HRI protein kinase in cytoplasmic heme sensing and mitochondrial stress response: Relevance to hematological and mitochondrial diseases

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Most iron in humans is bound in heme used as a prosthetic group for hemoglobin. Heme-regulated inhibitor (HRI) is responsible for coordinating heme availability and protein synthesis. Originally characterized in rabbit reticulocyte lysates, HRI was shown in 1976 to phosphorylate the α-subunit of eukaryotic initiation factor 2, revealing a new molecular mechanism for regulating protein synthesis. Since then, HRI research has mostly been focused on the biochemistry of heme inhibition through direct binding and heme sensing in balancing heme and globin synthesis to prevent proteotoxicity in erythroid cells. Beyond inhibiting translation of highly translated mRNAs, eukaryotic initiation factor 2α phosphorylation also selectively increases translation of certain poorly translated mRNAs, notably activating transcription factor 4 mRNA, for reprogramming of gene expression to mitigate stress, known as the integrated stress response (ISR). In recent years, there have been novel mechanistic insights of HRI–ISR in oxidative stress, mitochondrial function, and erythroid differentiation during heme deficiency. Furthermore, HRI–ISR is activated upon mitochondrial stress in several cell types, establishing the bifunctional nature of HRI protein. The role of HRI and ISR in cancer development and vulnerability is also emerging. Excitingly, the UBR4 ubiquitin ligase complex has been demonstrated to silence the HRI–ISR by degradation of activated HRI proteins, suggesting additional regulatory processes. Together, these recent advancements indicate that the HRI–ISR mechanistic axis is a target for new therapies for hematological and mitochondrial diseases as well as oncology. This review covers the historical overview of HRI biology, the biochemical mechanisms of regulating HRI, and the biological impacts of the HRI–ISR pathway in human diseases.

In the 1950–1960s, heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase was discovered and characterized as an inhibitor of translation in reticulocyte (Retic) lysates known as heme-controlled repressor (1–5). In 1976, over 20 years before the discovery of its kinase activity, Ranu and London (6) purified the protein and designated it as HRI, heme-regulated inhibitor. HRI was the first member of the eIF2α kinase family to be investigated and thus given the gene symbol of *Eif2ak1*. There are now four well-characterized members of this kinase family, each sensing unique stress signals in the tissues where they are prevalently expressed (Fig. 1*A*). HRI is highly expressed in erythroid cells and responds to heme availability and mitochondrial stress. The other eIF2α kinase family members are PKR (*Eif2ak2*), which is induced by interferon in response to viral infections, general control nonderepressible 2 (GCN2) (*Eif2ak4*), which is highly expressed in the liver and senses nutrient starvations and ribosome collision, whereas protein kinase R–like endoplasmic reticulum kinase (PERK) (*Eif2ak3*) is highly expressed in the pancreas and other secretory tissues and is activated in response to endoplasmic reticulum stress (Fig. 1*A*) (7).

All four eIF2α kinases phosphorylate a common substrate, the α-subunit of eIF2 at the conserved Ser51 residue. The molecular mechanism of inhibition of translational initiation by the phosphorylation of eIF2α has been studied extensively (7, 8). In brief, the GTP in the eIF2–GTP complex is hydrolyzed to GDP during translation. The recycling of eIF2 for another round of translational initiation therefore requires the exchange of its bound GDP for GTP, which is catalyzed by the rate-limiting initiation factor eIF2B. The GDP–GTP exchange activity of eIF2B is inhibited by phosphorylated eIF2α (eIF2αP). The eIF2 is a translation initiation factor that requires association with GTP to deliver initiator tRNA to ribosomes during protein synthesis, and thus phosphorylation of eIF2 prevents initiation of another round of translation (9). Lowering of global protein synthesis during stress would conserve resources and reprogram gene expression.

In addition to inhibiting translation of highly translated mRNAs, eIF2αP also selectively increases those poorly translated mRNAs in nonstressed conditions through upstream ORFs (uORFs) located in the 5′-leaders of their mRNAs, most notably the activating transcription factor 4 (ATF4) mRNA. Increased ATF4 protein production is important for inducing the transcriptional expression of genes involved in mitigation and adaptation to stress (Fig. 1*A*). Because this coordinated translational regulation can occur in response to multiple eIF2α kinases during a range of stress, this pathway has been termed the integrated stress response (ISR) (7, 8, 10, 11). However, when the stress is insurmountable, prolonged activation of ISR can also lead to programmed cell death.

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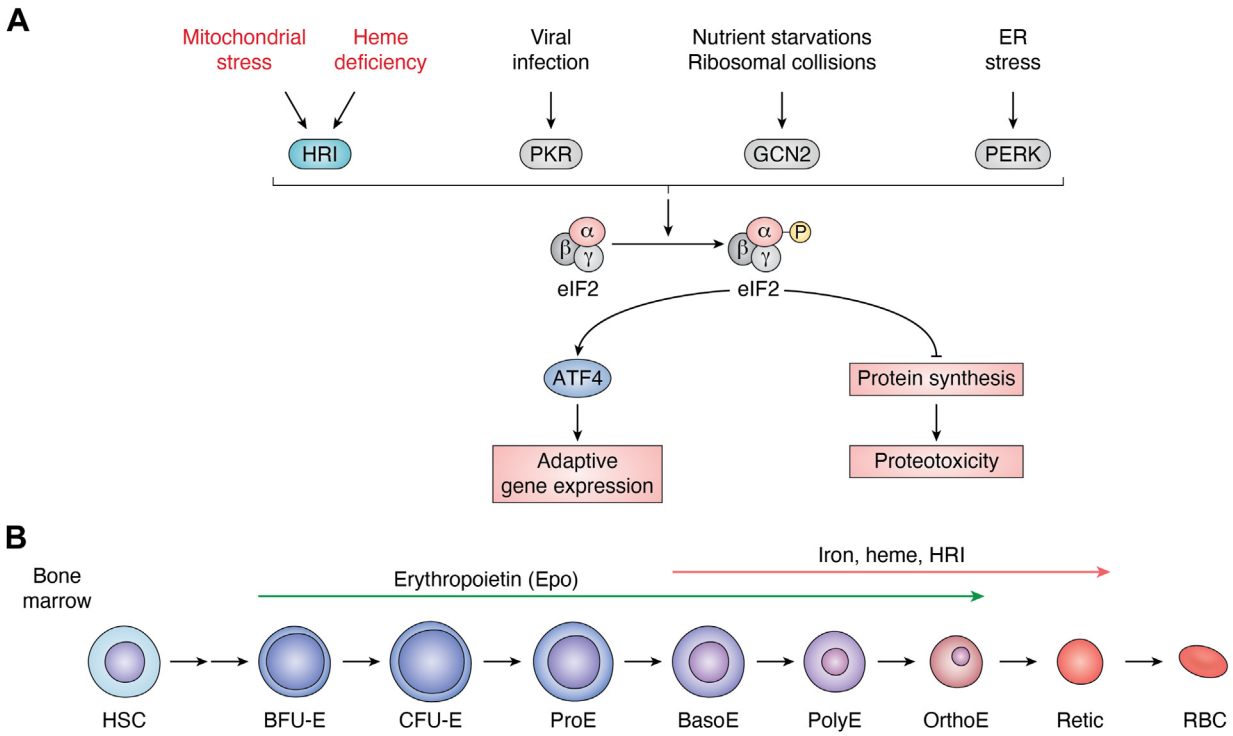

Figure 1. The integrated stress response of the eIF2α kinase family and the function of HRI in erythroid differentiation stages. *A*, integrated stress response. In mammalian cells, four eIF2α kinases are activated in response to major stress, heme deficiency, viral infection, nutrient starvations, ribosome collisions, endoplasmic reticulum, and mitochondrial stress. Activated eIF2α kinases phosphorylate eIF2α and inhibit protein synthesis to conserve energy. Decreased protein synthesis also reduces problematic folding of newly synthesized proteins and helps to restore proteostasis. Activation of PERK and HRI are essential to mitigate proteotoxicity of unfolded proteins in endoplasmic reticulum and cytosol, respectively. Phosphorylation of eIF2α also selectively enhances the translation of *Atf4* mRNA. ATF4 protein then initiates an adaptive gene expression to mitigate stress. *B*, specification of erythropoiesis. Adult erythropoiesis occurs at the bone marrow and starts with hematopoietic stem cells (HSCs). Burst-forming unit-erythroid (BFU-E) cells are the first committed erythroid progenitors that respond to erythropoietin (Epo), the erythroid growth factor. The erythroid differentiation follows the linear trajectory to mature red blood cells. Iron, heme, and HRI play essential roles at the terminal differentiation staring at the basophilic erythroblast stage when hemoglobin production is initiated. BasoE, basophilic erythroblast; CFU-E, colony-forming unit-erythroid; eIF2α, the alpha subunit of eukaryotic initiation factor 2; HRI, heme-regulated inhibitor; OrthoE, orthochromatic erythroblast; PERK, protein kinase R–like endoplasmic reticulum kinase; PolyE, polychromatic erythroblast; ProE, proerythroblast; RBC, red blood cell; Retic, reticulocyte.

Iron deficiency (ID) anemia is a prevalent disease worldwide and a major contributor to the global burden of anemia (12). Most of the iron in the human body is bound in heme, where its inclusion in hemoglobin accounts for as much as 70% of the total iron content (13). In addition to serving as a prosthetic group for hemoglobin, heme also acts as a signaling molecule for gene expression by modulating HRI-mediated translation (14, 15). HRI is a unique heme-sensing hemoprotein with two heme-binding domains, and its kinase activity is inhibited by heme (16–18). During erythroid differentiation, HRI coordinates the translation of globin mRNAs with the availability of heme to ensure the efficient production of large amounts of hemoglobin in erythroid precursors (19, 20). In HRI deficiency, excess globins synthesized during heme deficiency precipitate and cause proteotoxicity (Fig. 1*A*) (20, 21).

Since these initial findings, significant advancements of the HRI–ISR pathway have been made in both erythroid and nonerythroid systems. Studies of mutant mice defective for HRI–ISR and genome-wide unbiased approaches for profiling *in vivo* translation and the transcriptome have revealed novel functions of HRI in alleviation of oxidative stress, and in homeostasis of mitochondrial function, erythroid differentiation, as well as erythropoietin (Epo) signaling in primary murine erythroblasts (EBs), the nucleated hemoglobin producing erythroid precursors (11, 22). Most importantly, HRI regulates both cytoplasmic and mitochondrial protein synthesis (11, 22).

HRI has also been identified as the eIF2α kinase responsible for the induction of ISR during mitochondrial stress of electron transport chain (ETC) dysfunction through a novel mechanism of activation by the mitochondrial intermembrane space protein DELE1 (23, 24). The roles of HRI–ISR in brain neuronal cells and neuropathy in the context of mitochondrial diseases as well as neurodegeneration are currently under active investigation. In addition, the HRI–ISR signaling pathway is elevated in patient tumors compared with normal tissues. Roles of HRI–ISR in cancer development and vulnerability have been demonstrated, uncovering new mechanisms of regulating HRI signaling by cytochrome *c* and BIRC6-mediated proteasome degradation of HRI (25, 26).

Another recent discovery involves silencing of HRI–ISR signaling. Little is known about mechanisms inactivating eIF2α kinases either by their dephosphorylation or

degradation. Excitingly, an E3 ubiquitin ligase UBR4 complex has been identified to target uniquely activated phosphorylated HRI, but not the other three eIF2α kinases, for proteasome degradation (26, 27). Of interest, mutations in human UBR4 cause ataxia and early onset dementia by disrupting degradation of activated HRI and therefore, the inability to silence the HRI–ISR during mitochondria stress or heme deficiency (27). Together, these recent findings suggest that the regulation and functional significance of HRI extends beyond that of heme and oxidative stress regulation in hematology, and HRI signaling may be promising therapeutic targets for neurodegenerative and mitochondrial diseases as well as oncology.

Considering these recent advancements in expanding the roles and biological significance of HRI–ISR signaling, it is timely to review the historical background of HRI biology in protein synthesis and the biochemical characterization of heme regulation, along with recent findings of HRI–ISR in stress erythropoiesis. New discoveries of HRI–ISR in mitochondrial stress and the molecular mechanism of silencing of HRI–ISR signaling by E3-ligase–targeted HRI degradation will then be presented. The review will conclude with the biological significance of HRI–ISR in mitochondrial and neuronal diseases as well as in cancers. In addition, this review emphasizes the molecular mechanisms of activation and silencing of HRI–ISR under various stress and pathological conditions, highlighting the bifunctional nature of HRI protein for heme sensing and mitochondrial stress response. The roles of HRI in the regulation of fetal hemoglobin production (28) and innate immune response (29) have been reviewed recently and are not included in this review.

Historical review of HRI biology

The timeline of major HRI discoveries over the last 70 years is summarized in Table 1. The journey leading to the discovery of HRI began in the mid-1950s with the observation that inorganic iron stimulates protein synthesis in immature erythroid cells (1–3). Subsequent work in the mid-1960s demonstrated that heme, not iron, is the true effector for the stimulation of protein synthesis (4). In 1969, HRI protein was formally demonstrated in Retic lysates (5). The development of cell-free Retic lysates for *in vitro* translation was critical for advancing our understanding of heme regulation of HRI since mitochondria, which are required for heme biosynthesis, are removed during the preparation of Retic lysates. Protein synthesis in Retic lysates is, therefore, dependent on the addition of heme. In the absence of added heme, protein synthesis in Retic lysates continues for the first 5 to 10 min followed by an abrupt decline and shutoff of protein synthesis with disaggregation of polysomes (4, 30–32). The addition of heme permits protein synthesis to continue for a prolonged duration, restoring polysomes and protein synthesis even after initial shutoff of protein synthesis and hence, demonstrating that the addition of heme can reverse the inhibition of protein synthesis in heme deficiency (4, 32–34).

The inhibition of protein synthesis in heme deficiency is associated with a marked decrease in the formation of preinitiation complex consisting of 40S ribosomal subunit and associated eIF2–GTP adjoined to initiator tRNA (35). In the late 1970s, extensive biochemical characterization and purification demonstrated that HRI is a heme-regulated eIF2α kinase (32, 36–40) and that heme binds directly to HRI and inhibits both the autokinase and eIF2α kinase activities

Table 1
Timeline of major HRI discoveries

Year	Discovery	References
1956–1958	Iron stimulates globin synthesis in rabbit reticulocytes and BM cells *in vitro*	(1–3)
1965–1968	Heme, not iron, is the effector for the stimulation of globin synthesis at the level of translation initiation	(4)
1969	Discovery of HRI, the inhibitor of protein synthesis in heme deficiency	(5, 6, 30–32)
1976	HRI is a heme-regulated eIF2α kinase	(32, 36–40)
1978	Heme inhibits autokinase and eIF2α kinase activities of purified HRI	(34, 39)
1981	Heme binding directly to purified HRI	(41)
1989–1992	Heme promotes intersubunit disulfide formation between HRI homodimer	(16, 34, 63)
1991	Cloning of the HRI complementary DNA	(42)
1994	HRI is highly expressed in the erythroid lineage	(43)
1998	HRI purifies as a hemoprotein, two heme-binding sites	(16)
2000–2001	Two heme-binding domains, N terminus and kinase insertion. Two heme molecules per HRI monomer	(17, 18)
2001	HRI knockout mice, heme, arsenite, and cytoplasmic stress in erythroid cells	(20, 55)
2005	HRI responsible for arsenite-induced stress granule formation and cell survival in murine embryonic fibroblast cells	(90)
2005	HRI genetic modifier of erythropoietic protoporphyria and β-thalassemia	(21)
2007	HRI expression and function in macrophages	(47)
2011	HRI roles in cancers	(129)
2012	HRI–ISR in primary erythroid precursors	(75)
2018	HRI coordinated translation by eIF2αP and mTORC1 during iron-restricted erythropoiesis	(22)
2018	HRI–ISR represses fetal hemoglobin globin production	(28)
2019	Genome-wide translational regulation *in vivo* by HRI during heme deficiency: protein homeostasis and mitochondrial function	(11)
2019	HRI–ISR role for innate immune response	(48)
2020	HRI functions in neurons	(124)
2020	HRI–ISR is activated by mitochondrial stress in a heme-independent manner	(23, 24)
2022	Activation of HRI–ISR by cytochrome *c* in persistent cancer cells	(25)
2023–2024	Degradation of activated HRI *via* the UBR4 E3 ligase complex	(26, 27)

(34, 39, 41) (Table 1). Cloning of the complementary DNA encoding HRI from rabbit Retic was accomplished in 1991, revealing homology to PKR and yeast GCN2 in the conserved kinase domains, unique N-terminal domain, and the kinase insert (KI) between the two kinase catalytic domains (42). The availability of HRI complementary DNA not only provides an invaluable tool to further study the biochemistry and molecular biology of HRI but also led to the cloning of murine HRI genomic DNA for the subsequent generation of HRI knockout mice in 2001 (20) (Table 1), a powerful tool for investigating the biological functions of HRI *in vivo*.

Studies using $Hri^{-/-}$ mice establish that HRI is responsible for the inhibition of protein synthesis during heme deficiency (20). $Hri^{-/-}$ Retics have decreased levels of eIF2αP and increased rates of protein synthesis in comparison to $Hri^{+/+}$ Retics. Importantly, protein synthesis in $Hri^{-/-}$ Retics is no longer dependent on heme. Furthermore, HRI is activated in the Retics of mice deficient in ferrochelatase, the last enzyme that incorporates Fe into protoporphyrin IX to form heme, providing *in vivo* evidence that heme, not iron, regulates HRI (21). Together, generation and characterization of the $Hri^{-/-}$ mice and $Hri^{-/-}$ Retics confirmed the role of this eIF2α kinase in the heme regulation of mRNA translation and provided insights into the key processes by which heme availability is coordinated with the synthesis of globin to form hemoglobin (Fig. 1*A*).

Expression of HRI in tissues and cell types

Measurements of levels of mRNA, protein, and kinase activity of HRI in murine tissues have demonstrated that HRI is differentially expressed among tissues, with highest levels in blood and the bone marrow (BM) erythroid precursor cells (43). Specification of erythropoiesis in the BM as illustrated in Figure 1*B* starts with burst-forming unit erythroid and then progresses to colony-forming unit erythroid, proerythroblasts (ProEs), basophilic erythroblasts (BasoEs), polychromatic erythroblasts, orthochromatic erythroblasts, and enucleated Retics, which move out from the BM to blood stream and further mature to red blood cells (RBCs) upon removal of ribosomes and mitochondria. Levels of HRI increase during erythropoiesis with higher expression in EBs (44) in which iron and heme play critical roles (Fig. 1*B*). Starting at the BasoE stage, HRI is the predominant eIF2α kinase and is expressed at two orders of magnitude higher than the other three eIF2α kinases (11, 45). Thus, the timing and magnitude of HRI expression are central for its role in the production of hemoglobin and RBCs.

Single-cell RNA-Seq (scRNA-Seq) technology has since provided further details of *Hri* mRNA expression not only among tissues but also in specific cell types within these tissues. While *Hri* mRNA expression was originally thought to be erythroid tissue specific, it is now appreciated to be more broadly expressed. CZ CellxGene Discover, a data platform that provides curated and interoperable scRNA-Seq data, was recently reported (46) and is available at cellxgene.cziscience.com. scRNA-Seq analyses of both human and murine tissues from this data platform demonstrate that *Hri* mRNA expression is highest in yolk sac and BM where the primitive and definitive erythropoiesis reside, respectively (Fig. 2*A*). Within hematopoietic lineages, *Hri* mRNA is expressed in 40% of erythroid cells as compared with 17% of myeloid cells and 14% of plasma cells (Fig. 2*B*). Expression of *Hri* mRNA in myeloid cells is consistent with earlier work showing the roles of HRI in BM-derived macrophages for iron homeostasis, macrophage maturation (47) and innate immune signaling (48). Interestingly, HRI is also highly expressed in embryos during development (Fig. 2*A*), an observation that may be linked to smaller liter sizes of $Hri^{-/-}$ mice as compared with WT mice.

scRNA-Seq of lineage-depleted murine BM cells (removal of the mature blood cells) also reveal that HRI expression increases during differentiation starting from ProE with higher expression in EBs (Fig. 2*C*) (Zhang S. and Chen J.-J., unpublished results, 2019). Levels of *Atf4* mRNA are increased in ProE and BasoE but decline significantly later during differentiation when transcription wanes (Fig. 2*D*). On the other hand, *Hri* mRNA expression continues through the Retic stage to regulate protein synthesis. In BasoE, *Hri* and *Atf4* mRNAs are highly expressed and are among the most efficiently translated mRNAs, indicating that *Atf4* mRNA is poised for preferential translation by HRI-directed eIF2αP to promote terminal erythroid differentiation (11).

HRI expression in the brain is far lower than that in erythroid cells, with only 2% of the brain cells expressing HRI (Fig. 2*A*), and is universally low (2–3%) in all cell types, including neural cells, neurons, macroglia cells, oligodendrocytes, and astrocytes (Fig. 2*E*). These observations underscore the complexity of brain cells and suggest that the expression and function of HRI is only necessary in specific types of neurons.

When examining *Hri* mRNA expression closely in the specific cell types of various tissues outside the erythroid lineage, it is noted that *Hri* mRNA is expressed mainly in epithelial cells as well as the hemopoietic myeloid cells and leukocytes in whole tissues (46) (Chen J.-J., personal observation, 2024). An example of prevalent *Hri* mRNA expression in epithelial cells of murine large intestine (49) is shown in Figure 2*F*. The significance of HRI expression in epithelial cells will be discussed later in the context of mitochondrial stress as well as cancer development and vulnerability.

Molecular mechanism of heme regulation of HRI

Since its discovery, heme is viewed as being central to the regulation of HRI activity. The current understanding of heme regulation of HRI is summarized and illustrated in Figure 3. Briefly, HRI senses intracellular heme concentrations through direct binding of heme to its two heme-binding domains. Heme promotes intersubunit disulfide bond formation in HRI homodimers and inhibits kinase activity. Release of heme during heme deficiency facilitates autophosphorylation of HRI and induction of its eIF2α kinase activity (Fig. 3). These three major biochemical events in heme regulation of HRI are described in more detail later.

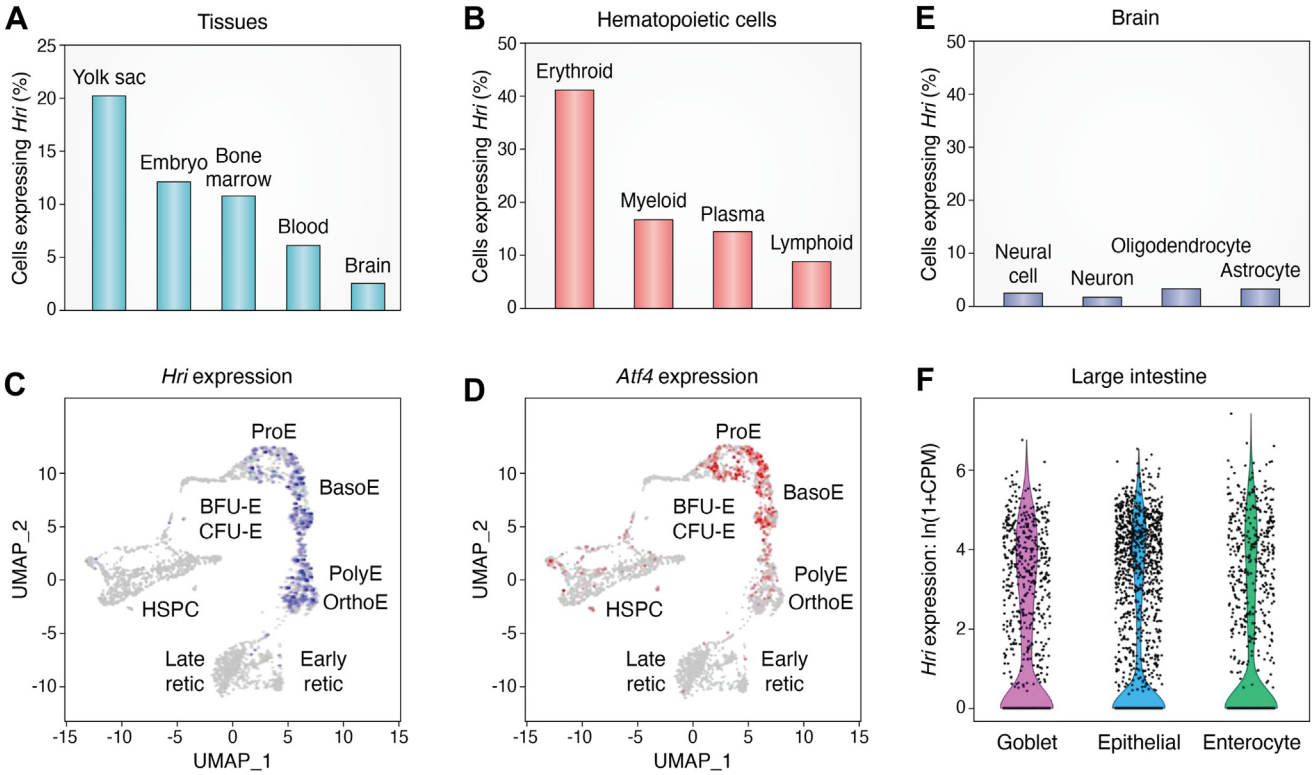

Figure 2. Expression of *Hri* mRNA in tissues and during erythropoiesis. *A, B,* and *E,* scRNA-Seq analysis of *Hri* mRNA expression in human tissues, bone marrow hematopoietic cells, and brain cells, respectively. The percentages of cells expressing *Hri* mRNA in each cell type as indicated are plotted on the *Y*-axis. Datasets are derived from CZ CellxGene Discover (cellxgene.cziscience.com), a data platform that provides curated and interoperable scRNA-Seq data (46). *C* and *D*, relative expression levels of *Hri* and *Atf4* mRNAs in lineage-depleted cells of mouse bone marrow at steady-state erythropoiesis (22) by scRNA-Seq. Uniform manifold approximation and projections (UMAP_1 and UMP_2) were used for dimensionality reduction and visualization for scRNA-Seq data (Zhang S. and Chen J-J., unpublished). *F*, scRNA-Seq analysis of *Hri* mRNA expression in cell types of murine large intestines. HSPC, hematopoietic stem and progenitor cell; scRNA-Seq, single-cell RNA sequencing.

Unique hemoprotein with two distinct heme-binding domains

Like other eIF2α kinases, HRI protein has a prominent insert sequence between its two kinase domains, and the HRI protein can be divided into five regions: the N terminus, the kinase I, KI, kinase II, and the C terminus (Fig. 3*A*). Both the N terminus and KI can bind heme, whereas kinase I, kinase II, and C terminus do not bind heme (Fig. 3*A*). Furthermore, the N terminus, but not the KI, purifies as a pink-colored hemoprotein when these portions of HRI are expressed as recombinant proteins in the presence of 5 μM heme. These findings indicate that the heme is stably incorporated into the N terminus with a high affinity (17).

Recombinant full-length HRI expressed in Sf9 insect cells also purifies as a hemoprotein, supporting the idea that there are two types of heme-binding sites in the purified HRI. One type of heme-binding site is nearly saturated with stably bound endogenous heme at the N terminus, which copurifies with HRI. The second binding site in the KI binds heme reversibly (16) (Fig. 3, *A* and *B*). Further supporting this model, the N-terminally truncated Met2 (Δ1–103) and Met3 (Δ1–130) HRI do not purify as hemoprotein, demonstrating the loss of the stable heme binding in Met2 and Met3 HRI that are devoid of the N-terminal region. The stoichiometry of two heme molecules per HRI monomer was established by direct measurement of heme chromophore through alkaline pyridine treatment of homogeneous heme-saturated HRI (18) (Fig. 3*B*).

Although no significant change in the molecular size can be discerned by gel filtration upon heme binding to the purified HRI (34, 50, 51), HRI homodimer with two heme molecules bound to both N terminus (Fig. 3*B*, latent HRI) and the homodimer with additional two heme molecules bound to KI (Fig. 3*B*, inactive eIF2α kinase) can be resolved by non-denaturing native gel electrophoresis (16). Together, these results demonstrate that HRI is a unique hemoprotein with dual heme-binding domains, and that the KI domain contains the heme-binding site responsible for the reversible heme regulation of HRI (16, 17) (Fig. 3).

Activation of HRI by autophosphorylation

Both native and purified HRI from Retic lysates are homodimers (34, 50, 51). The C-terminal domain is necessary for HRI dimerization (52, 53). HRI is activated by intermolecular autophosphorylation of the homodimer (16, 18, 54, 55). Purified HRI undergoes multiple autophosphorylation events in heme deficiency (16, 18, 41, 56). There are eight major tryptic phosphopeptides in fully autophosphorylated HRI (53). Autophosphorylation of murine HRI at Thr485 in the activation loop is the final stage of autophosphorylation, and Thr485-P is essential for attaining eIF2α kinase activity (53) (Fig. 3*B*). Autophosphorylation and dimerization are conserved features for the ordered mechanism of activation of each of the eIF2α kinase family members.

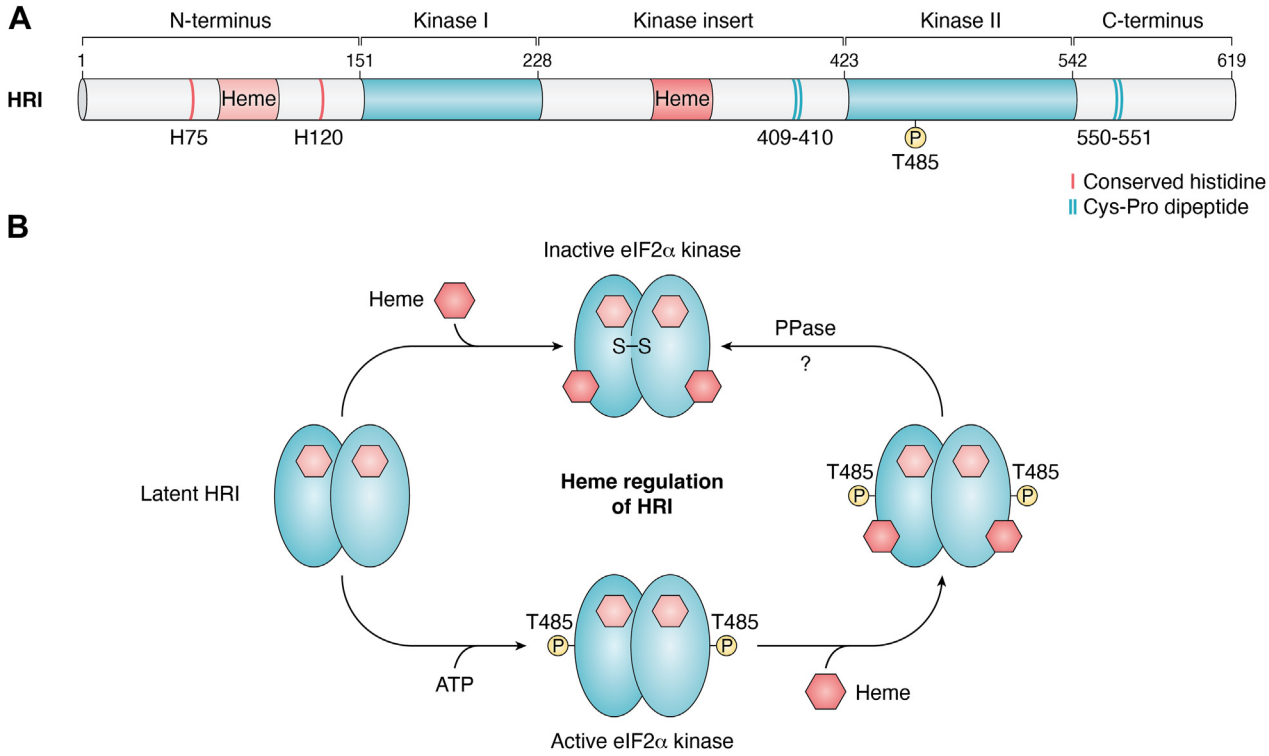

Figure 3. Protein domains of HRI and the activation of HRI in heme deficiency. *A*, HRI protein is composed of five regions: the unique N terminus of stable heme binding, two kinase catalytic regions (kinase I and kinase II), the kinase insert (KI) of reversible heme sensing, and the unique C terminus. The amino acid sequence of murine HRI is used here. Heme molecules are marked in *pink color* for the N terminus and in *darker pink* for the KI. The conserved histidine residues that coordinate the heme-iron molecule in the N terminus are marked by *pink lines*. Cys-Pro dipeptides, the putative heme-regulated motifs, are marked by *blue lines*. *B*, heme regulation of HRI. Latent heme-reversible HRI is a stable homodimer held together by noncovalent interactions. This form of HRI has one heme molecule stably bound per subunit to its N-terminal domain, indicated by *pink hexagon*. In heme abundance, one more heme molecule binds to the KI domain (indicated by *darker pink hexagon*) of each subunit of HRI, promotes intersubunit disulfide formation in the HRI homodimer, and inhibits kinase activity. In heme deficiency, autophosphorylation of HRI at Thr485 activates its eIF2α kinase activity. This activation of HRI is reversible by the addition of heme, which reduces eIF2αP and restores protein synthesis. However, the molecular mechanism for heme reversal is still unclear. It is possible that activated T485-P HRI may be dephosphorylated to inactivate its eIF2α kinase activity. eIF2α, the alpha-subunit of eukaryotic initiation factor 2; eIF2αP, phosphorylated eIF2α; HRI, heme-regulated inhibitor.

Inhibition of HRI kinase activities by heme

Both autokinase and eIF2α kinase activities of purified HRI are inhibited by submicromolar concentrations of heme with an apparent K_i (concentration at 50% inhibition) of 0.2 μM (16, 17). ATP binding to HRI is inhibited by prior treatment of HRI with heme in a concentration-dependent manner (57). These results support the idea that heme release is an early trigger for HRI activation. While N-terminally truncated HRI are active eIF2α kinases and autokinases, they are 10 times less sensitive to heme inhibition compared with the full-length HRI. These results suggest that there is cooperation between the two heme-binding domains in HRI to achieve efficient heme regulation of activity (17). In this regard, heme has been shown to stabilize the binding of the N terminus with N-terminally truncated HRI (58) as well as to stabilize the interaction of the N terminus with the KI (59).

In the N-terminal domain, conserved residues His75 and His120 were identified to be the proximal and distal heme ligand, respectively (60) (Fig. 3*A*). Mutation of His75 and His120 individually to Ala in full-length HRI resulted in decreased sensitivity to heme inhibition, similar to N-terminally truncated HRI (Bauer B., Ye A., and Chen J.-J., unpublished). HRI also contains two putative heme regulatory motifs (HRMs) (61), Cys-Pro (409–410) and Cys-Pro (550–551) in murine HRI (62), which are not present in the other three members of eIF2α kinases (Fig. 3*A*). However, the significance of HRM motifs in heme regulation of HRI is still not clear. HRM can bind heme and contains preferentially a basic amino acid followed by the conserved Cys-Pro dipeptides and a hydrophobic residue downstream (62). Substitutions of these two Cys residues individually to Ser had no significant effect on the heme responsiveness of HRI (17). Further, these two Cys residues are not conserved in chicken, xenopus, or fish HRI, indicating that both may be dispensable for the heme regulation of HRI. Igarashi *et al.* (59) suggested that His120 and Cys409 were important for binding heme ligands in the full-length HRI. It has also been suggested recently that heme regulation resides in the kinase regions (52). However, both studies used HRI expressed in *Escherichia coli*, and further validation is needed. In summary, while heme ligands, His75 and His120, in the N terminus are established, heme ligands for the reversible heme regulation in the KI are not yet clearly defined, and further investigation is needed.

Intersubunit disulfide bond formation upon heme binding

Heme promotes intersubunit disulfide bond formation in HRI homodimers (16, 34, 63) (Fig. 3*B*). There is a positive correlation between intersubunit disulfide formation of HRI and the inhibition of HRI kinase activities *in vitro* by various porphyrin compounds that are structurally similar to heme. Furthermore, there is a positive correlation between the abilities of various porphyrin compounds to promote intersubunit disulfide formation of HRI and the maintenance of protein synthesis, the reversal of the inhibition of protein synthesis, and the phosphorylation of eIF2α in rabbit Retic lysates (34). Together, these findings underscore the importance of free sulfhydryls and intersubunit disulfide bond formation in the regulation of HRI by heme (Fig. 3*B*).

In summary, these key biochemical events support the current model of heme regulation of HRI that is presented in Figure 3*B*. Latent HRI is a homodimeric hemoprotein with heme bound to the N terminus. In heme abundance, an additional molecule of heme binds to the KI domain of each monomer, promoting intersubunit disulfide bond formation to keep HRI inactive. In heme deficiency, HRI is activated by autophosphorylation, and the phosphorylation at threonine 485 in the activation loop is necessary for attaining the eIF2α kinase activity. This activation of HRI is reversible upon addition of heme. The exact mechanism for this reversal is not clear at present. It is likely that activated HRI can be dephosphorylated by a yet to be characterized phosphatase, leading to diminished activity. It has been demonstrated that the HRI can be dephosphorylated by alkaline phosphatase in cell lysates (55), and future studies should define critical protein phosphatases involved in modulating the phosphorylation and function of HRI.

HRI–ISR in the erythroid system

The molecular mechanisms by which heme regulates erythropoiesis are still not fully understood. A model for inducing heme deficiency in mice has been developed using dietary ID to study the functions of HRI and heme deficiency in whole-animal physiology. Since iron is recycled very efficiently, mice need to be weaned early at day 21 and fed an iron-deficient diet to achieve ID. Using this protocol, WT mice develop classical microcytic hypochromic ID anemia without noticeable pathology in other tissues (20) (Fig. 4*A*). By contrast, *Hri*$^{-/-}$ mice present with an unusual macrocytic hyperchromic anemia, with reduced number of RBCs and proteotoxicity because of excess heme-free globin inclusions (20, 21) (Fig. 4*B*). These *in vivo* studies support the model that HRI coordinates translation of globin mRNAs with the availability of heme in erythroid precursors to prevent proteotoxicity and for adaptation to ID (20, 21).

Beyond the regulation of globin mRNA translation, HRI also promotes erythroid differentiation and reduces ineffective erythropoiesis (IE) during ID (20) and in β-thalassemia (21), an inherited human disease with defect in β-globin productions resulting in accumulations of unpaired α-globin and anemia. Furthermore, HRI is necessary to inhibit the heme biosynthesis to reduce toxic protoporphyrin IX level and phenotypic severity in erythropoietic protoporphyria (21). To investigate the mechanisms by which eIF2αP regulates erythroid differentiation and IE *in vivo*, an erythroid-specific *eIF2α* Ala51 knockin mouse model (*eAA* mice), which harbors the substitution of the phosphorylation site Ser51 with Ala, has been generated (22). The *eAA* mice and those deleted for the ISR target *Atf4* were also studied in ID and compared with WT and *Hri*$^{-/-}$ mice (22). The phenotypes of these mice in ID are summarized in Figure 4 and are more fully described in detail later. An understanding of the global impact of HRI-mediated translational regulation on erythropoiesis has also emerged from ribosome profiling (Ribo-Seq) studies of EBs from WT and *Hri*$^{-/-}$ mice in ID (11). Ribo-Seq is a powerful tool to interrogate translation genome wide in an unbiased manner (64), providing new insights of HRI–ISR in terminal erythropoiesis (Fig. 4), which are also described in this section.

Severe anemia, IE, and oxidative stress in mice defective in HRI–ISR

During ID, *Hri*$^{-/-}$, *eAA*, and *Atf4*$^{-/-}$ mice were more anemic with reduced RBC numbers and lower blood hemoglobin levels than WT mice (Fig. 4*A*). In addition, *Hri*$^{-/-}$, *eAA*, and *Atf4*$^{-/-}$ mice developed splenomegaly, inhibition of erythroid differentiation starting at the BasoE stage with elevated serum Epo levels, and increased reactive oxygen species (ROS) levels in erythroid cells from BM, spleen, and blood during ID (22) (Fig. 4, *B* and *C*). These findings demonstrate that both eIF2αP and ATF4 in the HRI–ISR pathway are required to mitigate IE by promoting erythroid differentiation and reducing ROS level during ID.

Similar to *Hri*$^{-/-}$ mice, *eAA* mice also developed the unusual macrocytic hyperchromic anemia in ID (20, 22) (Fig. 4*B*). By contrast, *Atf4*$^{-/-}$ mice, which possess functional HRI, displayed microcytic hypochromic anemia similar to WT mice (22) (Fig. 4*C*). Furthermore, insoluble globin inclusions were visible in the blood samples from *Hri*$^{-/-}$ and *eAA* mice but not from *Atf4*$^{-/-}$ mice (Fig. 4, *B* and *C*). Thus, eIF2αP and translational control, but not ATF4, is essential to reduce proteotoxicity from cytoplasmic unfolded heme-free globin and is also required for the development of characteristic microcytic hypochromic anemia during ID. The similar erythroid phenotypes of *eAA* and *Hri*$^{-/-}$ mice demonstrate that the major physiological function of HRI in the erythroid lineage, if not all, is mediated by eIF2αP (Fig. 4*B*). It is to be emphasized that eIF2αP facilitates *Atf4* mRNA translation *in vivo* (22), but its expression does not appear to be critical for maintenance of proteostasis during ID. However, this enhanced ATF4 expression is necessary for terminal erythroid differentiation and oxidative homeostasis to be further discussed later.

Preferential translation of Atf4 *mRNA* in vivo *by HRI*

Beginning at BasoE, erythropoiesis is finely regulated by iron, heme, and HRI (15, 65, 66) (Fig. 1*B*). Ribo-Seq studies of BasoE from fetal livers of WT and *Hri*$^{-/-}$ embryos (11) demonstrate that *Atf4* mRNA is the most differentially

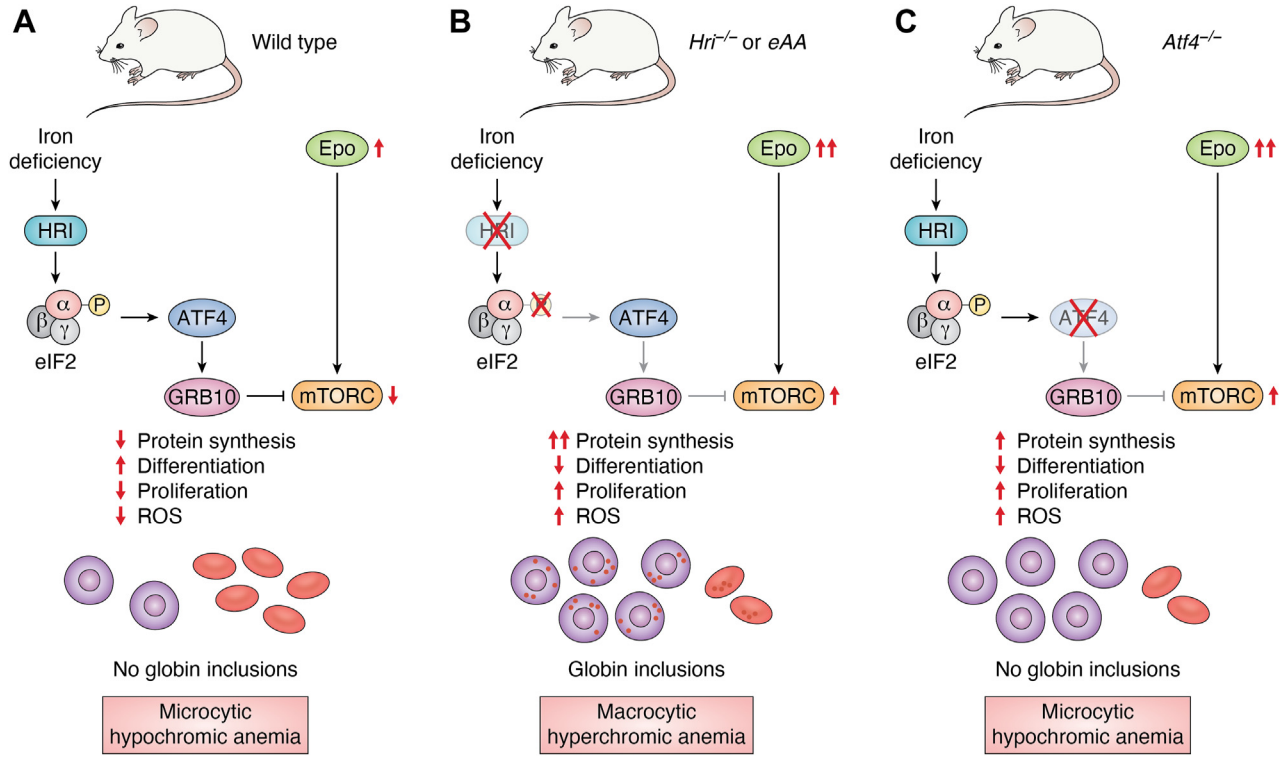

Figure 4. Genome-wide *in vivo* gene expression governed by heme and HRI–ISR during iron-restricted erythropoiesis. The regulation of gene expression and signaling pathways by heme and HRI are schematically illustrated. Summaries of the phenotypes of WT mice (*A*), *Hri*$^{-/-}$ or *eAA* mice (*B*), and *Atf4*$^{-/-}$ mice (*C*) during iron deficiency (ID) are presented. In WT mice shown in *A*, heme deficiency induced by dietary ID activates HRI, resulting in increased eIF2αP and inhibition of general protein synthesis in both the cytoplasm and mitochondria *via* decreased translation of ribosomal protein mRNAs from both cellular compartments. This reduced translation mitigates accumulation of unfolded proteins in both the cytoplasm and mitochondria to prevent proteotoxicity. Furthermore, selectively enhanced translation of *Atf4* mRNA by eIF2αP induces gene expression necessary for reprograming mitochondrial metabolism, reducing oxidative stress, and promoting erythroid differentiation. GRB10 highly induced by ATF4 represses mTORC1 signaling and acts as a negative feedback response to Epo signaling, which is elevated as the consequence of ID anemia. In the case of *Hri*$^{-/-}$ and *eAA* mice shown in *B*, the deficiency of HRI or eIF2αP, respectively (as indicated by the *red X mark*) results in the loss of ISR while maintains the mTORC1 signaling. This leads to increased protein synthesis and the accumulation of globin protein inclusions. On the other hand, deficiency of ATF4 in the case of *Atf4*$^{-/-}$ mice shown in *C* only affects protein synthesis by the mTORC1 pathway and not the eIF2αP pathway. The preservation of the HRI–eIF2αP pathway is sufficient to reduce protein synthesis to avert globin protein inclusions in erythroid cells. However, the loss of ATF4 alone is sufficed in inhibiting erythroid differentiation, promoting cell proliferation, and elevating ROS levels, similar to HRI or eIF2αP deficiency (*B* and *C*). Thus, eIF2αP, ATF4, and Epo–mTORC1 pathways are coordinated by HRI for the adaptation to effective erythropoiesis during heme deficiency. ATF4, activating transcription factor 4; eIF2αP, phosphorylated eukaryotic initiation factor 2; Epo, erythropoietin; GRB10, growth factor receptor–bound protein 10; HRI, heme-regulated inhibitor; ISR, integrated stress response; mTORC1, mechanistic target of rapamycin complex 1.

translated mRNA in BasoE between WT and *Hri*$^{-/-}$ during ID (11). *Atf4* mRNA has two well-characterized uORFs in its 5′-leader sequence, including uORF1, which encodes three amino acids and is translated regardless of stress and increased eIF2αP levels, and an inhibitory uORF2 that is suggested to be bypassed during stress (7, 67, 68). *Atf4* uORF1 had exceptionally high ribosome occupancy *in vivo*. However, uORF2 and the canonical ORF of *Atf4* mRNA were poorly translated in HRI deficiency, demonstrating HRI-enhanced translation of *Atf4* mRNA *via* uORFs *in vivo* in primary BasoE (11).

ATF4 is central for induced transcription of ISR-target genes. In RNA-Seq studies, significantly more genes are differentially expressed in the comparison of WT cells in ID *versus* WT cells in iron sufficiency (232 genes) than the comparison of *Hri*$^{-/-}$ cells in ID *versus* *Hri*$^{-/-}$ cells in iron sufficiency (37 genes), demonstrating the near-absolute requirement for HRI in upregulating the transcriptional response to ID (11). ATF4 target genes (69) are highly induced during ID in EBs and dependent on HRI. These upregulated ATF4 target genes are of ISR signature pathway (10) to reprogram cellular metabolism for adaptation to oxidative stress and to promote erythroid differentiation (11) (Fig. 4).

Crosstalk of HRI–ISR with Epo–mTORC1 signaling

Mechanistic target of rapamycin complex 1 (mTORC1) signaling is another key pathway in regulating translation initiation upon growth factor stimulation and nutrient supply (70, 71). The mTORC1 can further enhance translation of mRNAs containing 5′-terminal oligopyrimidine motifs involved in cytosolic and mitochondrial protein synthesis. The activity of mTORC1 in blood cells was reduced under ID. Mice with constitutively activated mTORC1 in the hematopoietic lineage exhibit macrocytic hyperchromic anemia with splenomegaly and IE (72), similar to the phenotypes of *Hri*$^{-/-}$ and *eAA* mice during ID (22). Interestingly, HRI–ISR mutant mice displayed elevated mTORC1 activity during ID, specifically in the erythroid lineage (22) (Fig. 4, *B* and *C*). Inhibition of mTORC1 activity by the drugs, rapamycin and INK128, not only increased RBC numbers and hemoglobin levels but also

reduced IE in *Hri*$^{-/-}$ mice in ID. However, inhibiting mTORC1 activity did not significantly reduce globin inclusions or ROS in blood Retics or RBCs of *Hri*$^{-/-}$ mice, demonstrating an essential and unique role of HRI–ISR in inhibiting globin mRNA translation and oxidative stress in the late stages of erythroid differentiation (22) (Fig. 4).

Growth factor receptor–bound protein 10 (GRB10), the most highly induced ATF4 target gene in BasoE (11) (Fig. 4), is part of a feedback mechanism to inhibit mTORC1 signaling in response to insulin and stem cell factor (73, 74). Reduction of GRB10 expression by shRNAs in fetal liver erythroid progenitors increased cell numbers of differentiating EBs and inhibited terminal erythroid differentiation (11), recapitulating the hallmarks of IE in *Hri*$^{-/-}$ mice during ID (20, 22, 75). Thus, ATF4-induced GRB10 expression serves as a feedback mechanism for Epo signaling by HRI–ISR to inhibit cell proliferation and enable differentiation during ID (Fig. 4).

In summary, HRI coordinates two key pathways for translational regulation, ISR and mTORC1 signaling, to achieve effective iron-restricted erythropoiesis (Fig. 4). HRI–eIF2αP is necessary for adaptation to microcytic hypochromic anemia and to inhibit globin mRNA translation, thereby reducing proteotoxicity. Induction of ATF4 and GRB10 by HRI–eIF2αP is required to repress Epo-stimulated mTORC1 signaling for homeostatic control of erythropoiesis by heme. The repression of mTORC1 pathway by HRI in heme deficiency is reminiscent to the scenario of the repression of mTORC1 by GCN2 upon amino acid deprivation (7, 76, 77). However, the mechanisms of repression mTORC1 signaling are likely different, employing distinct ATF4 target genes, *Grb10* in the case of HRI (11) and sestrin2/REDD1 for GCN2 (77, 78). In addition, mTORC1 and its downstream target eIF4E-BP have been shown to coordinate translation of globin mRNAs with the availability of nonessential amino acids (79). mTORC1-mediated translation also regulates mitochondrial biogenesis at earlier stages of erythropoiesis from hematopoietic stem and progenitor cell to ProE. Together, these studies underscore the unique requirement of mTORC1 signaling in erythropoiesis and the interplay between HRI–ISR and mTORC1 to maintain protein homeostasis and terminal erythroid differentiation (Fig. 4).

Maintenance of ribosome homeostasis and mitochondrial function

Beyond ISR mRNAs, translation of mRNAs encoding ribosomes and mitochondrial proteins is the other most significantly upregulated cellular components in HRI-deficient and iron-deficient states. Notably, in Ribo-Seq studies, 56 ribosomal protein (RP) mRNAs were more highly translated in iron and HRI deficiencies. Among them, translation of the 18 RP mRNAs lacking 5′-terminal oligopyrimidine motifs was upregulated by the depletion of HRI but not by mTORC1 (11). The mTORC1-independent translation of RP mRNAs requiring GCN2-mediated eIF2αP has also been observed in several cancer cell lines (80).

Translation of 163 mRNAs of nuclear-encoded mitochondrial proteins was also upregulated in iron and HRI deficiencies, including transcripts for mitochondrial RPs, components for oxidative phosphorylation (encoding for complexes I through V), and matrix proteins (11). Importantly, this increased translational efficiency of mitochondrial proteins was accompanied by enhanced mitochondrial protein synthesis. The mTORC1 pathway contributed to about 50% of mitochondrial protein synthesis in both WT and *Hri*$^{-/-}$ primary EBs. Mitochondrial protein synthesis was still elevated in *Hri*$^{-/-}$ EBs upon inhibition of mTORC1 activity (11). Therefore, HRI–eIF2αP contributes directly to regulation of mitochondrial protein synthesis during ID, in addition to its role in repressing mTORC1 activity (Fig. 4).

While WT erythroid cells were able to maintain a normal oxygen consumption rate in ID, *Hri*$^{-/-}$ erythroid cells displayed decreased basal and maximal respiration under both iron-sufficient and iron-deficient conditions (11). The impaired mitochondrial respiration in *Hri*$^{-/-}$ erythroid cells was not because of the reduced mitochondrial mass or DNA content in these cells. Instead, HRI is necessary to maintain mitochondrial respiration by inhibiting mitochondrial protein synthesis even in heme sufficiency. This finding agrees with the model whereby activation of HRI serves to mitigate mitochondrial stress in nonerythroid human carcinoma cell lines (23, 24, 81), which will be described further.

Notably, dietary iron repletion in iron-deficient WT, *Hri*$^{-/-}$, *eAA*, and *Atf4*$^{-/-}$ mice rapidly restores blood RBC numbers and hemoglobin levels in *Hri*$^{-/-}$, *eAA*, and *Atf4*$^{-/-}$ mice to levels similar to WT mice (22). Most importantly, HRI hyperphosphorylation in the spleen of *eAA* and *Atf4*$^{-/-}$ mice is greatly reduced upon iron repletion. Furthermore, mTORC1 activity is significantly reduced upon iron repletion in erythroid cells of *Hri*$^{-/-}$, *eAA*, and *Atf4*$^{-/-}$ mice concomitant with reduced splenomegaly and serum Epo levels as well as increased erythroid differentiation. Together, these findings demonstrate that HRI–ISR is silenced upon iron repletion and the restoration of heme concentrations (22). In summary, genome-wide Ribo-Seq studies provide support for the idea that HRI regulates three signaling pathways, eIF2αP, ATF4, and mTORC1, *via* translation in both cytosolic and mitochondrial cellular compartments. Coordination of these signaling pathways by HRI serves to prevent proteotoxicity and oxidative stress, maintain mitochondrial function, as well as enable effective erythropoiesis during ID (Fig. 4).

Dephosphorylation of eIF2αP in the recovery of anemia

The steady state of eIF2αP *in vivo* is regulated by the equilibrium between activities of eIF2α kinases and eIF2αP phosphatase (PPase1). The constitutively expressed repressor of eIF2α phosphorylation (CReP) (82) and stress-induced GADD34 (83–86) are the two regulatory proteins that recruit eIF2αP to PPase1 for dephosphorylation. Dephosphorylation of eIF2αP is employed under all stress conditions to silence ISR mediated by all four members of eIF2α kinases.

Under normal conditions, *GADD34*$^{-/-}$ mice develop mild microcytic anemia (87). In contrast to the complete restoration of anemia upon iron repletion in *Hri*$^{-/-}$, eAA, and *Atf4*$^{-/-}$ mice

(22), *Gadd34*$^{-/-}$ mice do not recover completely from ID anemia upon iron repletion because of the sustained high eIF2αP level and inhibition of globin synthesis (87). *Crep*$^{-/-}$ mice have significant more severe phenotypes, including growth retardation, impaired erythropoiesis, and postnatal death. Furthermore, the anemia of *Crep*$^{-/-}$ embryos cannot be rescued by deletion of *Hri* or *Perk* alone (88). In addition to HRI, other eIF2α kinases may also contribute to erythropoiesis prior to the BasoE stage to regulate cell proliferation (44). Thus, both inadequate eIF2αP signaling (as in HRI, eIF2αP, and ATF4 deficiencies) (20, 22, 89) and excessive eIF2αP signaling (as in GADD34 and CReP deficiencies) are associated with anemia. These findings underscore the delicate balance of maintaining proper eIF2αP levels, which is referred recently as adaptive zone (7), during erythropoiesis by transient and dynamic activation of eIF2α kinases and dephosphorylation of eIF2αP by PPase1.

Activation of HRI in mitochondrial stress: the OMA1–DELE1–HRI pathway

Besides heme deficiency, increased eIF2αP triggered by oxidative stress induced by arsenite exposure is mediated mainly by HRI both in erythroid cells (55) and nonerythroid murine embryonic fibroblasts (90). In contrast to heme deficiency, activation of HRI by arsenite requires intact Retics, not mitochondrial-depleted lysates used for *in vitro* translation, and occurs under heme-sufficient conditions (55). *Hri*$^{-/-}$ EBs suffer from increased ROS and apoptosis upon acute oxidative stress induced by arsenite. Induction of antioxidant genes upon acute oxidative stress in EBs depends on both HRI and ATF4 (75). For over 20 years, the mechanism of the activation of HRI by arsenite was not understood. One puzzling observation was that *Hri*$^{-/-}$ mice succumbed to death rapidly upon arsenite treatment but not by the process involving anemia (90). The recent demonstration that HRI is activated by mitochondrial stress, especially ETC dysfunction, finally resolved this mystery (23, 24).

It has been known for some time that mitochondrial stress and in particular ETC dysfunction activates the ISR with distinct gene expression signatures (91–93). What was not clear was which eIF2α kinase is responsible and how the kinase is activated. To answer this question, studies by Guo *et al.* and Fessler *et al.* (23, 24) used a screen featuring ISR translation reporters derived from *Atf4* mRNA and its target gene *Ddit3* (Chop) combined with genome-wide CRISPR/Cas9 knockouts in human cell lines that were subjected to inhibition of ETC by oligomycin and carbonyl cyanide-chlorophenylhydrazine. HRI was discovered to be the sole eIF2α kinase activated upon ETC dysfunction, and its activation was shown to involve DELE1, a mitochondrial intermembrane space protein with little known function, and a mitochondrial inner membrane protease, OMA1 (94, 95) (Fig. 5*A*). Upon treatment with ETC inhibitors, DELE1 was discovered to be cleaved by OMA1 (23) to generate the smaller C-terminal fragment designated as DELE1s, which migrates out from mitochondria to the cytoplasm. DELE1s then binds to HRI, resulting in the activation of HRI by autophosphorylation (Fig. 5*A*).

Activation of HRI by DELE1 has been demonstrated biochemically *in vitro* in the presence of heme (23), indicating that this process is distinct from the well-characterized heme regulation of HRI (Fig. 5*A*). Further biochemical and cryo-EM structural studies showed that oligomerization of DELE1s was important for the activation of HRI (96). Interestingly, DELE1s interacts with HRI through the N-terminal heme-binding domain in the full-length protein and did not bind the N-terminally truncated HRI (deletion of 1–160 amino acids). In addition, N-terminal 160 amino acids were sufficient to bind and coimmunoprecipitated with DELE1s (96).

The OMA1–DELE1–HRI pathway, which connects mitochondrial stress to cytosolic ISR upon ETC disruption, is summarized and schematically illustrated in Figure 5*A*. The exact molecular details of the activation of HRI by DELE1s remain to be investigated. Since OMA1–DELE1–HRI pathway operates in heme sufficiency, heme molecules and intersubunit disulfide are kept in the model (Fig. 5*A*). Thr485-P is essential for the activation of the eIF2α kinase activity of HRI and is therefore illustrated in Figure 5*A*. One plausible mechanism of DELE1s in the activation of HRI is that binding of DELE1s to HRI N terminus may prevent the interaction of the N terminus with KI domain of HRI such that HRI is no longer inhibited by heme. The N terminus serves as an autoinhibitory domain to keep HRI from autophosphorylation at Thr485 in the activation loop. Future structural analyses of HRI are needed to discern the mechanisms of DELE1 activation of HRI and the validity of release of the autoinhibitory model.

Silencing of HRI–ISR signaling by UBR4 E3 ubiquitin ligase complex

HRI–ISR signaling is also subjected to be regulated by processes that control the levels of the HRI protein. Recently, two groups, Cervia *et al.* (26) and Haakonsen *et al.* (27), uncovered the degradation of HRI *via* UBR4 ubiquitination complex. One of these studies will be discussed in greater details later in the context of epithelial cancers and focused on the role of BIRC6 of the UBR4 ubiquitination complex for cancer cell fitness (26). The other seminal investigation of UBR4 is motivated by the findings that UBR4 mutations cause ataxia and early onset dementia (97–99).

UBR4, known for its role in the N-end rule pathway (100), facilitates degradation of aggregation-prone nascent polypeptides (101). UBR4 is particularly important when mitochondrial function was compromised from defects either in the biogenesis and function of the ETC or mitochondrial protein import. HRI and the cleaved DELE1s are UBR4 target substrates (27) (Fig. 5*B*). In addition, loss of eIF2α, the eIF2B subunit eIF2B4, or CReP results in synthetic lethality with the UBR4 deletion, supporting the idea that UBR4 downregulates the ISR pathway (27). Importantly, inactive HRI K196R mutant that does not undergo autophosphorylation at Thr485 is not degraded. Thus, UBR4 selectively targets the activated phosphorylated HRI and the cleaved DELE1s for ubiquitination, subsequent proteasome degradation, and the eventual silencing of HRI–ISR.

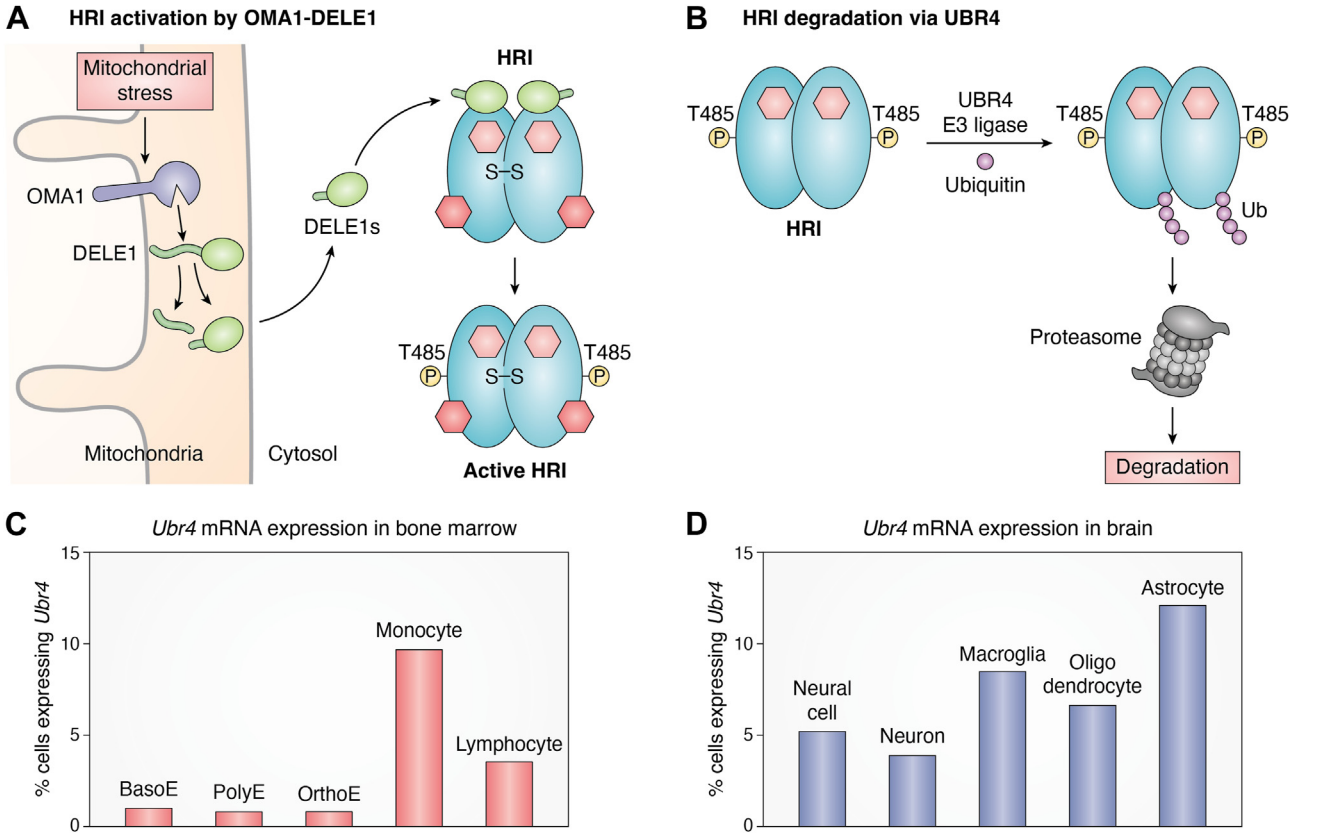

Figure 5. Activation and degradation of HRI during mitochondrial stress. *A*, activation of HRI in mitochondrial stress, the OMA1–DELE1–HRI pathway. During mitochondrial stress, mitochondrial inner membrane protease OMA1 is activated and in turn cleaves DELE1 at the N terminus. The cleaved C-terminal DELE1, designated as DELE1s, migrates from the mitochondrial intermembrane space to cytosol and binds to the N terminus of HRI, resulting in the autophosphorylation and activation of HRI. The subsequent ISR activation ensures the recovery from mitochondrial stress. *B*, ubiquitination of the HRI protein by the UBR4 E3 ligase complex. Active phosphorylated HRI, but not the inactive HRI, is ubiquitinated by UBR4 E3 ligase complex. The N-terminal heme-binding domain of HRI is necessary for UBR4 binding and subsequent ubiquitination. Ubiquitinated HRI is then subjected to degradation by the proteasome complex. *C* and *D*, scRNA-Seq analysis of *Ubr4* mRNA expression in human bone marrow hematopoietic cells and brain cells, respectively. The percentages of cells expressing *Ubr4* mRNA in each cell type as indicated are plotted on the *Y*-axis. Datasets are derived from CZ CellxGene Discover (cellxgene.cziscience.com) (46). HRI, heme-regulated inhibitor; scRNA-Seq, single-cell RNA sequencing.

Each of the two helical motifs in the N-terminal domain of HRI can mediate the recognition of HRI by the UBR4 complex. Notably, the helical HRI and DELE1s degrons closely resemble mitochondrial presequences that mediate protein transport into the organelle. The UBR4 complex therefore not only degrades DELE1s and HRI but also targets unimported mitochondrial proteins that accumulate in the cytoplasm during import stress. It is proposed that unimported mitochondrial precursors divert UBR4 from DELE1s and HRI to sustain ISR signaling (27), which inhibits both cytoplasmic and mitochondrial protein synthesis (11), and resolve the stress before silencing DELE1–HRI–ISR.

In summary, degradation of HRI and the subsequent silencing of ISR mediated by UBR4 has been demonstrated in the neuronal mitochondrial stress response (27) and epithelial cancer vulnerability (26). However, degradation of HRI by UBR4 in erythroid cells remains to be investigated. Interestingly, levels of *Ubr4* mRNA are extremely low in hemoglobin-producing EBs as compared with *Ubr4* mRNA expression in monocytes and lymphocytes in BM (Fig. 5*C*) as well as in brain cells (Fig. 5*D*). The low expression of *Ubr4* mRNA in erythroid precursors raises the question whether HRI protein is degraded in these cells by UBR4 ubiquitin ligase.

HRI–ISR in mitochondrial diseases and neural system

The original discovery of the activation of HRI by the OMA1–DELE1 pathway upon mitochondrial ETC dysfunction was made with immortalized cell lines derived from cancerous tissues (23, 24). Many of these cell lines originated from epithelial cells, which form the outer protective layers for organs. As described previously (Fig. 2*F*), HRI expression is prevalent in epithelial cells, second to erythroid cells as revealed by scRNA-Seq. Activation of HRI by OMA1–DELE1 pathway has also reported by the disruption of mitochondrial import or processing (102), mitophagy (103), mitochondrial DNA integrity (104), along with mitochondrial dysfunction occurring during severe ID (105). Importantly, DELE1 induction of HRI–ISR has been demonstrated in primary cells derived from human patients with mitochondrial disease or in those derived from mice with related mitochondrial defects. Mitochondrial diseases with neurological manifestation

stemming from inherited gene mutations or inhibition of proteasomes are presented in this section to illustrate the linkage between HRI–ISR activation and mitochondrial disease as well as the protective role of HRI–ISR in myocytes and neurons.

Chchd10 *mutations*

CHCHD10 is a small mitochondrial intermembrane space protein that is important for maintaining inner membrane cristae integrity and ETC function (106). Dominant missense substitutions in CHCHD10 protein cause an array of neuromuscular disorders including autosomal dominant isolated mitochondrial myopathy (R15S and G58R) (107), cardiomyopathy (G58R) (108), myopathy with amyotrophic lateral sclerosis/frontotemporal dementia (S59L) (109), adult-onset spinal muscular atrophy (G66V) (110), and amyotrophic lateral sclerosis (R15L) (111). Similar to patients, $Chchd10^{G58R}$ knockin mice are smaller, exhibit myopathy with smaller leg muscles and decreased strength/endurance, diminish mitochondrial complex I and IV activities, and die prematurely (108). The G58R substitution results in aggregations of CHCHD10 protein and the subsequent activation OMA1, which is critical for the survival of $CHCHD10^{G58R}$ mice. Global gene expression analysis of $Chchd10^{G58R}$ mouse hearts shows enrichment for the signature of HRI–ISR. Increased eIF2αP and DELE1s are observed in lysates of hearts and skeletal muscles from $Chchd10^{G58R}$ mice as compared with lysates from WT mice, supporting activation and a protective role for the OMA1–DELE1–HRI pathway in primary muscle cells.

Cox10 *mutations*

Mitochondrial DNA encodes 13 polypeptides that are essential components of oxidative phosphorylation and ETC (112). Mitochondrial disorders are among the most frequently inherited diseases of cell metabolism because of defects in ETC (113). Cytochrome *c* oxidase (COX) is the terminal enzyme complex of ETC transferring electrons from reduced cytochrome *c* to oxygen. Isolated COX deficiency is the most common cause of ETC defects in human. Mutations in *Cox10*, which is encoded by nuclear DNA and is one of the assembly factors of COX, cause Leigh syndrome, infantile hypertrophic cardiomyopathy, and fatal early onset neurological syndrome (107, 114, 115). A cardiac-specific knockout mice of *Cox10* exhibit mitochondrial cardiomyopathy, which is associated with ETC deficiency, lysosomal defects, and an aberrant mitochondrial morphology. Ablation of *Oma1* or *Dele1* in $Cox10^{-/-}$ mice aggravates cardiomyopathy. ISR induction, which depends on OMA1 and DELE1, is also necessary to protect against the ferroptosis incurred from oxidative stress in $Cox10^{-/-}$ heart tissues *via* ATF4-induced glutathione metabolism (116). Together, these studies indicate that the OMA1–DELE1–ISR pathway is operative in heart muscle cells and serves to mitigate oxidative stress upon COX10 deficiency in mice.

Afg3l2 *mutations*

More recently, the role of the OMA1–DELE1–ISR pathway in the central nervous system and in particular the cerebellum has also been reported (117). AFG3L2 is a mitochondrial protease, which forms homo-oligomers and hetero-oligomers in the inner membrane for the quality control and the degradation of nonassembled and damaged proteins (118). Biallelic mutations in the encoded proteolytic domain of AFG3L2 cause spastic ataxia type 5 (SPAX5), a severe childhood onset condition with the symptoms of cerebellar ataxia, spasticity resulting in significantly impaired ambulation, oculomotor apraxia, dystonia, and myoclonic epilepsy (119, 120). $Afg3l2^{-/-}$ mice resemble SPAX5 patients, presenting with a severe neurological syndrome with ataxia and spasticity that leads to early lethality (121). Mutations in *Afg3l2* lead to an inability to appropriately clear mitochondrial-encoded proteins in SPAX5 patient fibroblasts, resulting in the activation of OMA1 and mitochondrial fragmentation (117).

Importantly, activation of OMA1–DELE1–HRI–ISR has been demonstrated in the cerebellum of $Afg3l2^{-/-}$ mice and in fibroblasts from two patients (117). Silencing of DELE1 or HRI in patient fibroblasts impairs the cell growth of SPAX5 cultures and reduces eIF2αP. Augmentation of ISR signaling by Sephin-1, a selective inhibitor of the stress-induced eIF2αP phosphatase GADD34, which furthers the levels of eIF2αP (122), increases cell viability in SPAX5 fibroblasts and improves survival and dendritic arborization of $Afg3l2^{-/-}$ Purkinje neurons *ex vivo*. Moreover, Sephin-1 treatment prolongs the lifespan of $Afg3l2^{-/-}$ mice, improves their motor performances, and restores the health of Purkinje neurons with respect to mitochondrial morphology and respiratory capacity. This elegant study uncovered the protective function of OMA1–DELE1–HRI in cerebellum and Purkinje neurons under the settings of a neurodegenerative disease, SPAX5.

HRI activation in hippocampal neurons upon proteasome inhibition

Accumulation of cytoplasmic denatured proteins under heat shock, misfolding of proteins, or inhibition of the proteasome activity is another stress condition that can act through HRI–ISR (123). In neurons, there is extensive regulation of the synaptic proteome *via* synthesis and degradation that are critical for neural stimulation. However, the mechanism for balancing protein synthesis and degradation is still poorly understood. One recent study demonstrated that inhibition of protein synthesis upon treatment with proteasome inhibitors in murine primary hippocampal neurons is due solely to HRI-mediated eIF2α phosphorylation (124). Among the four eF2α kinases, *Hri* mRNA is most abundant in hippocampal neurons. Notably, HRI is expressed in the neurons at much lower levels than in erythroid or liver cells and is constitutively active in neurons because of the low heme concentration. Importantly, inhibition of protein synthesis in these tissues by proteasome inhibitors is reversible with the addition of exogenous heme.

The HRI protein has a short half-life of 4.2 h in neurons and is stabilized upon treatment with proteasome inhibitors.

Interestingly, HRI mRNA contains several rare codons, which normally impair translational efficiency. However, treatment with proteasome inhibitors and the resulting inhibition of protein synthesis enhances the availability of tRNAs for rare codons and thus increases *Hri* mRNA translation. Both processes contributed to increased HRI protein levels resulting in inhibition of protein synthesis to maintain the proteome in hippocampal neurons (124).

In summary, HRI–ISR activation by OMAI–DELE1 pathway has been highlighted in the primary cardiomyocytes and neuronal cells derived from three inherited human mitochondrial diseases, supporting the idea that HRI–ISR axis may be exploited for therapeutic targets in treating mitochondrial diseases. Furthermore, activation of HRI–ISR uniquely by proteasome inhibitors in primary murine hippocampal neurons reveals the previously unappreciated short half-life of HRI protein because of proteasome degradation, a new pathway of regulating HRI that is also observed in neurodegeneration and cancers.

HRI–ISR in cancer treatment and development

ISR is also upregulated in cancers for adaptation to hypoxic and nutrient-deprived environments (69, 125, 126). Often PERK or GCN2 are activated in cancers and serve to enhance tumor progression. However, HRI–ISR has emerged as an additional ISR pathway that is induced in some cancers (127, 128) and is a molecular target of certain anticancer agents, including 1-(benzo[*d*][1,2,3]thiadiazol-6-yl)-3-(3,4-dichlorophenyl)urea (BTdCPU) and ONC201, which are currently in clinical trials (Table 2). The use of proteasome inhibitor (bortezomib) and apoptosis inhibitors (BH3-mimetics) also suggests critical roles of HRI in cancers and for the drug resistance. Finally, analysis of cancer vulnerability by CRSPR/Cas9 loss-of-function screenings in carcinoma cell lines uncover the role of HRI in cancer development by a novel ubiquitination cascade mechanism. These studies are summarized in Table 2 and are described later in further details with the emphasis on defined molecular mechanisms of modulating HRI activity and expression in cancers.

HRI small-molecule activators: carcinomas and multiple myeloma

BTdCPU was identified as an inhibitor of eIF2 ternary complex formation (129) and specifically activates HRI among the four eIF2α kinases. Although BTdCPU was shown to bind to HRI in the earlier study (129), recently, it was demonstrated that BTdCPU also induced mitochondrial depolarization, suggesting that this compound instead activates HRI by the OMA1–DELE1 pathway (130). BTdCPU inhibits *in vitro* cell proliferation of several carcinoma cell lines and tumor growth *in vivo* of mice xenografted with MCF-7 human breast cancer cells.

In multiple myeloma, dexamethasone treatment induces the ISR and apoptosis. However, dexamethasone is only effective in about 40% of newly diagnosed patients. Interestingly, activation of HRI–ISR by BTdCPU in dexamethasone-resistant multiple myeloma cell lines promotes apoptosis in these cells. Furthermore, BTdCPU is also cytotoxic for dexamethasone-resistant primary multiple myeloma cells with no significant adverse effects on cells from healthy donors (131). ONC201, which is a small molecule currently in early phase clinical trials, kills many solid tumor cells by activating ISR mediated by HRI and PKR to induce apoptosis (132).

Proteasome inhibitor: pancreatic carcinoma

Proteasome inhibitor, bortezomib (Velcade), has been developed for cancer therapy. As described previously, HRI can be activated by proteasome inhibitors (123, 124). In human pancreatic carcinoma cell lines, it has been noted that cells resistant to bortezomib treatment have higher levels of eIF2αP to mitigate protein aggregates and ROS (133). Furthermore, HRI is the predominant eIF2α kinase activated in pancreatic carcinoma cell lines for adaptation to bortezomib-induced stress. Knockdown of HRI expression promotes cell death in

Table 2
Summary of HRI–ISR in cancers

Year	Cancer type	HRI–ISR activity	References
2011	Human carcinoma cell lines, breast (MCF-7), melanoma (CRL-2813), lung (A549), and prostate (PC-3)	HRI activation by BTdCPU	(129)
	Mice xenograft MCF-7 breast cancer cells	Inhibition of cell proliferation	
2016	Solid tumors, HCT-116 colon cell line, and mice xenografts	HRI activation by ONC201, antiproliferative and apoptosis	(132)
2017	Dexamethasone-resistant multiple myeloma cell lines and patient primary cells	HRI activation by BTdCPU apoptosis	(131)
2018	Pancreatic carcinoma cell lines resistant to bortezomib treatment	Higher HRI–eIF2αP levels correlate with reduction of protein aggregation, ROS, and resistance to bortezomib treatment	(133)
2021	B-lineage acute lymphoblastic leukemia (B-ALL) resistant to BH3-mimetic, ABT-203	Activation of HRI by DHA *via* removal of heme in HRI and the killing of cells HRI–eIF2αP inhibits translation of MCL-1 mRNA	(140)
2022	MDS-RS	Chronic activation of HRI–ISR in MDS-RS inhibits terminal erythroid differentiation	(135)
2022	BH3-memetic–tolerant persistent cancer cell lines	Activation of HRI–ISR by cytochrome *c* released from mitochondria necessary for the establishment of persistent cells	(25)
2022	Patient carcinoma samples, lung, breast, colon, gastric, and ovary	Elevated *Hri* mRNA expression compared to normal controls Patients with HRI-high tumors have decreased survival	(25, 26)
2023	Carcinoma cell lines	HRI expression is elevated in tumors compared with normal controls. Degradation of HRI by BIRC6-mediated ubiquitination ensures the survival of cancer cells	(26)

bortezomib-resistant cells, consistent with the critical role of HRI in maintaining protein homeostasis.

Myelodysplastic syndromes: persistent activation of HRI–ISR

Myelodysplastic syndromes with ringed sideroblast (MDS-RS) are hematological malignancies with splicing factor SF3B1 mutations often occurring in elderly populations (134, 135). Despite mechanistic insights into tumorigenesis induced by SF3B1 mutations, effective therapies for MDS-RS have yet to be developed. A recent scRNA-Seq study from MDS-RS patient BM samples demonstrates that SF3B1 mutations arrest terminal erythroid differentiation at the orthochromatic erythroblasts stage, as compared with control healthy donors (136). Furthermore, the chronic persistent upregulation of HRI–ISR pathway is prominent in ProE, BasoE, and polychromatic erythroblasts from MDS-RS patient samples because of heme deficiency. Deletion of HRI by CRISPR/Cas9 in three MDS-RS patient-derived primary EBs rescues erythroid differentiation with increased red cell production and heme biosynthesis. Thus, unresolved chronic activation of HRI–ISR in MDS-RS is maladaptive, and inhibition of HRI–ISR may offer a new therapeutic strategy for these patients.

BH3-mimetic induced apoptosis: Mcl-1 *mRNA translation*

Antiapoptotic proteins of the BCL-2 family, such as MCL-1, contain four BCL-2 homology (BH1–4) domains, which are often amplified in many types of cancers and serve to support tumor survival (137). By contrast, BH3-only family proteins contain one BH3 domain and are proapoptotic by a process initiated Bax/Bak signaling. BH3-mimetic small-molecule drugs have been developed and used to inhibit antiapoptotic members, permitting BH3-only proapoptotic members to trigger apoptosis (138). Illustrating these ideas, B-lineage acute lymphoblastic leukemia with poor prognosis is often characterized by MCL-1 amplification and resistance to the BH3-mimetic drug, ABT-263. Interestingly, the antimalarial drug, dihydroartemisinin (DHA), inhibited translation of *Mcl-1* mRNA and worked synergistically with BH3-mimetics (139). CRISPR/Cas9 screening revealed that loss of heme biosynthetic genes rendered resistance to DHA-induced cell death and that DHA treatment activates HRI–eIF2αP to inhibit translation of the *Mcl-1* mRNA (140). HRI is necessary for the synergistic killing of DHA with BH3-mimetics. Mechanistically, DHA disrupts heme binding to recombinant HRI protein, resulting in loss of the hemoprotein signature visible light absorption spectra. This study underscores the importance of HRI-mediated repression of *Mcl-1* translation in modulating apoptotic sensitivity of leukemic cells and implicates the feasibility of DHA as an activator of HRI for therapeutic purposes.

BH3-mimetic–tolerant persistent cancer cells: HRI activation by cytochrome c

Recent studies have yielded intriguing insights into the development of the drug-tolerant persistent cancer cells upon treatment with BH3-mimetics involving HRI–ISR (25). By gene expression profiling, the drug-unresponsive phenotype shares notable similarities to epithelial-to-mesenchymal transition (141), endowing persistent cells with temporary survival advantages against drug treatment. Central to the apoptotic pathway is the release of cytochrome *c* from the mitochondrial intermembrane space to cytosol *via* mitochondrial outer membrane permeabilization controlled by antiapoptotic and proapoptotic proteins (137, 142, 143) (Fig. 6*A*).

ScRNA-Seq analysis of BH3-mimetic persistent cells revealed transient signature gene expression of ISR and apoptosis, which was dependent on HRI and cytochrome *c*. Interestingly, heme did not inhibit HRI in the presence of cytochrome *c*, implicating activation of HRI by cytochrome *c* in the presence of heme (Fig. 6*A*). Furthermore, HRI and cytochrome *c* coimmunoprecipitated following incomplete mitochondrial outer membrane permeabilization and releasing of sublethal amount of cytochrome *c*, supporting the direct interactions between HRI and cytochrome *c*. The involvement of OMA1–DELE1–HRI pathway in the development of persistent cells remains to be investigated.

In support of the clinical significance of this finding, analysis of scRNA-Seq data from lung carcinoma samples (144) reveals increased HRI gene set scores in the patient samples with residual disease as compared with treatment naive or those with progressive disease. In addition, HRI expression is significantly higher in cancer samples, mainly carcinomas from lung, breast, colon, gastric, and ovary, as compared with normal tissues. Most importantly, patients with HRI-high expression tumors display decreased survival compared with patients with HRI-low tumors, consistent with the role of HRI–ISR in the formation of persistent cells (25) (Fig. 6*B*).

Cancer vulnerability and HRI protein degradation

Genome-scale approaches for loss-of-function screens (RNA interference and CRISPR/Cas9) have been employed to systematically identify genes that are essential for the proliferation and survival of many cancer cell lines (145, 146). Most of these studies identify single genes required for cancer cell fitness in specific contexts. Recently, an analysis of a cancer dependency dataset composed of CRISPR/Cas9 loss-of-function screens of 1086 carcinoma cell lines has identified a novel coessential gene module of the ubiquitination cascade that regulates ISR and survival of carcinomas (26). Specifically, this module is composed of four proteins, KCMF1, UBR4, UBA6, and BIRC6 (Fig. 6*B*). While UBA6 and BIRC6 are strongly essential in only 3.5% and 4.1% of the cell lines, respectively, KCMF1 and UBR4 are strongly essential in 68.0% and 65.1% of the cell lines, respectively. KCMF1 and UBR4 are heterodimeric E3 ligases and are essential for a large proportion of cancer types, indicating broader biological functions beyond the BIRC6 module.

Cell lines derived from epithelial cells, in which HRI expression is prevalent, are more dependent on the BIRC6 module than mesenchymal tissue–derived cancer cell lines. Depletion of BIRC6 activated HRI–ISR, which was prevented by ISRIB, a small-molecule inhibitor of ISR (147). Proteomic

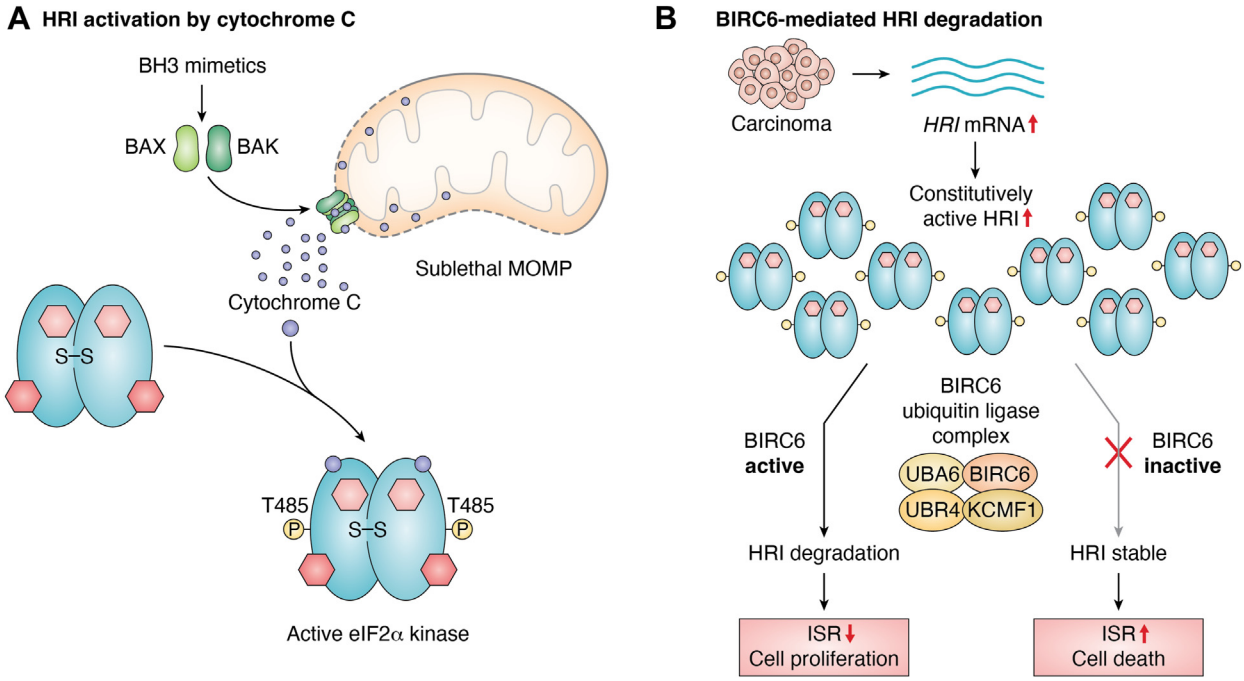

Figure 6. HRI–ISR in cancer development and vulnerability. *A*, activation of HRI by cytochrome *c*. Upon treatment with BH3-mimetics, BAX and BAK form pores causing mitochondrial outer membrane permeabilization (MOMP). Under sublethal MOMP conditions that do not result in apoptosis, the release of cytochrome *c* from the mitochondrial intermembrane space to cytosol activates HRI under heme sufficiency. The HRI–ISR pathway enables the formation of persistent cells resistant to BH3-mimetics. A is modified from articles (25, 143). *B*, BIRC6 mediated HRI degradation in carcinomas. *Hri* mRNA expression is increased in carcinoma cancer cells resulting in increased HRI protein levels. In epithelial carcinoma cells, HRI is constitutively active because of low cellular heme concentrations. This elevated active HRI can induce excessive ISR, causing cell death in the absence of BIRC6-dependent module of the ubiquitination cascade, which contains additional three proteins, UBA6, UBR4, and KCMF1, to target HRI for ubiquitination and subsequent degradation by proteosome. Carcinoma cells employ BIRC6 ubiquitin ligase complex to control HRI protein level and thus proper ISR activation for their survival and proliferation. HRI, heme-regulated inhibitor; ISR, integrated stress response.

analysis of BIRC6 depletion in the presence of ISRIB reveals that HRI is the most significantly upregulated protein. Moreover, the increase in HRI protein levels is accompanied by decreased ubiquitination upon depletion of UBA6, KCMF1, or UBR4 (Fig. 6*B*) and upon inhibition of proteosome with MG132. HRI protein has 2.6-fold longer half-life in BIRC6-depleted cells as compared with control cells increased from 3.5 to 9.0 h (Fig. 6*B*). The short half-life of HRI protein (4.2 h) was also noted in primary hippocampal neuronal cells described previously (124).

In addition, *Hri* mRNA expression is strongly elevated in tumors as compared with control normal samples and is strongly correlated with expression levels of UBA6, BIRC6, and KCMF1. Thus, a novel mechanism of BIRC6-mediated HRI degradation has evolved in certain epithelial-derived cancers to silence HRI–ISR, which is constitutively active because of low concentrations of heme in these cells (Fig. 6*B*). Intriguingly, the BIRC6 ubiquitination module only degrades HRI and not the other three eIF2α kinases. Most significantly, this is the first report of the downregulation of eIF2α kinases at the protein level to silence ISR (26).

In summary, activation of the HRI–ISR pathway by small molecules, BTdCPU and ONC201, is effective in killing cancer cells, and HRI–ISR axis may be further developed for cancer therapeutics (Table 2). On the other hand, under the native *in vivo* environments, HRI–ISR provides growth advantages for carcinomas. HRI is constitutively active in carcinomas because of low heme concentrations in contrast to erythroid cells. In fact, HRI-high expression correlates with poor prognosis for patient survival in two recent studies highlighted here (25, 26) (Fig. 6*B* and Table 2). Furthermore, *Hri* mRNA is elevated in cancers though the mechanism responsible remains to be investigated.

Concluding remarks

Since its discovery as an inhibitor of hemoglobin synthesis, increasing biological functions and significance have been ascribed to HRI both in the erythroid and nonerythroid systems. In the erythroid system, HRI is a master regulator of translation, coordinating ISR and mTORC1 pathways for effective erythropoiesis under heme deficiency (11, 22) and in thalassemia (21, 148). Most notably, HRI regulates not only cytoplasmic but also mitochondrial protein synthesis. HRI is also necessary for mitochondrial respiration even in heme sufficiency (11). These findings are relevant to the broader significance of the recently discovered OMA1–DELE1–HRI pathway during mitochondrial stress in several non–erythroid cell types, most notably epithelial and neuronal cells. This HRI-mediated ISR is beneficial in mitochondrial diseases, mitigating the clinical severities in myopathies, neuropathies, ataxia, and neurodegeneration.

Equally fascinating is the emerging role of HRI in carcinogenesis, especially in carcinomas (25), consistent with prevalent HRI expression in epithelial cells surrounding the organs

as revealed by scRNA-Seq. Interestingly, *Hri* mRNA is elevated in patient tumors as compared with normal tissues (25, 149). In the erythroid system, *Hri* mRNA is induced by heme and GATA1 during terminal erythropoiesis. In the future, it will be important to investigate the mechanisms responsible for the increased *Hri* mRNA expression in tumors.

Since HRI can be constitutively active in some cancers because of low cellular heme concentrations in nonerythroid cells, a new mechanism of silencing HRI–ISR by HRI degradation *via* the BIRC6 module of UBR4 E3 ligase enables the survival of some subtypes of epithelial-derived cancer cells (26). Inspired by UBR4 mutations in human patients causing ataxia and early onset dementia, UBR4 ligase–mediated HRI degradation has also been demonstrated in neuronal cells (27). Interestingly, the N-terminal stable heme-binding domain of HRI is required for interactions with both cleaved DELE1s and UBR4 for seemingly opposing actions, activation and degradation, respectively. To date, there is no published three-dimensional protein structures of HRI. In the future, it will be most instructive to our understanding of mechanisms of the activation of HRI in heme deficiency and during mitochondrial stress by obtaining HRI structures in the presence and absence of heme as well as other effectors (DELE1s and UBR4) by cryo-EM.

UBR4 is the first E3 ligase reported for eIF2α kinases and is uniquely specific to activated phosphorylated HRI. In erythroid cells, where the heme concentration is higher and dynamically regulated because of high demands of hemoglobin synthesis, silencing of HRI–ISR signaling by heme has been demonstrated in iron-deficient mice upon iron repletion (22). While the half-life of HRI protein is short in neurons and cancer cell lines, the turnover of HRI in erythroid cells has not yet been measured and would be of great interest. It will also be important to investigate whether degradation of HRI by UBR4 or other E3 ligases occurs in erythroid cells, in which expression of *Ubr4* and *Birc6* mRNAs is very low.

Both PERK and GCN2 can also be activated by proteasome inhibitors. This raises an interesting question as why HRI is preferentially activated by proteasome inhibitors in neurons and certain cancer cells presented in this review. One possibility is that HRI is constructively active in these cells because of low concentrations of heme. The other is that HRI protein is short lived with half-life of around 4 h. So, proteasome inhibitors stabilize active autophosphorylated HRI protein. This is also consistent with the observation of the UBR4-mediated degradation of HRI specifically but not other three eIF2α kinases. Silencing of the ISR mediated by other three eIF2α kinases will be an important subject for future research.

These recent advancements of HRI–ISR open new directions for HRI research in stress responses to anemia, mitochondrial dysfunction, neurodegeneration, and carcinogenesis, which are also hallmarks of aging process. The HRI–ISR signaling pathway may present opportunities for new therapeutics in inherited human diseases and upon aging.

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Abbreviations—The abbreviations used are: ATF4, activating transcription factor 4; BasoE, basophilic erythroblast; BH, BCL-2 homology domain; BM, bone marrow; BTdCPU, 1-(benzo[d][1,2,3]thiadiazol-6-yl)-3-(3,4-dichlorophenyl)urea; COX, cytochrome c oxidase; DHA, dihydroartemisinin; EB, erythroblast; *eAA* mice, erythroid-specific *eIF2α* Ala51 knockin mouse model; eIF2α, the alpha-subunit of eukaryotic initiation factor 2; eIF2αP, phosphorylated eIF2α; Epo, erythropoietin; ETC, electron transport chain; GRB10, growth factor receptor–bound protein 10; GCN2, general control nonderepressible 2; HRI, heme-regulated inhibitor; HRM, heme regulatory motif; ID, iron deficiency; IE, ineffective erythropoiesis; ISR, integrated stress response; KI, kinase insert; MDS-RS, myelodysplastic syndrome with ringed sideroblast; mTORC1, mechanistic target of rapamycin complex 1; PERK, protein kinase R–like endoplasmic reticulum kinase; ProE, proerythroblast; RBC, red blood cell; Ribo-Seq, ribosome profiling; ROS, reactive oxygen species; RP, ribosomal protein; scRNA-Seq, single-cell RNA sequencing; SPAX5, spastic ataxia type 5; uORF, upstream ORF.

References

1. Kruh, J., and Borsook, G. (1956) Hemoglobin synthesis in rabbit reticulocytes *in vitro*. *J. Biol. Chem.* **220**, 905–915
2. Kassenaar, A., Morell, H., and London, I. M. (1957) The incorporation of glycine into globin and the synthesis of heme in vitro in duck erythrocytes. *J. Biol. Chem.* **229**, 423–435
3. Morell, H., Savoie, J. C., and London, I. M. (1958) The biosynthesis of heme and the incorporation of glycine into globin in rabbit bone marrow *in vitro*. *J. Biol. Chem.* **233**, 923–929
4. Grayzel, A. I. P., Horchner, P., and London, I. M. (1966) The stimulation of globin synthesis by heme. *Proc. Natl. Acad. Sci. U. S. A.* **55**, 650–655
5. Maxwell, C. R., and Rabinovitz, M. (1969) Evidence for an inhibitor in the control of globin synthesis by hemin in a reticulocyte lysate. *Biochem. Biophys. Res. Commun.* **35**, 79–85
6. Ranu, R. S., and London, I. M. (1976) Regulation of protein synthesis in rabbit reticulocyte lysates: purification and initial characterization of the cyclic 3':5'-AMP independent protein kinase of the heme-regulated translational inhibitor. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 4349–4363
7. Wek, R. C., Anthony, T. G., and Staschke, K. A. (2023) Surviving and adapting to stress: translational control and the integrated stress response. *Antioxid. Redox Signal.* **39**, 351–373
8. Costa-Mattioli, M., and Walter, P. (2020) The integrated stress response: from mechanism to disease. *Science* **368**. https://doi.org/10.1126/science.aat5314
9. Krishnamoorthy, T., Pavitt, G. D., Zhang, F., Dever, T. E., and Hinnebusch, A. G. (2001) Tight binding of the phosphorylated alpha subunit of

initiation factor 2 (eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. *Mol. Cell Biol.* **21**, 5018–5030
10. Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., *et al.* (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* **11**, 619–633
11. Zhang, S., Macias-Garcia, A., Ulirsch, J. C., Velazquez, J., Butty, V. L., Levine, S. S., *et al.* (2019) HRI coordinates translation necessary for protein homeostasis and mitochondrial function in erythropoiesis. *eLife* **8**. https://doi.org/10.7554/eLife.46976
12. Camaschella, C. (2019) Iron deficiency. *Blood* **133**, 30–39
13. Chung, J., Chen, C., and Paw, B. H. (2012) Heme metabolism and erythropoiesis. *Curr. Opin. Hematol.* **19**, 156–162
14. Chen, J. J. (2007) Regulation of protein synthesis by the heme-regulated eIF2alpha kinase: relevance to anemias. *Blood* **109**, 2693–2699
15. Chen, J. J., and Zhang, S. (2019) Heme-regulated eIF2alpha kinase in erythropoiesis and hemoglobinopathies. *Blood* **134**, 1697–1707
16. Chefalo, P., Oh, J., Rafie-Kolpin, M., Kan, B., and Chen, J.-J. (1998) Heme-regulated eIF-2α kinase purifies as a hemoprotein. *Eur. J. Biochem.* **258**, 820–830
17. Rafie-Kolpin, M., Chefalo, P. J., Hussain, Z., Hahn, J., Uma, S., Matts, R. L., *et al.* (2000) Two heme-binding domains of heme-regulated eIF-2α kinase: N-terminus and kinase insertion. *J. Biol. Chem.* **275**, 5171–5178
18. Bauer, B. N., Rafie-Kolpin, M., Lu, L., Han, A., and Chen, J. J. (2001) Multiple autophosphorylation is essential for the formation of the active and stable homodimer of heme-regulated eIF-2α kinase. *Biochemistry* **40**, 11543–11551
19. Crosby, J. S., Chefalo, P. J., Yeh, I., Ying, S., London, I. M., Leboulch, P., *et al.* (2000) Regulation of hemoglobin synthesis and proliferation of differentiating erythroid cells by heme-regulated eIF-2α kinase. *Blood* **96**, 3241–3247
20. Han, A. P., Yu, C., Lu, L., Fujiwara, Y., Browne, C., Chin, G., *et al.* (2001) Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *EMBO J.* **20**, 6909–6918
21. Han, A. P., Fleming, M. D., and Chen, J. J. (2005) Heme-regulated eIF2alpha kinase modifies the phenotypic severity of murine models of erythropoietic protoporphyria and beta-thalassemia. *J. Clin. Invest.* **115**, 1562–1570
22. Zhang, S., Macias-Garcia, A., Velazquez, J., Paltrinieri, E., Kaufman, R. J., and Chen, J. J. (2018) HRI coordinates translation by eIF2alphaP and mTORC1 to mitigate ineffective erythropoiesis in mice during iron deficiency. *Blood* **131**, 450–461
23. Guo, X., Aviles, G., Liu, Y., Tian, R., Unger, B. A., Lin, Y. T., *et al.* (2020) Mitochondrial stress is relayed to the cytosol by an OMA1-DELE1-HRI pathway. *Nature* **579**, 427–432
24. Fessler, E., Eckl, E. M., Schmitt, S., Mancilla, I. A., Meyer-Bender, M. F., Hanf, M., *et al.* (2020) A pathway coordinated by DELE1 relays mitochondrial stress to the cytosol. *Nature* **579**, 433–437
25. Kalkavan, H., Chen, M. J., Crawford, J. C., Quarato, G., Fitzgerald, P., Tait, S. W. G., *et al.* (2022) Sublethal cytochrome c release generates drug-tolerant persister cells. *Cell* **185**, 3356–3374.e3322
26. Cervia, L. D., Shibue, T., Borah, A. A., Gaeta, B., He, L., Leung, L., *et al.* (2023) A ubiquitination cascade regulating the integrated stress response and survival in carcinomas. *Cancer Discov.* **13**, 766–795
27. Haakonsen, D. L., Heider, M., Ingersoll, A. J., Vodehnal, K., Witus, S. R., Uenaka, T., *et al.* (2024) Stress response silencing by an E3 ligase mutated in neurodegeneration. *Nature* **626**, 874–880
28. Khandros, E., and Blobel, G. A. (2024) Elevating fetal hemoglobin: recently discovered regulators and mechanisms. *Blood* **144**, 845–852
29. Girardin, S. E., Cuziol, C., Philpott, D. J., and Arnoult, D. (2021) The eIF2alpha kinase HRI in innate immunity, proteostasis, and mitochondrial stress. *FEBS J.* **288**, 3094–3107
30. Waxman, H. S., and Rabinovitz, M. (1965) Iron supplementation in vitro and the state of aggregation and function of reticulocyte ribosomes in hemoglobin synthesis. *Biochem. Biophys. Res. Commun.* **19**, 538–545
31. Waxman, H. S., and Rabinovitz, M. (1966) Control of reticulocyte polyribosome content and hemoblogin synthesis by heme. *Biochim. Biophys. Acta* **129**, 369–379
32. Ranu, R. S., Levin, D. H., Delaunay, J., Ernst, V., and London, I. M. (1976) Regulation of protein synthesis in rabbit reticulocyte lysates: characteristics of inhibition of protein synthesis by a translational inhibitor from heme-deficient lysates and its relationship to the initiation factor which binds Met-tRNAf. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 2720–2724
33. Zucker, W. V., and Schulman, H. M. (1968) Stimulation of globin-chain initiation by hemin in the reticulocyte cell-free system. *Proc. Natl. Acad. Sci. U. S. A.* **59**, 582–589
34. Yang, J. M., London, I. M., and Chen, J.-J. (1992) Effects of hemin and porphyrin compounds on intersubunit disulfide formation of heme-regulated eIF-2α kinase and the regulation of protein synthesis in reticulocyte lysates. *J. Biol. Chem.* **267**, 20519–20524
35. Legon, S., Jackson, R. J., and Hunt, T. (1973) Control of protein synthesis in reticulocyte lysates by haemin. *Nat. New Biol.* **241**, 150–152
36. Hunt, T., Vanderhoff, G. A., and London, I. M. (1972) Control of globin synthesis: the role of heme. *J. Mol. Biol.* **66**, 471–481
37. Balkow, K., Hunt, T., and Jackson, R. J. (1975) Control of protein synthesis in reticulocyte lysates: the effect of nucleotide triphosphates on formation of the translational repressor. *Biochem. Biophys. Res. Commun.* **67**, 366–374
38. Levin, D. H., Ranu, R. S., Ernst, V., and London, I. M. (1976) Regulation of protein synthesis in reticulocyte lysates: phosphorylation of methionyl-tRNA$_f$ binding factor by protein kinase activity of the translational inhibitor isolated from heme-deficient lysates. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 3112–3116
39. Trachsel, H., Ranu, R. S., and London, I. M. (1978) Regulation of protein synthesis in rabbit reticulocyte lysates: purification and characterization of heme-reversible translational inhibitor. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3654–3658
40. Farrell, P., Balkow, K., Hunt, T., Jackson, R. J., and Trachsel, H. (1977) Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. *Cell* **11**, 187–200
41. Fagard, R., and London, I. M. (1981) Relationship between the phosphorylation and activity of the heme-regulated eIF-2a kinase. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 866–870
42. Chen, J.-J., Throop, M. S., Gehrke, L., Kuo, I., Pal, J. K., Brodsky, M., *et al.* (1991) Cloning of the cDNA of the heme-regulated eukaryotic initiation factor 2α (eIF-2α) kinase of rabbit reticulocytes: homology to yeast GCN2 protein kinase and human double-stranded-RNA-dependent eIF-2a kinase. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7729–7733
43. Crosby, J. S., Lee, K., London, I. M., and Chen, J.-J. (1994) Erythroid expression of the heme-regulated eIF-2α kinase. *Mol. Cell Biol.* **14**, 3906–3914
44. Liu, S., Bhattacharya, S., Han, A., Suragani, R. N., Zhao, W., Fry, R. C., *et al.* (2008) Haem-regulated eIF2alpha kinase is necessary for adaptive gene expression in erythroid precursors under the stress of iron deficiency. *Br. J. Haematol.* **143**, 129–137
45. Kingsley, P. D., Greenfest-Allen, E., Frame, J. M., Bushnell, T. P., Malik, J., McGrath, K. E., *et al.* (2013) Ontogeny of erythroid gene expression. *Blood* **121**, e5–e13
46. [preprint] CZI Single-Cell Biology Program, Abdulla, S., Aevermann, B., Assis, P., Badajoz, S., and Bell, S. M. (2023) CZ CELL×GENE Discover: a single-cell data platform for scalable exploration, analysis and modeling of aggregated data. *bioRxiv*. https://doi.org/10.1101/2023.10.30.563174
47. Liu, S., Suragani, R. N., Wang, F., Han, A., Zhao, W., Andrews, N. C., *et al.* (2007) The function of heme-regulated eIF2alpha kinase in murine iron homeostasis and macrophage maturation. *J. Clin. Invest.* **117**, 3296–3305
48. Abdel-Nour, M., Carneiro, L. A. M., Downey, J., Tsalikis, J., Outlioua, A., Prescott, D., *et al.* (2019) The heme-regulated inhibitor is a cytosolic sensor of protein misfolding that controls innate immune signaling. *Science* **365**. https://doi.org/10.1126/science.aaw4144
49. Tabula Muris Consortium, Overall coordination, Logistical coordination, Organ collection and processing, Library preparation and sequencing, Computational data analysis, *et al.* (2018) Single-cell

transcriptomics of 20 mouse organs creates a Tabula Muris. *Nature* **562**, 367–372
50. Gross, M., and Rabinovitz, M. (1972) Control of globin synthesis by heme: factors influencing formation of an inhibitor of globin chain initiation in reticulocyte lysates. *Biochim. Biophys. Acta* **287**, 340–352
51. Hunt, T. (1979) The control of protein synthesis in rabbit reticulocyte lysates. In: Russel, T. R., Brew, K., Schultz, J., Haber, H., eds. *Miami Winter Symposium: From Gene to Protein*, Academic Press, New York: 321–345
52. Ricketts, M. D., Emptage, R. P., Blobel, G. A., and Marmorstein, R. (2022) The heme-regulated inhibitor kinase requires dimerization for heme-sensing activity. *J. Biol. Chem.* **298**, 102451
53. Rafie-Kolpin, M., Han, A. P., and Chen, J. J. (2003) Autophosphorylation of threonine 485 in the activation loop is essential for attaining eIF2alpha kinase activity of HRI. *Biochemistry* **42**, 6536–6544
54. Chefalo, P. J., Yang, J. M., Ramaiah, K. V. A., Gehrke, L., and Chen, J.-J. (1994) Inhibition of protein synthesis in insect cells by baculovirus-expressed heme-regulated eIF-2α kinase. *J. Biol. Chem.* **269**, 25788–25794
55. Lu, L., Han, A. P., and Chen, J. J. (2001) Translation initiation control by heme-regulated eukaryotic initiation factor 2alpha kinase in erythroid cells under cytoplasmic stresses. *Mol. Cell Biol.* **21**, 7971–7980
56. Gross, M., and Mendelewski, J. (1978) An association between the formation of the hemin-controlled translational repressor and the phosphorylation of a 100,000 molecular weight protein. *Biochim. Biophys. Acta* **520**, 650–663
57. Chen, J.-J., Pal, J. K., Petryshyn, R., Kuo, I., Yang, J. M., Throop, M. S., *et al.* (1991) Amino acid microsequencing of the internal tryptic peptides of heme-regulated eukaryotic initiation factor 2 α-subunit kinase: homology to protein kinases. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 315–319
58. Yun, B. G., Matts, J. A., and Matts, R. L. (2005) Interdomain interactions regulate the activation of the heme-regulated eIF 2 alpha kinase. *Biochim. Biophys. Acta* **1725**, 174–181
59. Igarashi, J., Murase, M., Iizuka, A., Pichierri, F., Martinkova, M., and Shimizu, T. (2008) Elucidation of the heme binding site of heme-regulated eukaryotic initiation factor 2alpha kinase and the role of the regulatory motif in heme sensing by spectroscopic and catalytic studies of mutant proteins. *J. Biol. Chem.* **283**, 18782–18791
60. Inuzuka, T., Yun, B. G., Ishikawa, H., Takahashi, S., Hori, H., Matts, R. L., *et al.* (2004) Identification of crucial histidines for heme binding in the N-terminal domain of the heme-regulated eIF2alpha kinase. *J. Biol. Chem.* **279**, 6778–6782
61. Lathrop, J. T., and Timko, M. P. (1993) Regulation by heme of mitochondrial protein transport through a conserved amino acid motif. *Science* **259**, 522–525
62. Zhang, L., and Guarente, L. (1995) Heme binds to a short sequence that serves a regulatory function in diverse proteins. *EMBO J.* **14**, 313–320
63. Chen, J.-J., Yang, J. M., Petryshyn, R., Kosower, N., and London, I. M. (1989) Disulfide bond formation in the regulation of eIF-2a kinase by heme. *J. Biol. Chem.* **264**, 9559–9564
64. Ingolia, N. T., Ghaemmaghami, S., Newman, J. R., and Weissman, J. S. (2009) Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science* **324**, 218–223
65. Chiabrando, D., Mercurio, S., and Tolosano, E. (2014) Heme and erythropoieis: more than a structural role. *Haematologica* **99**, 973–983
66. Muckenthaler, M. U., Rivella, S., Hentze, M. W., and Galy, B. (2017) A red carpet for iron metabolism. *Cell* **168**, 344–361
67. Pavitt, G. D., and Ron, D. (2012) New insights into translational regulation in the endoplasmic reticulum unfolded protein response. *Cold Spring Harb. Perspect. Biol.* **4**, a012278
68. Chen, J. J. (2014) Translational control by heme-regulated eIF2alpha kinase during erythropoiesis. *Curr. Opin. Hematol.* **21**, 172–178
69. Pakos-Zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A., and Gorman, A. M. (2016) The integrated stress response. *EMBO Rep.* **17**, 1374–1395
70. Thoreen, C. C., Chantranupong, L., Keys, H. R., Wang, T., Gray, N. S., and Sabatini, D. M. (2012) A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature* **485**, 109–113
71. Thoreen, C. C. (2017) The molecular basis of mTORC1-regulated translation. *Biochem. Soc. Trans.* **45**, 213–221
72. Knight, Z. A., Schmidt, S. F., Birsoy, K., Tan, K., and Friedman, J. M. (2014) A critical role for mTORC1 in erythropoiesis and anemia. *eLife* **3**, e01913
73. Plasschaert, R. N., and Bartolomei, M. S. (2015) Tissue-specific regulation and function of Grb10 during growth and neuronal commitment. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 6841–6847
74. Yan, X., Himburg, H. A., Pohl, K., Quarmyne, M., Tran, E., Zhang, Y., *et al.* (2016) Deletion of the imprinted gene Grb10 promotes hematopoietic stem cell self-renewal and regeneration. *Cell Rep.* **17**, 1584–1594
75. Suragani, R. N., Zachariah, R. S., Velazquez, J. G., Liu, S., Sun, C. W., Townes, T. M., *et al.* (2012) Heme-regulated eIF2alpha kinase activated Atf4 signaling pathway in oxidative stress and erythropoiesis. *Blood* **119**, 5276–5284
76. Anthony, T. G., McDaniel, B. J., Byerley, R. L., McGrath, B. C., Cavener, D. R., McNurlan, M. A., *et al.* (2004) Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eIF2 kinase GCN2. *J. Biol. Chem.* **279**, 36553–36561
77. Ye, J., Palm, W., Peng, M., King, B., Lindsten, T., Li, M. O., *et al.* (2015) GCN2 sustains mTORC1 suppression upon amino acid deprivation by inducing Sestrin2. *Genes Dev.* **29**, 2331–2336
78. Xu, D., Dai, W., Kutzler, L., Lacko, H. A., Jefferson, L. S., Dennis, M. D., *et al.* (2020) ATF4-Mediated upregulation of REDD1 and Sestrin2 suppresses mTORC1 activity during prolonged leucine deprivation. *J. Nutr.* **150**, 1022–1030
79. Chung, J., Bauer, D. E., Ghamari, A., Nizzi, C. P., Deck, K. M., Kingsley, P. D., *et al.* (2015) The mTORC1/4E-BP pathway coordinates hemoglobin production with L-leucine availability. *Sci. Signal.* **8**, ra34
80. Li, B. B., Qian, C., Gameiro, P. A., Liu, C. C., Jiang, T., Roberts, T. M., *et al.* (2018) Targeted profiling of RNA translation reveals mTOR-4EBP1/2-independent translation regulation of mRNAs encoding ribosomal proteins. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E9325–E9332
81. Condon, K. J., Orozco, J. M., Adelmann, C. H., Spinelli, J. B., van der Helm, P. W., Roberts, J. M., *et al.* (2021) Genome-wide CRISPR screens reveal multitiered mechanisms through which mTORC1 senses mitochondrial dysfunction. *Proc. Natl. Acad. Sci. U. S. A.* **118**. https://doi.org/10.1073/pnas.2022120118
82. Jousse, C., Oyadomari, S., Novoa, I., Lu, P., Zhang, Y., Harding, H. P., *et al.* (2003) Inhibition of a constitutive translation initiation factor 2alpha phosphatase, CReP, promotes survival of stressed cells. *J. Cell Biol.* **163**, 767–775
83. Novoa, I., Zeng, H., Harding, H. P., and Ron, D. (2001) Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J. Cell Biol.* **153**, 1011–1022
84. Kojima, E., Takeuchi, A., Haneda, M., Yagi, A., Hasegawa, T., Yamaki, K., *et al.* (2003) The function of GADD34 is a recovery from a shutoff of protein synthesis induced by ER stress: elucidation by GADD34-deficient mice. *FASEB J.* **17**, 1573–1575
85. Connor, J. H., Weiser, D. C., Li, S., Hallenbeck, J. M., and Shenolikar, S. (2001) Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. *Mol. Cell Biol.* **21**, 6841–6850
86. Brush, M. H., Weiser, D. C., and Shenolikar, S. (2003) Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Mol. Cell Biol.* **23**, 1292–1303
87. Patterson, A. D., Hollander, M. C., Miller, G. F., and Fornace, A. J., Jr. (2006) Gadd34 requirement for normal hemoglobin synthesis. *Mol. Cell Biol.* **26**, 1644–1653
88. Harding, H. P., Zhang, Y., Scheuner, D., Chen, J. J., Kaufman, R. J., and Ron, D. (2009) Ppp1r15 gene knockout reveals an essential role for translation initiation factor 2 alpha (eIF2alpha) dephosphorylation in mammalian development. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 1832–1837

89. Masuoka, H. C., and Townes, T. M. (2002) Targeted disruption of the activating transcription factor 4 gene results in severe fetal anemia in mice. *Blood* **99**, 736–745
90. McEwen, E., Kedersha, N., Song, B., Scheuner, D., Gilks, N., Han, A., *et al.* (2005) Heme-regulated inhibitor kinase-mediated phosphorylation of eukaryotic translation initiation factor 2 inhibits translation, induces stress granule formation, and mediates survival upon arsenite exposure. *J. Biol. Chem.* **280**, 16925–16933
91. Bao, X. R., Ong, S. E., Goldberger, O., Peng, J., Sharma, R., Thompson, D. A., *et al.* (2016) Mitochondrial dysfunction remodels one-carbon metabolism in human cells. *eLife* **5**. https://doi.org/10.7554/eLife.10575
92. Viader, A., Sasaki, Y., Kim, S., Strickland, A., Workman, C. S., Yang, K., *et al.* (2013) Aberrant Schwann cell lipid metabolism linked to mitochondrial deficits leads to axon degeneration and neuropathy. *Neuron* **77**, 886–898
93. Quiros, P. M., Prado, M. A., Zamboni, N., D'Amico, D., Williams, R. W., Finley, D., *et al.* (2017) Multi-omics analysis identifies ATF4 as a key regulator of the mitochondrial stress response in mammals. *J. Cell Biol.* **216**, 2027–2045
94. Baker, M. J., Lampe, P. A., Stojanovski, D., Korwitz, A., Anand, R., Tatsuta, T., *et al.* (2014) Stress-induced OMA1 activation and autocatalytic turnover regulate OPA1-dependent mitochondrial dynamics. *EMBO J.* **33**, 578–593
95. Ehses, S., Raschke, I., Mancuso, G., Bernacchia, A., Geimer, S., Tondera, D., *et al.* (2009) Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. *J. Cell Biol.* **187**, 1023–1036
96. Yang, J., Baron, K. R., Pride, D. E., Schneemann, A., Guo, X., Chen, W., *et al.* (2023) DELE1 oligomerization promotes integrated stress response activation. *Nat. Struct. Mol. Biol.* **30**, 1295–1302
97. Conroy, J., McGettigan, P., Murphy, R., Webb, D., Murphy, S. M., McCoy, B., *et al.* (2014) A novel locus for episodic ataxia:UBR4 the likely candidate. *Eur. J. Hum. Genet.* **22**, 505–510
98. Choi, K. D., Kim, J. S., Kim, H. J., Jung, I., Jeong, S. H., Lee, S. H., *et al.* (2017) Genetic variants associated with episodic ataxia in korea. *Sci. Rep.* **7**, 13855
99. Monies, D., Abouelhoda, M., AlSayed, M., Alhassnan, Z., Alotaibi, M., Kayyali, H., *et al.* (2017) The landscape of genetic diseases in Saudi Arabia based on the first 1000 diagnostic panels and exomes. *Hum. Genet.* **136**, 921–939
100. Tasaki, T., Kim, S. T., Zakrzewska, A., Lee, B. E., Kang, M. J., Yoo, Y. D., *et al.* (2013) UBR box N-recognin-4 (UBR4), an N-recognin of the N-end rule pathway, and its role in yolk sac vascular development and autophagy. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 3800–3805
101. Yau, R. G., Doerner, K., Castellanos, E. R., Haakonsen, D. L., Werner, A., Wang, N., *et al.* (2017) Assembly and function of heterotypic ubiquitin chains in cell-cycle and protein quality control. *Cell* **171**, 918–933.e920
102. Fessler, E., Krumwiede, L., and Jae, L. T. (2022) DELE1 tracks perturbed protein import and processing in human mitochondria. *Nat. Commun.* **13**, 1853
103. Chakrabarty, Y., Yang, Z., Chen, H., and Chan, D. C. (2024) The HRI branch of the integrated stress response selectively triggers mitophagy. *Mol. Cell* **84**, 1090–1100.e1096
104. Fu, Y., Sacco, O., DeBitetto, E., Kanshin, E., Ueberheide, B., and Sfeir, A. (2023) Mitochondrial DNA breaks activate an integrated stress response to reestablish homeostasis. *Mol. Cell* **83**, 3740–3753.e3749
105. Sekine, Y., Houston, R., Eckl, E. M., Fessler, E., Narendra, D. P., Jae, L. T., *et al.* (2023) A mitochondrial iron-responsive pathway regulated by DELE1. *Mol. Cell* **83**, 2059–2076.e2056
106. Liu, Y. T., Huang, X., Nguyen, D., Shammas, M. K., Wu, B. P., Dombi, E., *et al.* (2020) Loss of CHCHD2 and CHCHD10 activates OMA1 peptidase to disrupt mitochondrial cristae phenocopying patient mutations. *Hum. Mol. Genet.* **29**, 1547–1567
107. Ajroud-Driss, S., Fecto, F., Ajroud, K., Lalani, I., Calvo, S. E., Mootha, V. K., *et al.* (2015) Mutation in the novel nuclear-encoded mitochondrial protein CHCHD10 in a family with autosomal dominant mitochondrial myopathy. *Neurogenetics* **16**. https://doi.org/10.1007/s10048-014-0421-1
108. Shammas, M. K., Huang, X., Wu, B. P., Fessler, E., Song, I. Y., Randolph, N. P., *et al.* (2022) OMA1 mediates local and global stress responses against protein misfolding in CHCHD10 mitochondrial myopathy. *J. Clin. Invest.* **132**. https://doi.org/10.1172/JCI157504
109. Bannwarth, S., Ait-El-Mkadem, S., Chaussenot, A., Genin, E. C., Lacas-Gervais, S., Fragaki, K., *et al.* (2014) A mitochondrial origin for frontotemporal dementia and amyotrophic lateral sclerosis through CHCHD10 involvement. *Brain* **137**, 2329–2345
110. Penttila, S., Jokela, M., Bouquin, H., Saukkonen, A. M., Toivanen, J., and Udd, B. (2015) Late onset spinal motor neuronopathy is caused by mutation in CHCHD10. *Ann. Neurol.* **77**, 163–172
111. Johnson, J. O., Glynn, S. M., Gibbs, J. R., Nalls, M. A., Sabatelli, M., Restagno, G., *et al.* (2014) Mutations in the CHCHD10 gene are a common cause of familial amyotrophic lateral sclerosis. *Brain* **137**, e311
112. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., *et al.* (1981) Sequence and organization of the human mitochondrial genome. *Nature* **290**, 457–465
113. Fernandez-Vizarra, E., and Zeviani, M. (2021) Mitochondrial disorders of the OXPHOS system. *FEBS Lett.* **595**, 1062–1106
114. Valnot, I., von Kleist-Retzow, J. C., Barrientos, A., Gorbatyuk, M., Taanman, J. W., Mehaye, B., *et al.* (2000) A mutation in the human heme A:farnesyltransferase gene (COX10) causes cytochrome c oxidase deficiency. *Hum. Mol. Genet.* **9**, 1245–1249
115. Coenen, M. J., van den Heuvel, L. P., Ugalde, C., Ten Brinke, M., Nijtmans, L. G., Trijbels, F. J., *et al.* (2004) Cytochrome c oxidase biogenesis in a patient with a mutation in COX10 gene. *Ann. Neurol.* **56**, 560–564
116. Ahola, S., Rivera Mejias, P., Hermans, S., Chandragiri, S., Giavalisco, P., Nolte, H., *et al.* (2022) OMA1-mediated integrated stress response protects against ferroptosis in mitochondrial cardiomyopathy. *Cell Metab.* **34**, 1875–1891.e1877
117. Franchino, C. A., Brughera, M., Baderna, V., De Ritis, D., Rocco, A., Seneca, S., *et al.* (2024) Sustained OMA1-mediated integrated stress response is beneficial for spastic ataxia type 5. *Brain* **147**, 1043–1056
118. Quiros, P. M., Langer, T., and Lopez-Otin, C. (2015) New roles for mitochondrial proteases in health, ageing and disease. *Nat. Rev. Mol. Cell Biol.* **16**, 345–359
119. Pierson, T. M., Adams, D., Bonn, F., Martinelli, P., Cherukuri, P. F., Teer, J. K., *et al.* (2011) Whole-exome sequencing identifies homozygous AFG3L2 mutations in a spastic ataxia-neuropathy syndrome linked to mitochondrial m-AAA proteases. *PLoS Genet.* **7**, e1002325
120. Dosi, C., Galatolo, D., Rubegni, A., Doccini, S., Pasquariello, R., Nesti, C., *et al.* (2020) Expanding the clinical and genetic heterogeneity of SPAX5. *Ann. Clin. Transl. Neurol.* **7**, 595–601
121. Maltecca, F., Aghaie, A., Schroeder, D. G., Cassina, L., Taylor, B. A., Phillips, S. J., *et al.* (2008) The mitochondrial protease AFG3L2 is essential for axonal development. *J. Neurosci.* **28**, 2827–2836
122. Das, I., Krzyzosiak, A., Schneider, K., Wrabetz, L., D'Antonio, M., Barry, N., *et al.* (2015) Preventing proteostasis diseases by selective inhibition of a phosphatase regulatory subunit. *Science* **348**, 239–242
123. Yerlikaya, A., Kimball, S. R., and Stanley, B. A. (2008) Phosphorylation of eIF2alpha in response to 26S proteasome inhibition is mediated by the haem-regulated inhibitor (HRI) kinase. *Biochem. J.* **412**, 579–588
124. Alvarez-Castelao, B., Tom Dieck, S., Fusco, C. M., Donlin-Asp, P., Perez, J. D., and Schuman, E. M. (2020) The switch-like expression of heme-regulated kinase 1 mediates neuronal proteostasis following proteasome inhibition. *eLife* **9**. https://doi.org/10.7554/eLife.52714
125. Ye, J., Kumanova, M., Hart, L. S., Sloane, K., Zhang, H., De Panis, D. N., *et al.* (2010) The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. *EMBO J.* **29**, 2082–2096
126. Tameire, F., Verginadis, I. I., and Koumenis, C. (2015) Cell intrinsic and extrinsic activators of the unfolded protein response in cancer: mechanisms and targets for therapy. *Semin. Cancer Biol.* **33**, 3–15
127. Burwick, N., and Aktas, B. H. (2017) The eIF2-alpha kinase HRI: a potential target beyond the red blood cell. *Expert Opin. Ther. Targets* **21**, 1171–1177
128. Yerlikaya, A. (2022) Heme-regulated inhibitor: an overlooked eIF2alpha kinase in cancer investigations. *Med. Oncol.* **39**, 73

129. Chen, T., Ozel, D., Qiao, Y., Harbinski, F., Chen, L., Denoyelle, S., *et al.* (2011) Chemical genetics identify eIF2alpha kinase heme-regulated inhibitor as an anticancer target. *Nat. Chem. Biol.* **7**, 610–616
130. Perea, V., Baron, K. R., Dolina, V., Aviles, G., Kim, G., Rosarda, J. D., *et al.* (2023) Pharmacologic activation of a compensatory integrated stress response kinase promotes mitochondrial remodeling in PERK-deficient cells. *Cell Chem. Biol.* **30**, 1571–1584.e1575
131. Burwick, N., Zhang, M. Y., de la Puente, P., Azab, A. K., Hyun, T. S., Ruiz-Gutierrez, M., *et al.* (2017) The eIF2-alpha kinase HRI is a novel therapeutic target in multiple myeloma. *Leuk. Res.* **55**, 23–32
132. Kline, C. L., Van den Heuvel, A. P., Allen, J. E., Prabhu, V. V., Dicker, D. T., and El-Deiry, W. S. (2016) ONC201 kills solid tumor cells by triggering an integrated stress response dependent on ATF4 activation by specific eIF2alpha kinases. *Sci. Signal.* **9**, ra18
133. White, M. C., Schroeder, R. D., Zhu, K., Xiong, K., and McConkey, D. J. (2018) HRI-mediated translational repression reduces proteotoxicity and sensitivity to bortezomib in human pancreatic cancer cells. *Oncogene* **37**, 4413–4427
134. Papaemmanuil, E., Cazzola, M., Boultwood, J., Malcovati, L., Vyas, P., Bowen, D., *et al.* (2011) Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. *N. Engl. J. Med.* **365**, 1384–1395
135. Adema, V., Ma, F., Kanagal-Shamanna, R., Thongon, N., Montalban-Bravo, G., Yang, H., *et al.* (2022) Targeting the EIF2AK1 signaling pathway rescues red blood cell production in SF3B1-mutant myelodysplastic syndromes with ringed sideroblasts. *Blood Cancer Discov.* **3**, 554–567
136. Clough, C. A., Pangallo, J., Sarchi, M., Ilagan, J. O., North, K., Bergantinos, R., *et al.* (2022) Coordinated missplicing of TMEM14C and ABCB7 causes ring sideroblast formation in SF3B1-mutant myelodysplastic syndrome. *Blood* **139**, 2038–2049
137. Merino, D., Kelly, G. L., Lessene, G., Wei, A. H., Roberts, A. W., and Strasser, A. (2018) BH3-Mimetic drugs: blazing the trail for new cancer medicines. *Cancer Cell* **34**, 879–891
138. Montero, J., and Letai, A. (2018) Why do BCL-2 inhibitors work and where should we use them in the clinic? *Cell Death Differ.* **25**, 56–64
139. Budhraja, A., Turnis, M. E., Churchman, M. L., Kothari, A., Yang, X., Xu, H., *et al.* (2017) Modulation of Navitoclax sensitivity by dihydroartemisinin-mediated MCL-1 repression in BCR-ABL(+) B-lineage acute lymphoblastic Leukemia. *Clin Cancer Res* **23**, 7558–7568
140. Smith, K. H., Budhraja, A., Lynch, J., Roberts, K., Panetta, J. C., Connelly, J. P., *et al.* (2021) The heme-regulated inhibitor pathway modulates susceptibility of poor prognosis B-lineage acute leukemia to BH3-mimetics. *Mol. Cancer Res.* **19**, 636–650
141. Shen, S., Vagner, S., and Robert, C. (2020) Persistent cancer cells: the deadly survivors. *Cell* **183**, 860–874
142. Kalkavan, H., and Green, D. R. (2018) MOMP, cell suicide as a BCL-2 family business. *Cell Death Differ.* **25**, 46–55
143. Glover, H. L., Schreiner, A., Dewson, G., and Tait, S. W. G. (2024) Mitochondria and cell death. *Nat. Cell Biol.* **26**, 1434–1446
144. Maynard, A., McCoach, C. E., Rotow, J. K., Harris, L., Haderk, F., Kerr, D. L., *et al.* (2020) Therapy-induced evolution of human lung cancer revealed by single-cell RNA sequencing. *Cell* **182**, 1232–1251.e1222
145. Tsherniak, A., Vazquez, F., Montgomery, P. G., Weir, B. A., Kryukov, G., Cowley, G. S., *et al.* (2017) Defining a cancer dependency map. *Cell* **170**, 564–576.e516
146. McDonald, E. R., 3rd, de Weck, A., Schlabach, M. R., Billy, E., Mavrakis, K. J., Hoffman, G. R., *et al.* (2017) Project DRIVE: a compendium of cancer dependencies and synthetic lethal relationships uncovered by large-scale, deep RNAi screening. *Cell* **170**, 577–592.e510
147. Sidrauski, C., Tsai, J. C., Kampmann, M., Hearn, B. R., Vedantham, P., Jaishankar, P., *et al.* (2015) Pharmacological dimerization and activation of the exchange factor eIF2B antagonizes the integrated stress response. *eLife* **4**, e07314
148. Matte, A., Federti, E., Winter, M., Koerner, A., Harmeier, A., Mazer, N., *et al.* (2019) Bitopertin, a selective oral GLYT1 inhibitor, improves anemia in a mouse model of beta-thalassemia. *JCI Insight* **4**. https://doi.org/10.1172/jci.insight.130111
149. Leto, D. E., Morgens, D. W., Zhang, L., Walczak, C. P., Elias, J. E., Bassik, M. C., *et al.* (2019) Genome-wide CRISPR analysis identifies substrate-specific conjugation modules in ER-associated degradation. *Mol. Cell* **73**, 377–389.e311