severe burns than *Fech*^{m1Pas/m1Pas} controls but remained alive. Thus, HRI modifies the cutaneous photosensitivity phenotype of experimental EPP.

Altogether, our results demonstrate that both copies of HRI are necessary to reduce the severity of phenotype in the heme-deficient *Fech*^{m1Pas/m1Pas} mice in terms of their hematology, liver pathology, and photosensitivity.

Activation of HRI in reticulocytes of a murine model of β -thalassemia intermedia. HRI can be activated by denatured proteins in heme-supplemented reticulocyte lysates. In β -thalassemia, excess α -globin denatures and aggregates. We therefore examined whether HRI was activated in the blood cells of β -thalassemia mice (*Hbb*^{-/-}).

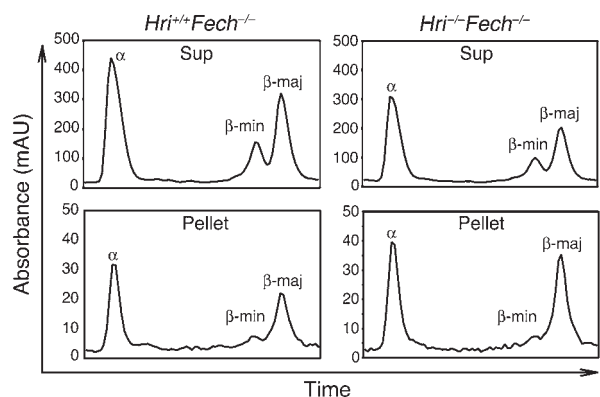


Figure 2
Inclusions in *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} rbc are composed of both α - and β -globins. The globin chain compositions in the soluble hemoglobin (sup) and the aggregated inclusions (pellet) were analyzed by HPLC. β -maj, β -major; β -min, β -minor.

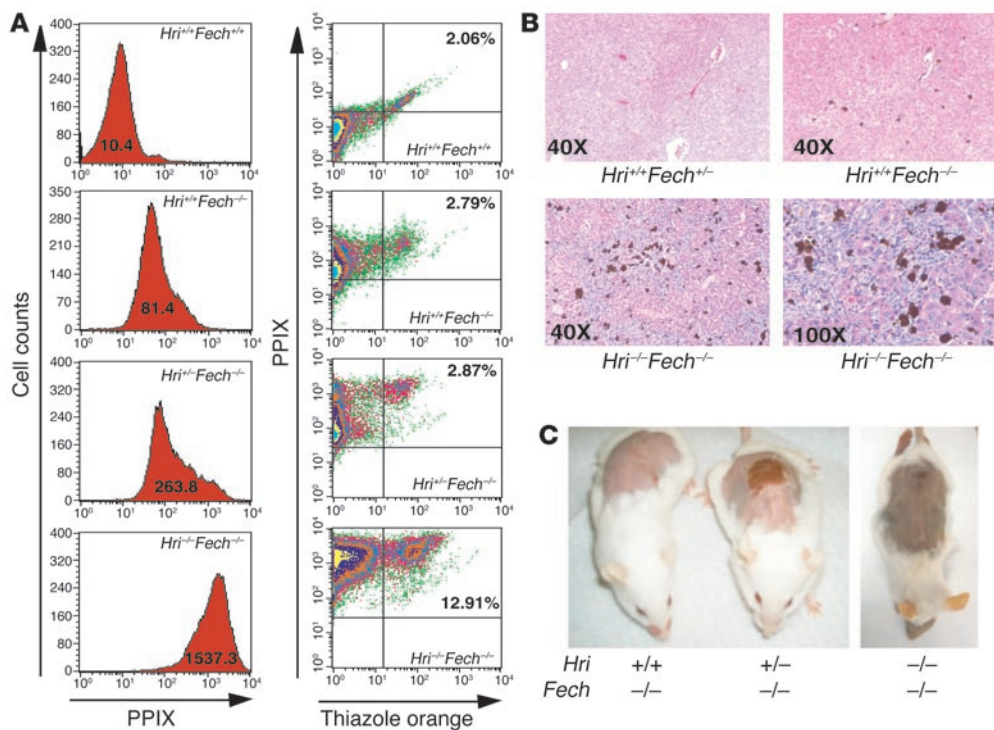


Figure 3 More severe pathology in HRI-deficient *Fech*^{m1Pas/m1Pas} mice. **(A)** Increased protoporphyrin level in the reticulocytes and rbc's of *Fech*^{m1Pas/m1Pas} mice deficient in HRI. The fluorescent porphyrin levels in blood cells were analyzed by FACS analysis (left panels). The numbers inside the peak represent the arbitrary mean values of PPIX in each genotype. FACS analyses of the fluorescent porphyrin in reticulocytes were performed after staining with thiazole orange (right panels). The values on the right upper quadrant are percentages of reticulocytes. Left panels: x axes are arbitrary units of fluorescent intensity. Right panels: x and y axes are arbitrary units of fluorescent intensity. **(B)** Liver pathology. The liver sections were stained with H&E. **(C)** Skin photosensitivity. The *Hri*^{-/-}*Fech*^{m1Pas/m1Pas} mice were extremely jaundiced, as can be seen by the yellow color in their ears, and were dead 6 hours after exposure to mercury vapor lamp. Photos were taken 4 days after exposure to the mercury vapor lamp for the *Hri*^{+/-}*Fech*^{m1Pas/m1Pas} mice and *Hri*^{+/+}*Fech*^{m1Pas/m1Pas} mice. Twelve- to sixteen-week-old mice were used for these experiments.

In wild-type reticulocytes, HRI was present mainly in the faster migrating, unphosphorylated, functionally inactive form (Figure 1A). However, in *Hbb*^{-/-} reticulocytes, a significant portion of HRI was activated and upshifted (data not shown).

Hri is essential for the survival of mice with β -thalassemia intermedia. To establish the physiological importance of HRI in β -thalassemia, *Hri*^{-/-} mice were bred with β -thalassemic *Hbb*^{-/-} animals. The genotypes of 162 F2 progeny from 17 litters were analyzed. None of the live-born F2 mice were *Hri*^{-/-}*Hbb*^{-/-} (Table 2). Mice representing the 8 other genotypes were born at nearly expected frequencies, apart from *Hri*^{+/-}*Hbb*^{-/-} mice (8.0% vs. 12.5%; see below for significance).

To determine when and why double mutant embryos die, *Hri*^{-/-}*Hbb*^{+/-} mice were interbred. Beginning at E15.5, double-knockout embryos were very pale and smaller in size, and these died uniformly by E18.5 (Figure 4A). When the hematocrit of one litter of embryos at E17.5 was examined, the double-knockout embryos had an average hematocrit of 13.7%, only one-third that of the β -major heterozygote genotypes (44.6%) and significantly less than the hematocrit of the *Hri*^{+/-}*Hbb*^{-/-} embryos (19.4% \pm 2.2%). This observation was confirmed in F1 intercross animals, which also allowed comparison to *Hri*^{+/-}*Hbb*^{-/-} mice (data not shown). Together, these results demonstrate that *Hri*^{-/-}*Hbb*^{-/-} embryos died of severe anemia.

Erythropoiesis in mice undergoes switching during embryonic development. Starting at E8, macrocytic, nucleated primitive

erythrocytes containing embryonic-globin ($\alpha_2\gamma_2$) are produced in the yolk sac (Figure 4B). Definitive (adult) erythropoiesis, which produces enucleated, normocytic erythrocytes (Figure 4C) expressing α -globin, β -major globin, and a small amount of β -minor globin (adult globins, $\alpha_2\beta^{\text{maj}}_2$ and $\alpha_2\beta^{\text{min}}_2$), begins at E10 in the fetal liver and several days later in the spleen and bone marrow; primitive erythropoiesis wanes at E13.

Because of this temporal pattern of cell type and globin switching, embryonic blood smears were examined. At E12.5 and E13.5

Table 2 F2 progeny of *Hri*^{-/-} and *Hbb*^{-/-} mice crosses

Genotype	n	%	Expected %
<i>Hri</i> ^{+/+} <i>Hbb</i> ^{+/+}	10	6.10	6.25
<i>Hri</i> ^{+/+} <i>Hbb</i> ^{+/-}	19	11.60	12.50
<i>Hri</i> ^{+/+} <i>Hbb</i> ^{-/-}	7	4.30	6.25
<i>Hri</i> ^{+/-} <i>Hbb</i> ^{+/+}	29	17.80	12.50
<i>Hri</i> ^{+/-} <i>Hbb</i> ^{+/-}	58	35.60	25.00
<i>Hri</i> ^{+/-} <i>Hbb</i> ^{-/-}	13	8.00	12.50
<i>Hri</i> ^{-/-} <i>Hbb</i> ^{+/+}	8	4.90	6.25
<i>Hri</i> ^{-/-} <i>Hbb</i> ^{+/-}	19	11.60	12.50
<i>Hri</i> ^{-/-} <i>Hbb</i> ^{-/-}	0	0	6.25

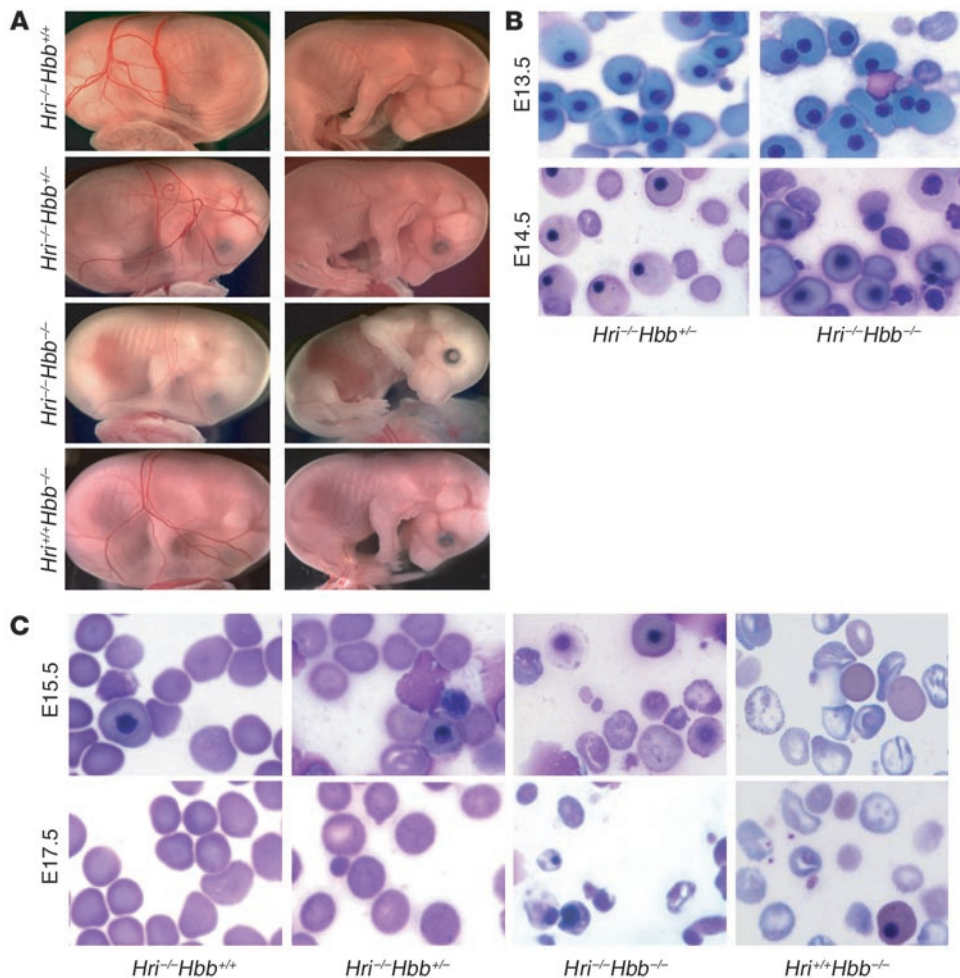


Figure 4
Severe anemia of $Hri^{-/-}Hbb^{-/-}$ embryos during definitive erythropoiesis. (A) E17.5 embryos with different β -major genotypes. (B) Wright-Giemsa stained embryonic blood smears of E13.5 and E14.5. Original magnification, $\times 1,000$. (C) Wright-Giemsa stained embryonic blood smears of E15.5 and E17.5. Original magnification, $\times 1,000$.

(Figure 4B), smears from all genotypes were morphologically indistinguishable. However, thereafter, and coinciding with the onset of definitive hematopoiesis, intraerythroid inclusions could be seen in all $Hbb^{-/-}$ embryos but were particularly prominent in $Hri^{-/-}Hbb^{-/-}$ embryos (Figure 4C). By E15.5, primitive erythropoiesis has ceased, and embryos rely entirely upon definitive erythropoiesis. At this stage, the rbc's from double-knockout animals were extremely fragile, with most of their globins present as large aggregates (Figure 4C). By E17.5, the few surviving $Hri^{-/-}Hbb^{-/-}$ embryos were severely growth retarded and profoundly anemic. The severity of the double-knockout phenotype suggests that HRI ordinarily mitigates the severity of β -thalassemia, probably by modulating protein synthesis in erythroid precursors to minimize the accumulation of the toxic α -globin precipitates.

HRI haploinsufficiency results in a more severe adult β -thalassemic phenotype. Complete blood analyses were performed on adult mice of the 8 viable genotypes. The results obtained from $Hri^{+/+}Hbb^{+/+}$, $Hri^{+/+}Hbb^{+/-}$, and $Hri^{+/+}Hbb^{-/-}$ mice were not statistically different from those of wild-type ($Hri^{+/+}Hbb^{+/+}$) mice (data not shown). Thus, the lack of 1 copy of the β -major globin gene did not cause any significant phenotype. This was also the case in $Hri^{-/-}Hbb^{+/+}$ mice (Figure 5A).

$Hbb^{-/-}$ mice have a severe hypochromic microcytic anemia with a reticulocytosis, similar to humans with β -thalassemia intermedia ($P < 0.001$, Figure 5A). Anemia was more severe in $Hbb^{-/-}$ mice lacking 1 copy of HRI, $Hri^{-/-}Hbb^{-/-}$ mice (Figure 5A), which had

significantly decreased rbc numbers and hemoglobin content ($P < 0.001$). Furthermore, thalassemic animals with a single copy of HRI had an MCV of 51.4 ± 3.72 fl, intermediate between that of wild type (57.3 ± 2.96 fl) and $Hri^{+/+}Hbb^{-/-}$ animals (44.12 ± 6.13 fl) ($P < 0.01$). This result further supports the notion that HRI is activated by denatured globins and contributes to the microcytosis seen in thalassemia.

Both light and electron microscopy demonstrated inclusion bodies in erythrocytes (Figure 5, B and C). Based upon a previous finding of morphologically similar inclusions in iron-deficient $Hri^{-/-}$ mice (24), we hypothesized that the accumulation of α -globin inclusions in β -thalassemia might normally be restricted by translational inhibition by activated HRI (24). Indeed, more inclusions were seen in thalassemic reticulocytes lacking 1 copy of HRI ($Hri^{-/-}Hbb^{-/-}$; Figure 5, B and C). When the amount of aggregated globins was quantitated, there were 2.24-fold more inclusions in $Hri^{-/-}Hbb^{-/-}$ mice than in $Hbb^{-/-}$ alone. Furthermore, the inclusions in the $Hbb^{-/-}$ blood samples were composed of nearly all α -globin chains despite the presence of β -minor globin in the soluble hemoglobin (Figure 6). These results demonstrate that activation of HRI is important in minimizing the accumulation of denatured α -globin aggregates, and importantly, that both copies of HRI are required for this function.

Thalassemia syndromes are often complicated by splenomegaly, cardiomegaly, and iron overload. Each of these parameters was

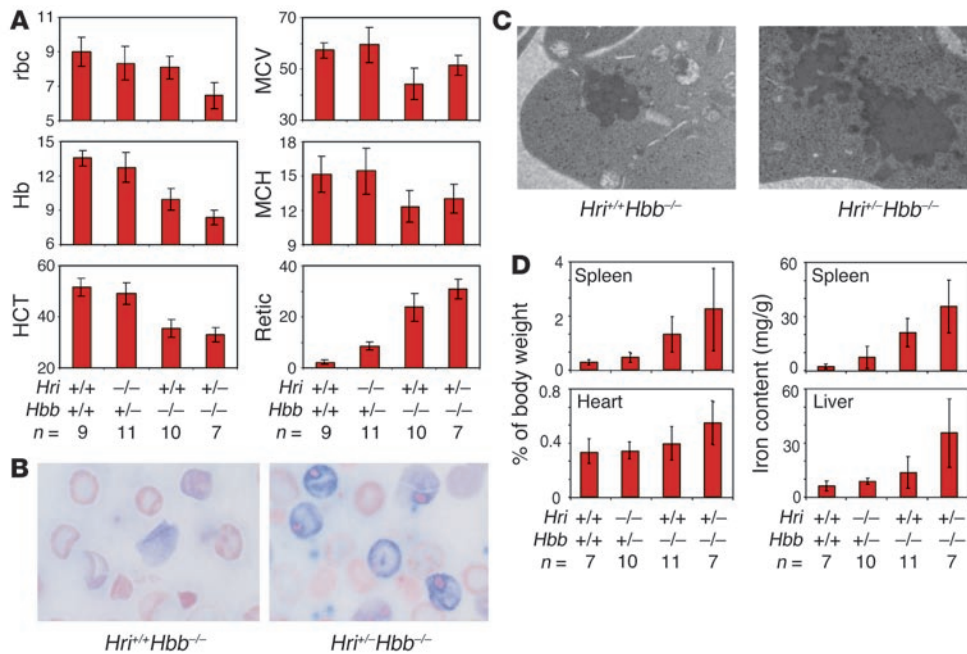


Figure 5 More severe pathology of β -thalassemic mice lacking 1 copy of HRI gene. (A) Complete blood cell counts in the adult mice. Data are presented as mean \pm SD. Units of the hematological parameters are the same as in Figure 1. HCT, hematocrit (expressed as percentages). (B) Wright-Giemsa stained peripheral blood smears. Inclusions are indicated by arrows. Original magnification, $\times 1000$. (C) Electron microscopic examination of inclusions in reticulocytes. Original magnification, $\times 22,000$. (D) Splenomegaly, cardiomegaly, and iron contents in spleen and liver. Tissue irons are expressed as μg iron/g of body weight. Data are presented as mean \pm SD. Twelve- to sixteen-week-old mice were used for these experiments.

examined in the $Hri^{-/-}Hbb^{+/-}$ F2 animals. Only β -thalassemic mice showed increases in their spleen to body weight ratios. Compared to nonthalassemic animals, a 3-fold increase was seen in $Hri^{+/-}Hbb^{-/-}$ mice, which was further exaggerated to an 8-fold difference in $Hri^{-/-}Hbb^{-/-}$ animals (Figure 5D, top left panel; $P < 0.05$). Consistent with their more severe anemia, there was also a significant increase in the heart sizes of $Hri^{-/-}Hbb^{-/-}$ animals (Figure 5D, lower left panel; $P < 0.05$). Splenic and hepatic iron overload secondary to the chronic anemia were also more severe in $Hri^{-/-}Hbb^{-/-}$ mice (Figure 5D, right panels; $P < 0.05$).

In addition, the lifespan of $Hri^{-/-}Hbb^{-/-}$ mice was compromised, and fertility was significantly decreased. When pregnancies were carried to term, the litters were small in number, and the pups were runted and pale, and died soon after birth. Importantly, none of the full-term pups was of $Hri^{-/-}Hbb^{-/-}$ genotype. $Hri^{-/-}Hbb^{+/-}$ mice, on the other hand, appeared normal in their hematology and pathophysiology (Figure 5, A and D) and were fertile. Thus, results from viable adult mice demonstrate that the lack of a single copy of HRI results in a clinically more severe form of β -thalassemia.

Discussion

The role of eIF2 α phosphorylation in the regulation of protein synthesis by a variety of stresses has been studied extensively in cultured cells (reviewed in ref. 4). The availability of genetically modified mice with a knock-in allele (Ser51Ala) of eIF2 α (33) and the knockout of each of the 4 eIF2 α kinases (24, 34–37) has begun to unveil the physiological importance of eIF2 α phosphorylation and the roles of individual eIF2 α kinases in the in vivo response to

stress. Each of the eIF2 α kinase null lines has a distinct phenotype. *Perk* null mice are compromised in their ability to respond to viral challenges (34, 38), and *Perk* null mice develop diabetes between 2 and 4 weeks of age (35). *Gcn2* null mice are also viable, but the viability is reduced upon amino acid starvation (36), and we previously demonstrated that the erythroid response to iron deficiency is abnormal in *Hri* null mice (24). The phenotypes of the individual eIF2 α kinase knockout mice provide the in vivo evidence of the regulation of each of these kinases by different stresses. The early postnatal lethal phenotype of eIF2 α Ser51Ala knockin mice in the absence of stress is, however, much more severe than would be expected from the phenotypes of individual eIF2 α kinase knockout mice. Extensive search of both the human and mouse genomes has yielded no evidence for other eIF2 α kinases in mammals. Thus, the severe phenotype of Ser51Ala knockin mice, which are unable to undergo eIF2 α

phosphorylation, suggests that there is an additional physiological function of the eIF2 α kinases yet to be discovered during development and in disease states.

In this report, we have further uncovered the protective function of HRI in 2 murine models of human intrinsic rbc disorders, EPP and β -thalassemia. In both cases, the presence of HRI reduces the severity of these diseases, and the maximal effect is gained when both copies of HRI are retained, as HRI haploinsufficiency resulted in more severe clinical and pathological manifestations.

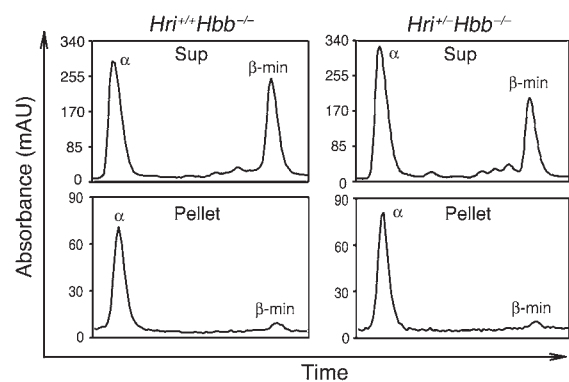


Figure 6 Inclusions in $Hbb^{-/-}$ rbc are composed of α -globin. The globin chain compositions in the soluble hemoglobin and the aggregated inclusions were analyzed by HPLC.

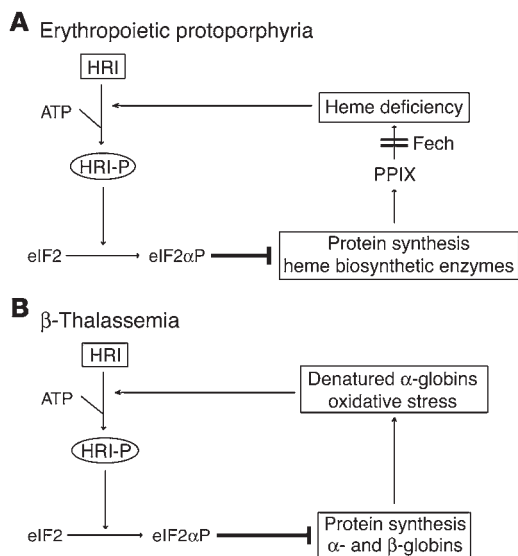


Figure 7
 Models of the essential roles of HRI in EPP and β -thalassemia. **(A)** In EPP, Fech deficiency results in heme deficiency that activates HRI. Activated HRI phosphorylates eIF2 α and inhibits protein synthesis in immature rbc. Most pertinently, the reduction of the synthesis of heme biosynthetic enzymes by HRI minimizes the accumulation of toxic PPIX, the culprit of EPP clinical symptoms. **(B)** In β -thalassemia intermedia, excess α -globin aggregates normally activate HRI to prevent further synthesis of the unpaired α -globins that precipitate. In the absence of HRI, the thalassemic rbc precursors continue to make α -globins in excess of β -globins, forming inclusions and consequently destroying the rbc precursors.

In both of these models, animals have a hypochromic, microcytic anemia, and HRI was activated. We have shown previously that HRI is required in the development of erythrocyte hypochromia and microcytosis in iron deficiency (24). Our present study provides further evidence that the morphologic hypochromia and microcytosis may be a general hallmark of HRI activation. Furthermore, increases in the accumulation of globin inclusions were seen in both EPP and β -thalassemia mice lacking HRI (Figures 1, 4, and 5). Thus, these results indicate that the protective role of HRI in these 2 diseases is mediated through the regulation of general protein synthesis as illustrated in Figure 7.

The excessive accumulation of PPIX in $Hri^{+/-}Fech^{m1Pas/m1Pas}$ compound mutants (Figures 1 and 3) demonstrates that downregulation of heme precursor synthesis through translational inhibition of the biosynthetic enzymes by activated HRI is an important adaptive mechanism for preventing the accumulation of toxic PPIX (Figure 7A). This observation may have important clinical implications. It is well established that a small fraction of EPP patients develop fatal hepatic pathology (reviewed in ref. 31). The molecular mechanism for this severe phenotypic expression is unknown but does not appear to be necessarily related to the specific disease allele (31). The severe pathology of the $Hri^{+/-}Fech^{m1Pas/m1Pas}$ mice reported here (Figures 1 and 3) indicates that HRI may be a significant modifier gene contributing to EPP disease severity, particularly in the development of hepatic pathology. Furthermore, $Hri^{+/-}Fech^{m1Pas/m1Pas}$ and $Hri^{+/-}Fech^{m1Pas/m1Pas}$ mice may be useful models for the study of severe EPP.

In β -thalassemia, there is an imbalance of α - and β -globin chains with excess of α -globin. The α -globin tetramers are relatively

unstable and form inclusions, which result in destruction of erythroid precursors (39). As illustrated in Figure 7B, HRI is normally activated in β -thalassemia and inhibits protein synthesis, including that of α -globin, the most abundant protein in β -thalassemic erythroid precursors. However, in the absence of HRI, protein synthesis continues, and a large excess of α -globin is made, resulting in numerous intraerythroid inclusions (Figures 4–6). It is well documented that the phenotypic severity of human β -thalassemia is quite diverse and largely related to the nature of the specific β -globin mutation(s) (reviewed in ref. 40). Allele-specific variability can be negated in mice. Our present study of $Hri^{+/-}Hbb^{-/-}$ mice and $Hri^{-/-}Hbb^{-/-}$ embryos demonstrates that HRI plays a critical role in modifying the β -thalassemic phenotype in mice, suggesting that HRI may be one of the modifier genes that influences the outcome of β -thalassemia in humans.

In summary, we have presented evidence for the protective function of HRI in 2 rbc disorders. However, the role of HRI may be broader and more general in other intrinsic rbc disorders, such as α -thalassemia, sickle cell anemia, and glucose 6-phosphatase dehydrogenase deficiency, among others. We previously showed that HRI is activated under stress conditions other than heme deficiency, such as oxidative stress, heat shock, and osmotic shock (25). Thus, translational regulation by HRI is important in safeguarding the amount of the globin proteins to be made under various conditions. It is critical to reduce excess synthesis of globin proteins under nonoptimal stress conditions or disease states. The rbc are packed with a very high concentration of hemoglobin. It is therefore important to keep globin synthesis in check. HRI serves as a checkpoint to prevent excess synthesis of globins, which may precipitate and may be detrimental to rbc precursors.

Methods

Mouse strains, breeding, and genotyping. All mouse production and experimentation was approved by the Committee on Animal Care at Massachusetts Institute of Technology. $Hri^{-/-}$ mice on an inbred B6, 129 mixed genetic background were generated in our laboratory as described previously (24). BALB/cJ- $Fech^{m1Pas/m1Pas}$ mice (26) and B6.129P2- $Hbb^{m1Unc/J}$ mice (27) were obtained from Jackson Laboratory. To generate double heterozygote F1 progeny, male $Hri^{-/-}$ mice were crossed with female $Fech^{m1Pas/m1Pas}$ or $Hbb^{-/-}$ mice. The double heterozygotes were interbred to produce the F2 progeny. A total of 159 $Hri^{+/-}Fech^{m1Pas/+}$ intercross progeny from 14 litters and 162 $Hri^{-/-}Hbb^{+/-}$ intercross progeny from 17 litters were analyzed. The Hri (24) and $Fech$ genotypes were determined by PCR. The genotypes of the β -major gene were deduced by analyzing the hemoglobins separated by isoelectric focusing gel electrophoresis (41).

For embryonic studies, $Hri^{-/-}Hbb^{+/-}$ mice were interbred, as $Hri^{+/-}Hbb^{-/-}$ mice were infertile. The date of copulation plug detection was defined as embryonic day E 0.5. Mice were sacrificed at different developmental stages, and the embryos were dissected. Blood samples were collected from either the amnionic vessels or cervical arteries of the embryos for preparation of the blood smears and the hematocrit measurements.

Hematological and pathological analyses. Complete blood analyses of the peripheral blood collected from the tail veins of mice aged 12–16 weeks were performed by the Division of Comparative Medicine at MIT using a Hemavet 800 instrument (CDC Technologies Inc.). Reticulocyte counts were determined on a FACS as described (42).

Peripheral blood cell morphology was examined on Wright-Giemsa stained smears. Tissues were fixed in formalin and processed for paraffin embedding and sectioning by the MIT Division of Comparative Medicine, using standard procedures. Nonheme-tissue iron was assayed as previously



described (43). Electron microscopy was performed on Karnovsky's fixed, EDTA-anticoagulated blood using standard methodologies in the Department of Pathology, Children's Hospital, Harvard Medical School.

Western blot analysis of the phosphorylation of HRI and eIF2 α . Blood samples were collected by heart puncture or tail vein and were washed twice with ice-cold phosphate-buffered saline supplemented with 5 mM glucose. Packed blood cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 16 μ g/ml benzamidine HCl, 10 μ g/ml phenanthroline, 10 μ g/ml aprotinin, 10 μ g/ml pepstatin A, and 1 mM PMSF (Cell Signaling Technology). Phosphorylation of HRI and eIF2 α in the blood samples was analyzed by Western blot as described previously (25).

Erythrocyte protoporphyrin concentrations. Erythrocyte protoporphyrin concentrations were determined on a FACSCalibur flow cytometer (BD) at an excitation wavelength of 488 nm as described previously (44). An emission wavelength of 650 nm was used to detect protoporphyrin-specific fluorescence.

Skin photosensitivity assay. The backs of mice were shaved, depilated, and exposed for 20 minutes to a mercury vapor lamp filtered with window glass to remove ultraviolet B irradiation (32). Mice were examined daily for the progression of the burns, and the severities of photosensitivity were recorded.

Analysis of globin chain composition in the inclusion bodies. The blood samples (20 μ l) were collected from tail veins. Blood cells were centrifuged and washed 2 times with cold PBS and then lysed in 200 μ l of 0.1 \times PBS on ice for 30 minutes. Lysates were centrifuged at 16,060 *g* for 30 minutes at 4 $^{\circ}$ C. The supernatants were mixed with 100 μ l of 60% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) and then diluted 20 fold with 20% ACN/0.1% TFA. The diluted supernatants were filtered through a 0.22 μ m filter, and 500 μ l of samples were used for HPLC analysis. Reverse-phase HPLC was performed on an \ddot{A} KTA Basic10 system (Amersham Biosciences) using a Vydac C4 column (25 cm by 0.46 cm, no. 214TP54; Grace Vydac). A linear gradient from 24% to 60% ACN containing 0.1% TFA was used with a run-

ning time of 120 minutes and at a flow rate of 1 ml/min. The elution of globin chains was monitored by absorbance at 214 nm. The data was analyzed with Unicorn 4.12 software (Amersham Biosciences).

For analysis of inclusions, the pellets were first washed with 0.1 \times PBS until the washing solution became colorless and free of trapped soluble hemoglobin. The washed pellets were dissolved in 500 μ l of 20% ACN/0.1% TFA. Samples were then processed for HPLC as described above.

Quantification of globins in the inclusion. For quantification of the inclusion bodies, hemolysates were prepared as described above. Lysates were centrifuged at 2,000 *g* for 30 minutes at 4 $^{\circ}$ C to remove the membrane and debris. The supernatant was centrifuged at 100,000 *g* for 3 hours at 4 $^{\circ}$ C to collect aggregated inclusions. Pellets were rinsed with 0.1 \times PBS to remove the trapped soluble hemoglobin and then were dissolved in SDS sample buffer. Globin in the pellets was then separated on 15% SDS-PAGE gels and stained with Coomassie Brilliant Blue R-250 (Pierce). The intensity of globin protein stain was quantitated using AlphaEasy FC software (Blue Sky Software Corp.).

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