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Article

HRG1 Is Essential for Heme Transport from the Phagolysosome of Macrophages during Erythrophagocytosis

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SUMMARY

Adult humans have about 25 trillion red blood cells (RBCs), and each second we recycle about 5 million RBCs by erythrophagocytosis (EP) in macrophages of the reticuloendothelial system. Despite the central role for EP in mammalian iron metabolism, the molecules and pathways responsible for heme trafficking during EP remain unknown. Here, we show that the mammalian homolog of HRG1, a transmembrane heme permease in *C. elegans*, is essential for macrophage iron homeostasis and transports heme from the phagolysosome to the cytoplasm during EP. HRG1 is strongly expressed in macrophages of the reticuloendothelial system and specifically localizes to the phagolysosomal membranes during EP. Depletion of *Hrg1* in mouse macrophages causes attenuation of heme transport from the phagolysosomal compartment. Importantly, missense polymorphisms in human *HRG1* are defective in heme transport. Our results reveal HRG1 as the long-sought heme transporter for heme-iron recycling in macrophages and suggest that genetic variations in HRG1 could be modifiers of human iron metabolism.

INTRODUCTION

In humans, terminal erythroid differentiation is the process by which the earliest morphologically recognizable erythroid precursor in the bone marrow, the proerythroblast, differentiates into a mature red blood cell (RBC) in the peripheral blood (Ji et al., 2011). This transformation requires the coordinated maturation of the nucleus and cytoplasm that eventuates in fully hemoglobinized mature RBCs, devoid of a nucleus and all other organelles (Chasis and Mohandas, 2008; Mohandas and Chasis, 2010). Defects in numerous pathways can disrupt this sequence of maturation, but none is as important as the synthesis of hemoglobin, which requires the stoichiometric assembly of four globin peptides (two α and two β chains) and four heme (iron-protoporphyrin IX) moieties. Fully one-third of the protein in an RBC is hemoglobin which contains more than a billion iron atoms in the form of heme (Knutson and Wessling-Resnick, 2003). Consequently, it is not surprising that inherited or acquired defects in hemoglobin synthesis, including hemoglobinopathies, iron deficiency, and the anemia of inflammation, are among the most prevalent human afflictions.

Defects in the synthesis or metabolism of any one of the three components—globin, iron, or protoporphyrin IX—lead to ineffective erythropoiesis. While globin and porphyrin synthesis are the province of the RBC precursor itself, iron metabolism requires systemic homeostatic control that regulates iron absorption in the intestine, iron storage in the liver and macrophages, and erythroid uptake and utilization. Importantly, most of the ~25 mg of iron required to synthesize the hemoglobin in over 360 billion RBCs each day (5 million per second) derives from recycling of heme-iron from aged RBCs catabolized by reticuloendothelial system (RES) macrophages (Bratosin et al., 1998). At steady state, dietary heme and nonheme iron absorption contributes only 1–2 mg per day to the systemic iron economy (Andrews, 1999; Hentze et al., 2010).

RBC recycling occurs by a process known as erythrophagocytosis (EP), which is accompanied by the enzymatic catabolism of RBC components within the macrophage phagolysosome, resulting in the breakdown of hemoglobin and the release of heme (Bratosin et al., 1998). Several lines of evidence support a cellular model in which the degradation of heme occurs in the cytosol via the catalytic activity of the smooth endoplasmic reticulum-associated enzymes heme oxygenases 1 and 2 (HMOX1/2), producing iron, biliverdin, and carbon monoxide (Gottlieb et al., 2012; Yanatori et al., 2010; Yoshida and Sato, 1989). The iron liberated from heme can be stored in ferritin (FTH1/FTL1) or transported out of the cell by the iron exporter ferroportin (FPN1) whose cell surface expression is controlled by the iron regulatory hormone hepcidin (HAMP1) (Weiss, 2009). This model of EP necessitates the transport of heme from

the lumen of the phagolysosome into the cytosol, although the identity of the hypothetical heme transporter on the phagolysosomal membrane has not been identified.

Notwithstanding the importance of their function to iron metabolism and erythropoiesis, identification and characterization of heme and other porphyrin transporters in mammals have proven to be difficult (Hamza and Dailey, 2012; Quigley et al., 2004; Severance et al., 2011). This is in part due to a lack of genetic and molecular tools but is also a consequence of the promiscuity of proteins capable of transporting heme with low affinity, which has confounded functional cloning experiments (Helias et al., 2012; Krishnamurthy et al., 2004, 2006; Qiu et al., 2007; Saison et al., 2012; Salojin et al., 2011; Shayeghi et al., 2005; Wang et al., 2012). Furthermore, the lack of in vivo experimental validation or in vivo phenotypes of those proteins identified in vitro or solely on the basis of in vivo expression patterns has been misleading or provided ambiguous results.

We exploited *C. elegans*, a heme auxotroph, to identify HRG1-related heme transporters (Rajagopal et al., 2008). Worms acquire heme from the environment and transport it throughout the organism, negating several of the variables that have historically confounded the mammalian heme transport field (Rao et al., 2005). Depletion of *hrg-1* in worms by RNAi leads to abnormal heme sensing and accumulation of heme analogs (Rajagopal et al., 2008). Transient knockdown of *zfhrg1* in zebrafish leads to profound erythropoietic defects, while ectopic expression of human *HRG1* in murine erythroleukemia cells leads to enhanced ZnMP uptake. HRG1 is conserved in vertebrates, and human and worm proteins bind heme (Rajagopal et al., 2008; Yuan et al., 2012). Furthermore, the strength of this interaction is low pH dependent, suggesting that it may function in an acidic microenvironment, such as the phagolysosome. Correspondingly, HRG1 localizes primarily to the endolysosome in nonpolarized cells. Here we show that the long-sought heme transporter for macrophage heme-iron recycling is the human homolog of *C. elegans* HRG1 and identify a polymorphism that may be a genetic modifier of heme metabolism in humans. Our studies in mice, zebrafish, yeast, and mammalian cell lines support this conclusion.

RESULTS

HRG1 Is Abundantly Expressed in Reticuloendothelial Macrophages

To identify the tissue distribution of HRG1, polyclonal antibodies were generated against the 18 amino acid cytoplasmic C terminus of human HRG1. Immunoblotting with the antisera detected an ~15 kDa band corresponding to the predicted molecular weight of HRG1 monomers, in various human and mouse cell lines (Figure 1A and see Figure S1A online). Knockdown of either human or mouse *HRG1* by siRNA resulted in a significant reduction in HRG1 protein, indicating that the signal detected by immunoblotting was authentic (Figure 1B and Figure S1B). Immunohistochemistry with HRG1 antibodies detected high levels of HRG1 in the macrophages of the spleen, liver, and bone marrow in wild-type 129SvEv/Tac mice and humans—tissues that constitute the reticuloendothelial macrophage system responsible for recycling the heme iron in effete RBCs (Figures 1C and 1D and Figures S1C and S1D). HRG1 was also detected as weak granular/vesicular staining within hepatocytes (Figures 1C and 1D, right panels), consistent with the nearly ubiquitous expression of *HRG1* mRNA and protein in different cell types and tissues (Rajagopal et al., 2008).

Hrg1 Is Recruited to Phagolysosomal Membranes during Erythrophagocytosis

To ascertain the function of HRG1 in mammals, bone marrow-derived macrophages (BMDMs) from mice were isolated and differentiated ex vivo. Consistent with the previously observed location of ectopic human HRG1::GFP in HEK293 cells, endogenous Hrg1 was localized primarily to the endolysosome in BMDM by immunofluorescence microscopy (Figure 1E). To simulate EP, we exposed BMDM to oxidized RBCs. Oxidation of RBCs exposes phosphatidylserine on the outer membrane leaflet, mimicking cellular senescence and enhancing susceptibility to phagocytosis by macrophages (Cambos and Scorza, 2011). Strikingly, the phagolysosomal membranes of macrophages surrounding the RBCs were greatly enriched with Hrg1 (Figure 1F). Quantitation of the fluorescence signal intensity revealed that Hrg1 was recruited to the phagolysosomal membranes specifically during phagocytosis of RBCs, but not latex beads (Figure 1G versus Figure 1H, and Figure 1I). Additionally, no signal was detected in BMDM either under resting conditions or during phagocytosis using pre-immune serum (Figures S1E and S1F). The enhancement in Hrg1 immunofluorescence was derived from the BMDMs and not the engulfed RBCs, as mature RBCs do not express detectable Hrg1 (Figure S1G). Furthermore, recruitment of Hrg1 to the phagolysosomal membranes was not an indirect consequence of heme or iron, as BMDMs exposed to opsonized latex beads in the presence of either heme:arginate (20 μM) or iron (Fe:NTA, 200 μM) did not influence Hrg1 trafficking (Figure S1H). In resting BMDMs, a negligible portion of Hrg1 overlapped with the lysosomal marker Lamp; however, during EP, Hrg1 and Lamp1 protein strongly colocalized on the phagolysosomal membrane (Figure S1I), supporting the notion that Hrg1 is specifically recruited to the phagolysosome during EP.

Hrg1 Is Regulated by Heme, Iron, and EP

We next determined whether EP regulates *Hrg1*. BMDM exposed to RBCs showed greater abundance of *Hrg1* mRNA and protein in a time-dependent manner (Figures 2A and 2B). This regulation was also dose dependent, as increasing the ratio of RBCs to macrophages showed a proportional increase in *Hrg1* levels (Figures 2C and 2D). To distinguish between the individual contributions of heme and iron on *Hrg1* expression, EP was performed in the presence of the iron chelator deferoxamine (DFO). Exposure of BMDM to DFO only partially suppressed *Hrg1* induction (Figure 2E). This time- and dose-dependent regulation of *Hrg1* was reproducible by heme and iron (Figures S2A–S2C). Furthermore, other genes critical for heme-iron recycling, namely *Hmox1*, essential for heme degradation, and *Fpn1*, required for iron export, showed a similar responsiveness to EP, heme, and iron, although the magnitude of fold-change was different (Figures S2D–S2G) (Delaby et al., 2008).

Although *Hrg1* mRNA is upregulated in BMDMs grown in the presence of heme-depleted media and supplemented with 100 μM iron, 0.5 mM succinyl acetone, an inhibitor of endogenous heme synthesis, suppressed *Hrg1* mRNA induction by

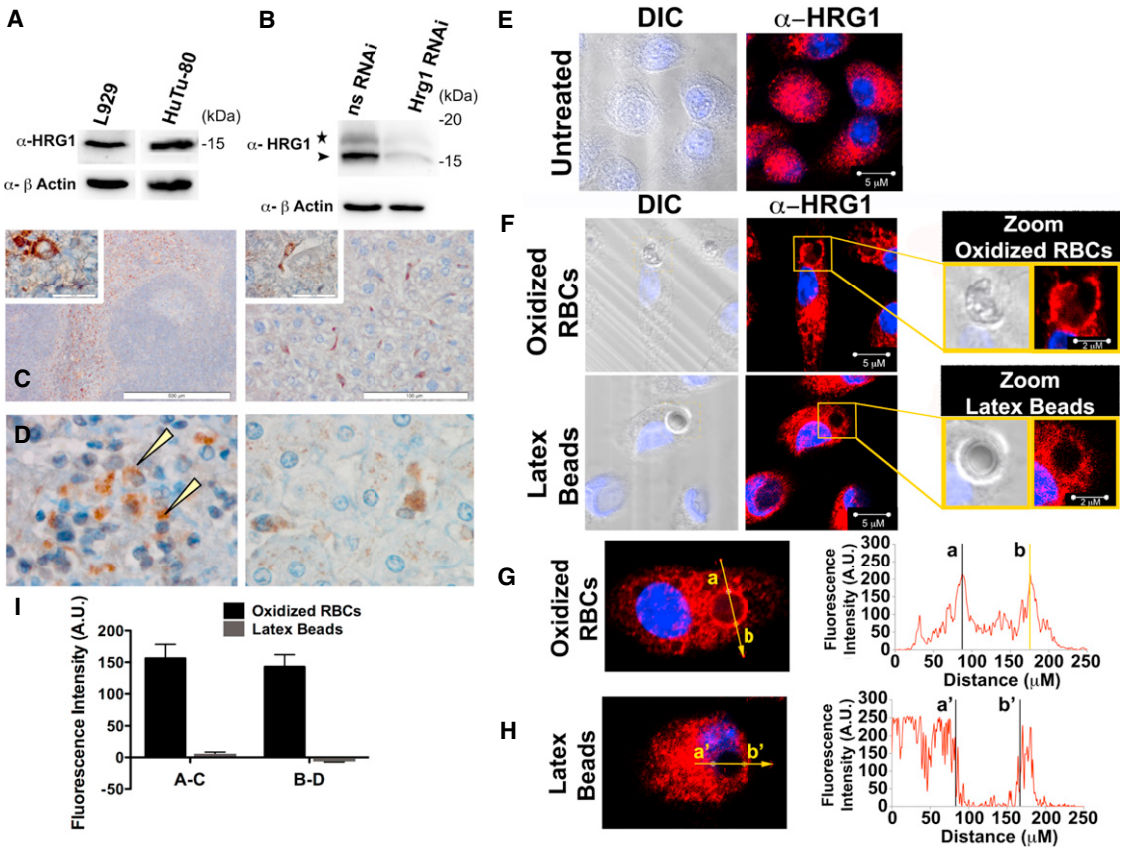

Figure 1. HRG1 Protein Is Expressed in Human and Mouse Macrophages of the Reticuloendothelial System

(A) Immunoblot of HRG1 protein in the mouse fibroblast cell line L929 and the human duodenal cell line HuTu-80.

(B) The α-HRG1 antibody detects mouse Hrg1 specifically. Immunoblot of Hrg1 protein in mouse bone marrow-derived macrophages (BMDM) transfected with either nonspecific RNAi (nsRNAi) or with *mHrg1* RNAi. The arrowhead indicates the predicted 15 kDa Hrg1 band. The star marks a doublet band that is also suppressed by *mHrg1* RNAi. For (A) and (B), actin is shown as a loading control.

(C and D) Immunohistochemistry of HRG1 expression in mouse spleen (C, left panel), mouse liver (C, right panel), human spleen (D, left panel), and human liver (D, right panel). Yellow and black arrowheads indicate HRG1 expresssion in macrophages and hepatocytes, respectively. HRG1 staining appears as a brown stain on a blue hematoxylin background. Final magnification is as follows: (C, left panel) 4× with 100× insert, (C, right) 20× with 100× insert, (D, left) 40×, and (D, right) 100×.

(E and F) Immunofluorescence imaging of endogenous Hrg1 localization in BMDM that were untreated (E) or fed either oxidized RBCs (F, upper panel) or latex beads (F, lower panel). Hrg1 protein was detected using a rabbit α-HRG1 antibody followed by α-rabbit Alexa 568. Nuclei were stained with DAPI. Right panel shows zoom-in pictures of the phagolysosome of BMDM fed RBCs or latex beads.

(G and H) Quantification of the red fluorescence intensity corresponding to Hrg1 protein localization at the phagolysosomal membrane of BMDM fed RBCs (markers a and b in G), or latex beads (markers a′ and b′ in H).

(I) Quantification of the peak of fluorescence at the phagolysosomal membrane in BMDM fed either oxidized RBCs or latex beads. For each condition, ten phagolysosomes were analyzed. The change in fluorescence intensity at the phagolysosomal membrane was assessed by calculating [fluorescence at membrane (points a and b) – fluorescence at point 5 μm away from membrane (points c and d)]. Quantification was performed using Zeiss Zen software for confocal microscopy. See also Figure S1.

iron (Figure 2F). This result suggests that *Hrg1* is regulated indirectly by iron via de novo heme synthesis.

We have previously shown that noniron metalloporphyrins act as heme analogs and are transported by HRG1 proteins (Rajagopal et al., 2008; Rao et al., 2005). To determine whether the effects of heme on Hrg1 expression could be reproduced by a metalloporphyrin, we exposed BMDM to zinc protoporphyrin IX (ZnPPIX). ZnPPIX is a physiologically relevant metalloporphyrin because its levels increase during iron-deficiency anemia (Labbé et al., 1999). Moreover, ZnPPIX is nonmetabolizable and inhibits heme degradation by binding to HMOX enzymes (Labbé et al., 1999). Hrg1 levels increased in a dose-dependent manner in BMDM that were exposed to ZnPPIX (Figure 2G). High concentrations of ZnPPIX, as expected, resulted in reduced ferritin levels, an indication that iron release from heme is attenuated due to inhibition of Hmox-dependent heme catabolism. These results affirm that iron via heme and noniron metalloporphyrins regulate Hrg1 in macrophages.

Hrg1 Is Regulated by Hemolysis In Vivo

To substantiate our in vitro cell biological studies in vivo, we injected mice with phenylhydrazine, which causes hemolytic anemia by damaging circulating erythrocytes, resulting predominantly in intravascular hemolysis (Itano et al., 1975). The

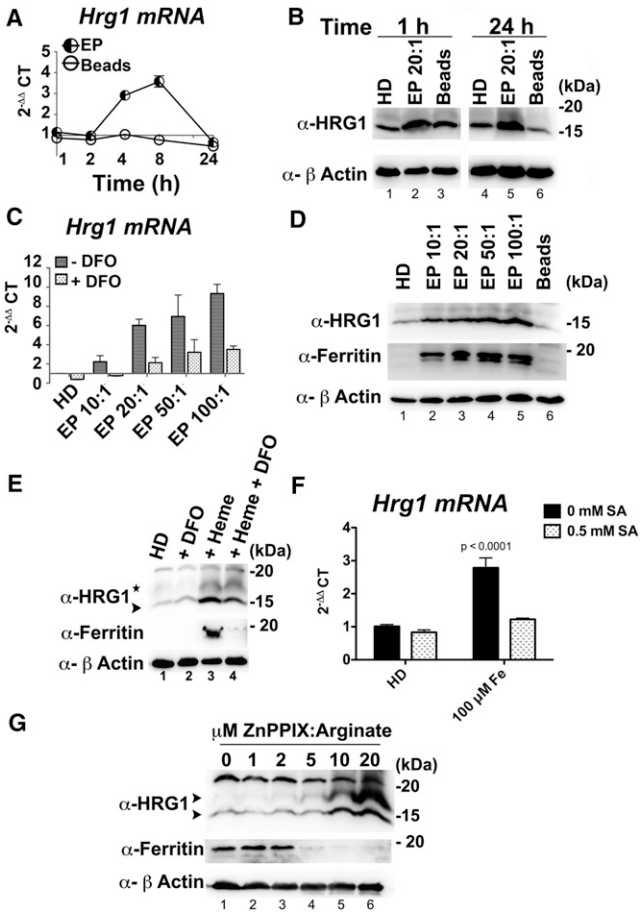

Figure 2. Hrg1 mRNA and Protein Are Regulated by Erythrophagocytosis and Heme in Mouse BMDM

(A) qRT-PCR time course analysis of *Hrg1* mRNA levels in BMDM fed either RBCs (EP, ●) or latex beads (○).

(B) Immunoblot of Hrg1 protein levels in BMDM 1 and 24 hr following feeding with RBCs or latex beads, or in time-matched controls grown in heme-depleted serum media (HD). Ferritin levels are shown as a control for increases in intracellular Fe levels in cells fed RBCs or grown in heme.

(C) qRT-PCR analysis of *Hrg1* mRNA levels in BMDM fed increasing numbers of RBCs, in the presence or absence of the iron chelator desferrioxamine (DFO, 100 μM). Increasing ratios of RBCs:BMDM were added to precultured BMDM. mRNA levels were assessed 4 hr following EP.

(D) Immunoblot of Hrg1 expression in BMDM fed increasing ratios of RBCs:BMDM. Samples were analyzed 24 hr following EP.

(E) Immunoblot of Hrg1 protein levels in BMDM exposed to 20 μM heme: arginate in the presence or absence of 100 μM DFO.

(F) qRT-PCR analysis of Hrg1 mRNA levels in BMDM treated with 100 μM Fe:NTA in the presence or absence of 0.5 mM succinyl acetone (SA).

(G) Immunoblot of Hrg1 protein levels in BMDM exposed to increasing concentrations of ZnPPIX:arginate.

(B, D, E, and G) Actin is shown as a loading control. For (A), (C), and (F), changes in mRNA levels were assessed as fold change compared to control samples from BMDM grown in HD media. Values were normalized to *Gapdh* mRNA levels (internal control). Error bars represent standard error of the mean (SEM). See also Figure S2.

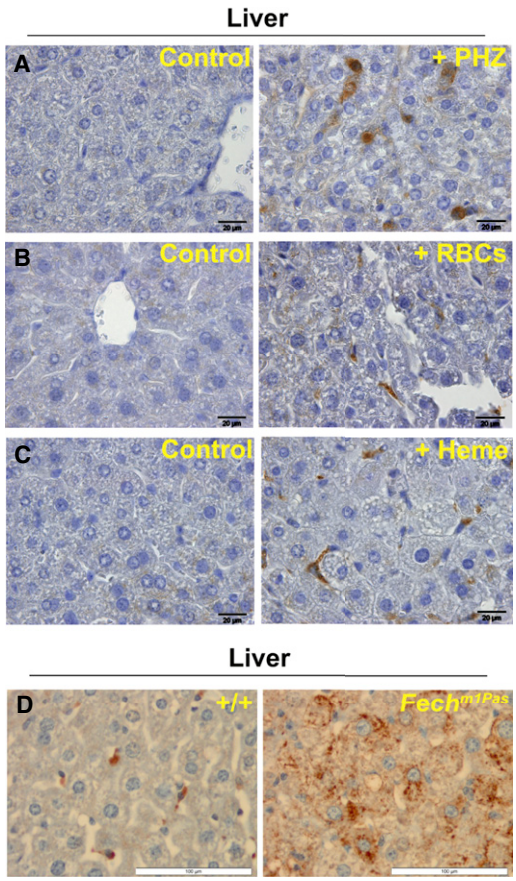

Figure 3. Hrg1 Protein Levels Are Increased in RES Macrophages of Mice Injected with Phenylhydrazine, Damaged RBCs, or Heme, and in the *Fech*^{m1Pas} Mouse

Hrg1 immunohistochemistry on mouse livers was performed 24 hr after treatment with PBS (A, left panel) or phenylhydrazine hydrochloride (PHZ, A, right panel), untreated RBCs (B, left panel) or oxidatively damaged RBC (B, right panel), vehicle (C, left panel), or heme (C, right panel). (D) Hrg1 IHC in livers from a wild-type mouse in which Hrg1 is localized primarily in macrophages (D, left panel) or a ferrochelatase-deficient (*Fech*^{m1Pas}) mouse in which it is highly expressed in hepatocytes (D, right panel). Hrg1 staining appears as a brown stain on a blue hematoxylin background. Original magnification is as follows: (A–C), 100×, (D) 20×. See also Figure S3.

released hemoglobin and heme are cleared from the circulation by haptoglobin and hemopexin, respectively, resulting in a concomitant net increase in intracellular heme within macrophages of the RES. Phenylhydrazine-treated mice showed a dramatic increase in Hrg1 within the resident macrophages of the liver and spleen (Figure 3A and Figure S3A). These results were highly reproducible and comparable when mice were injected with either oxidized RBCs or heme arginate to mimic a transient increase in intravascular heme (Figures 3B and 3C, Figures S3B and S3C).

To determine whether Hrg1 is regulated in a genetic animal model of anemia due to a defect in heme synthesis, we analyzed Hrg1 in tissues from mice with erythropoietic porphyria. The *Fech*^{m1Pas} mouse carries a recessive hypomorphic allele and expresses approximately 5% of wild-type ferrochelatase activity, resulting in a mild hemolytic anemia, associated with a vast excess of protoporphyrin IX production and tissue protoporphyrin IX accumulation (Davies et al., 2005). We found that Hrg1 was strikingly elevated in liver macrophages of homozygous *Fech*^{m1Pas} mice (Figure 3D), indicating that *Hrg1* is induced

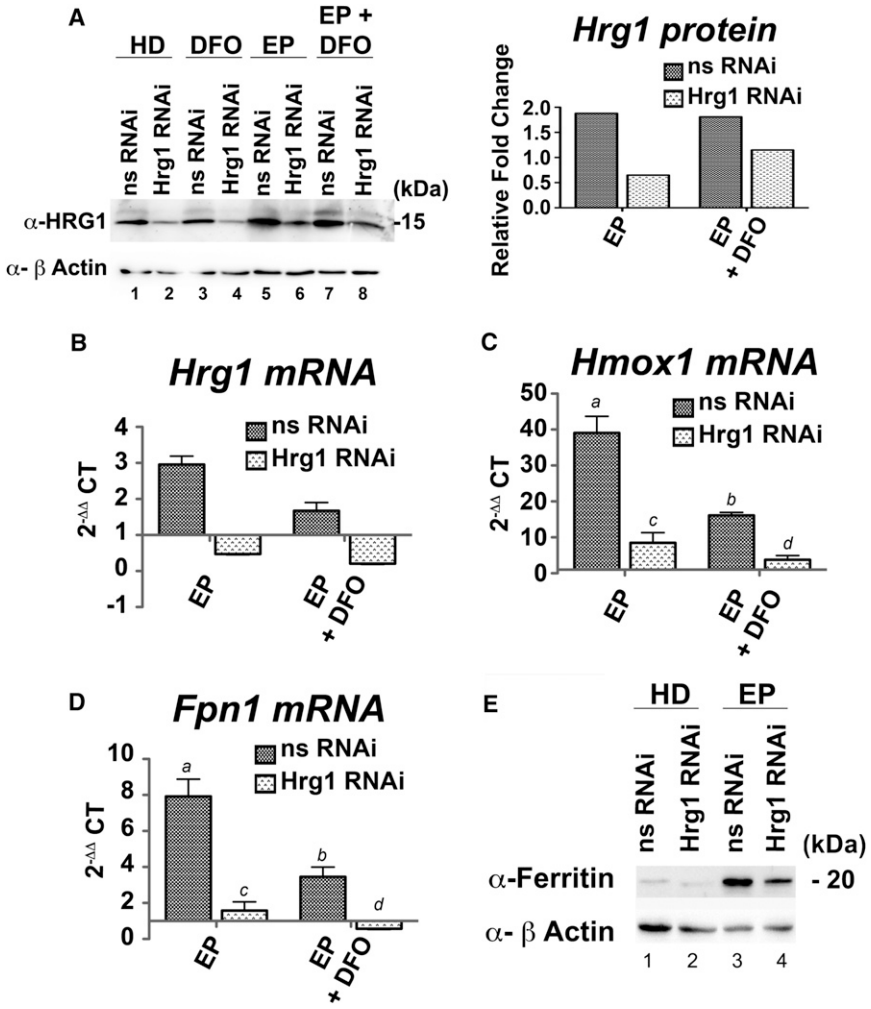

Figure 4. Hrg1 Mediates Heme Transport from the Phagolysosome during Erythrophagocytosis

(A) Immunoblot of Hrg1 protein levels in BMDM transfected with either nsRNAi or *Hrg1* RNAi and fed RBCs (EP, 20:1 RBC:BMDM) in the presence or absence of the iron chelator desferrioxamine. Actin is shown as a loading control. The right panel shows quantitation of the intensity of Hrg1 bands relative to nsRNAi HD controls and normalized to actin controls.

(B–D) qRT-PCR of *Hrg1* (B), *Hmox1* (C), and *Fpn1* (D) mRNA levels in BMDM transfected with nsRNAi or *Hrg1* RNAi. BMDM were fed RBCs (EP) in the presence or absence of the iron chelator desferrioxamine (DFO, 100 μM). Values represent the fold change relative to HD nsRNAi controls, normalized to *Gapdh* ($2^{-\Delta\Delta}$ CT). Samples were harvested 4 hr post-EP. Statistical analysis for (C) and (D) for EP and EP + DFO conditions are as follows: n = 6, nsRNAi versus *Hrg1* RNAi $p < 0.0001$. Error bars represent SEM. Values with different letter labels are significantly different ($p < 0.05$).

(E) Immunoblot of ferritin levels in BMDM treated with either nsRNAi or *Hrg1* RNAi and fed RBCs (EP, 10:1 RBC:BMDM). Actin is shown as loading control. See Figure S4D for Hrg1 immunoblot. See also Figure S4.

in vivo in conditions of protoporphyrin IX accumulation and chronic hemolysis.

Hrg1 Mediates Heme Transport from the Phagolysosome during Erythrophagocytosis

We hypothesized that Hrg1 recruitment to the phagolysosomal membranes may be connected to its function as a heme transporter. If this hypothesis were correct, then Hrg1 must transport heme from the lumen into the cytosol based on its membrane orientation and topology (Yuan et al., 2012), and downstream markers that are induced during EP would be dependent on Hrg1 function (Delaby et al., 2008; Marro et al., 2010). To substantiate this proposition, we knocked down *Hrg1* by siRNA. Because *Hmox1* and *Fpn1* mRNA inductions signify heme and iron availability during EP, we assessed the response of these markers to *Hrg1* siRNA. siRNA treatment resulted in greater than 75% depletion in *Hrg1* mRNA and protein levels in BMDM grown in heme-depleted culture media (Figures 4A and 4B, Figure S4A). Strikingly, *Hrg1* knockdown resulted in a significant attenuation of *Hmox1* and *Fpn1* mRNA induction during EP (Figures 4C and 4D, Figures S4B and S4C). This suppression prevailed in the presence of the iron chelator DFO, confirming that both heme and iron regulate *Hrg1*. Importantly, knockdown of *Hrg1* during EP resulted in a concomitant reduction in ferritin accumulation (Figure 4E and Figure S4D). These results unequivocally demonstrate that Hrg1 is essential for heme transport from the phagolysosome during EP and is genetically upstream from *Hmox1*, *Fpn1*, and ferritin, known markers of heme-iron recycling.

Missense Polymorphisms in Human *HRG1* Result in Defective Heme Transport

To determine whether genetic variations in human *HRG1* may be modifiers of iron metabolism, we searched the NHLBI Exome Sequencing Project (ESP 5400 and ESP 6500SI) and found three missense variants in *HRG1*—G73S (frequency in population, 0.0309%), S82L (0.0309%), and W115C (0.0279%). These three variants were present in European Americans (EAs) but not in African Americans (AAs). However, the ESP database can be biased because they selectively remove extremely rare variants and samples that have first- to third-degree kinship to ensure sampling of unrelated DNA. To identify additional missense variants, we sequenced coding regions and intron-exon boundaries in DNA obtained from patient cohorts with unexplained microcytic anemia. A single sequence variant in *HRG1/SLC48A1* was identified (c. 107C > T, P36L) in an AA proband who was also a compound heterozygote for mutations in *TMPRSS6* and thus had iron refractory iron deficiency anemia (IRIDA) (Finberg et al., 2008); the patient's unaffected mother was also a carrier

for the P36L variant and a TMPRSS6 allele. Furthermore, we sequenced Coriell Control DNA panels of 50 AA and 50 EA and found two additional AA samples that were carriers for the P36L variant. This polymorphism was not identified in EA.

Amino acid sequence alignments revealed that only tryptophan 115 and proline 36 are highly conserved in vertebrate HRG1 (Figure S5A). Topology modeling of vertebrate HRG1 predicts W115 to be located in the center of the fourth transmembrane domain (TMD) while P36 lies within a stretch of five amino acids (YRQPG) in the first exoplasmic (E1) loop between TMD 1 and TMD 2 (Figure 5A). The presence of a central turn-inducing proline within short luminal helical hairpins between TMD segments is a key topogenic determinant of membrane proteins (Monné et al., 1999).

To assess the significance of each of the amino acid substitution, we exploited growth assays in yeast. *Saccharomyces cerevisiae hem1Δ* strains are unable to grow in aerobic conditions due to a genetic defect in the heme biosynthesis enzyme δ-aminolevulinic acid synthase. However, the *hem1Δ* growth defect can be rescued by supplementation of the growth medium with either δ-aminolevulinic acid or low amounts of exogenous heme (0.25 μM) plus HRG1 expression (Yuan et al., 2012). Neither G73S nor S82L had any effect on the growth of *hem1Δ* yeast, while W115C showed a modest effect on growth (Figure S5B). However, the P36L variant showed a severe reduction in yeast growth compared to wild-type HRG1 at both 0.25 and 1 μM heme.

To further explore the functional consequence of the P36 substitution in HRG1, we generated single-copy integrants of *HRG1* in *hem1Δ* yeast which expressed equivalent levels of wild-type and P36L proteins (Figure S5C). The P36L variant was unable to rescue the growth of *hem1Δ*, even in the presence of 10 μM heme, whereas wild-type HRG1 restored growth of yeast with as little as 0.25 μM heme (Figure 5B). Unlike wild-type HRG1 ($p < 0.0001$), P36L did not alter intracellular heme levels ($p > 0.05$), as β-galactosidase activity remained unchanged in yeast expressing the *CYC1::lacZ* promoter-reporter fusion (Figure 5C). β-galactosidase activity reflects the availability of regulatory pools of cytoplasmic heme to activate *CYC1* expression (Yuan et al., 2012).

To evaluate the significance of the P36L substitution in vivo, we utilized a vertebrate animal model. We have previously reported that transient knockdown of *zfhrg1a* with antisense morpholino (MO2) in zebrafish embryos results in a severe erythropoietic defect that can be rescued by coinjection with *C. elegans HRG1* (Rajagopal et al., 2008). Coinjection of wild-type *zfhrg1a* with MO2 significantly rescued the morphant anemia phenotype ($p < 0.01$), but the P34L variant, the polymorphic ortholog of the human P36L variant, was unable to rescue the hematological defects ($p > 0.05$; Figure 5D, and Figure S5D).

A Hemoprotein Reporter in Mammalian Cells Interrogates Heme Availability and P36L Functionality

The results from yeast and zebrafish suggest that the P36L variant is defective in heme transport. To corroborate this finding during EP in mouse BMDM, we created a hemoprotein reporter which can be used in a robust assay to directly evaluate intracellular heme levels. We chose the horseradish peroxidase (HRP) enzyme because it contains a heme cofactor, and as such, perturbations in cellular heme levels result in a concomitant change in HRP activity that is easily measured by in-gel assays, in situ histochemistry, or plate assays (Veitch, 2004). To confine HRP to the secretory compartment, we incorporated a Golgi targeting sequence from galactosyltransferase into the amino terminus of HRP (Bard et al., 2006). HRP activity in HEK293 cell lines was directly proportional to both exogenous and endogenously synthesized heme (Figure 5E). Succinyl acetone significantly attenuated HRP activity, while supplementation of heme to the growth medium greatly enhanced HRP activity (Figure 5E). Importantly, HRP activity was directly dependent on heme because iron chelation by DFO did not attenuate HRP activity (Figure 5E). This result was reproducible in BMDM, as HRP activity was enhanced in a dose-dependent manner in response to heme supplementation in the growth medium (Figure 5F).

To test whether the P36L polymorphism alters heme transport, we transduced BMDM with adenoviral vectors driving the expression of HRG1, Golgi-HRP, or both (Figures S5E and S5F). As expected, the activity of HRP was significantly enhanced in the presence of either heme supplementation or EP in cells that overexpressed wild-type HRG1 (Figures 5G and 5H, $p < 0.0001$). Consistent with our studies in yeast and zebrafish, the P36L variant failed to activate HRP above vector controls under either condition ($p > 0.05$), suggesting that this variant is unable to function normally. Notably, this difference in function was not because of reduced expression or aberrant intracellular localization, as immunolocalization experiments revealed comparable levels and similar localization patterns of wild-type and P36L proteins in adenoviral transduced BMDM (Figure S5G). Altogether, our studies in yeast, zebrafish, and mouse macrophages provide compelling evidence that the P36L missense variant is a dysfunctional transporter.

DISCUSSION

The importance of iron recycling is emphasized by the fact that the majority of body iron required to produce new RBCs is acquired by recycling iron derived from heme contained within hemoglobin from senescent RBCs (Andrews, 1999). Although several genes involved in iron recycling have been identified, including *Nramp1* (Vidal et al., 1996), *Nramp2/DMT1* (Fleming et al., 1997; Gunshin et al., 1997), *heme oxygenase 1*, and *ferroportin 1* (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000), compelling cell biological evidence has supported the existence of a heme transporter on the macrophage phagolysosomal membranes, but none have been found to date. Studies have shown that proteolytic degradation of hemoglobin in the phagolysosome results in the release of heme, which is subsequently degraded by heme oxygenase enzymes to yield biliverdin, carbon monoxide, and iron (Maines, 1997). The catabolism of heme during EP (Marro et al., 2010) is mediated primarily by the induction of Hmox-1, an enzyme tethered to the endoplasmic reticulum membranes with the active site facing the cytosol (Gottlieb et al., 2012). This model dictates that heme must be transported from the phagolysosome into the cytosol for catabolism and iron release (Figure 5I). Evidence exists that cytosolic labile pools of heme increase following EP. For example, Delaby et al. (Delaby et al., 2008) have previously

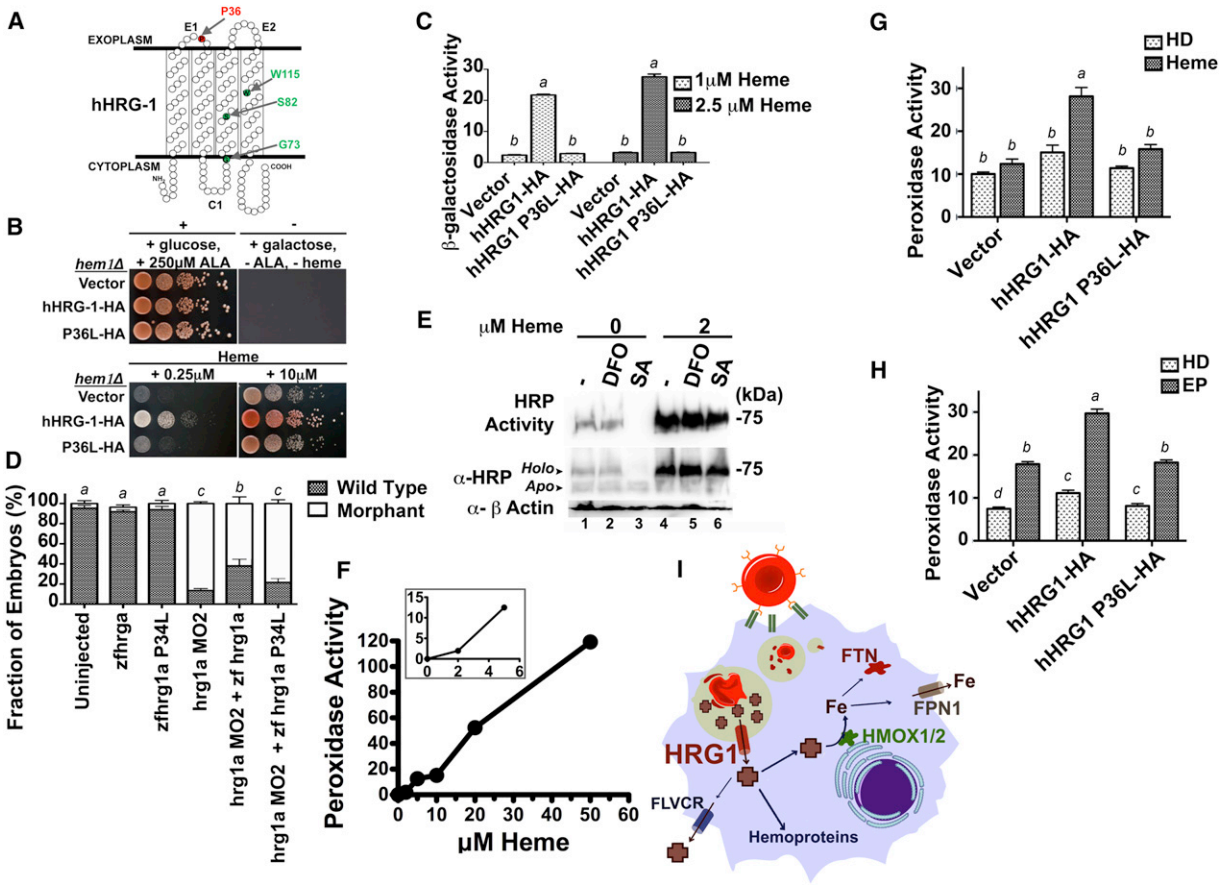

Figure 5. The P36L Polymorphism in Human and Zebrafish HRG1 Results in Defective Heme Transport in Yeast, Zebrafish, and BMDM

(A) Topology of human HRG1. The arrows indicate the polymorphic positions.

(B) The P36L human variant fails to rescue the growth of the *hem1*Δ(6D) yeast strain under low heme concentrations. *hem1*Δ(6D) yeast strains stably expressing either pYes-DEST52 vector control, hHRG1-HA, or hHRG1 P36L-HA were grown overnight in 2% w/v raffinose SC (−His) medium and spotted in serial dilution on 2% w/v raffinose SC (−His, + 0.4% w/v galactose) plates supplemented with 0.25 μM or 10 μM hemin. (+) positive control, + 0.4% w/v glucose, + 250 μM ALA; (−) negative control, + 0.4% galactose, −ALA, −hemin. Plates were incubated at 30°C for 3 days before imaging.

(C) The P36L polymorph is defective in heme transport in yeast. *hem1*Δ(6D) yeast strains stably expressing either pYes-DEST52 vector control, hHRG1-HA, or hHRG1 P36L-HA were cotransformed with pCYC1-LacZ and grown in 2% w/v raffinose SC (−His, −Trp) medium for 12 hr, washed, and grown in 2% w/v raffinose SC (−His, −Trp, + 0.4% w/v galactose) medium supplemented with 1 μM or 2.5 μM hemin. Galactosidase activity (Miller units) was measured in cell lysates. Statistical analysis is as follows: n = 3, vector versus hHRG1-HA $p < 0.0001$, vector versus P36L-HA $p > 0.05$; error bars represent SEM.

(D) The P34L variant of zebrafish *hrg1a* is unable to rescue the hematological defects associated with *hrg1a* MO2 (*hrg1a* MO2 + zf *hrg1a* P34L versus *hrg1a* MO2, $p > 0.05$) compared to wild-type *zfhrg1a* (*hrg1a* MO2 + zf *hrg1a* versus *hrg1a* MO2, $p < 0.01$). Zebrafish embryos injected with *hrg1a* MO2 (2.7 ng/ embryo) exhibit a hematological defect (*hrg1a* MO2 versus uninjected, $p < 0.001$; see Figure S8B). Rescue experiments were performed by coinjecting *hrg1a* MO2 with 120 pg/embryo of *hrg1a* mRNA or 120 pg/embryo of *hrg1a* P34L mRNA in one to two cell-stage embryos. Following *O*-dianisidine staining at 48 hpf (hours postfertilization), the numbers of affected embryos showing erythroid defects were counted; ~150 embryos were analyzed per morpholino treatment. The graph shows the percentage of morphants (erythroid defect) and wild-type (no erythroid defect) embryos. Error bars represent the SEM.

(E) Golgi HRP activity is dependent on heme. HEK293 cells were grown in HD media (−) and pretreated with 100 μM DFO or 0.5 mM succinyl acetone (SA) where indicated. Following transfection with a construct expressing either vector control or Golgi-HRP, cells were exposed to 2 μM heme:arginate for 24 hr prior to harvesting. Golgi-HRP activity from lysates was assessed using on-blot chemiluminescence following SDS-PAGE. HRP protein was detected on a separate immunoblot using a rabbit α-HRP antibody. Actin is shown as a loading control.

(F) Dose response of HRP activity in BMDM transduced with adenovirus expressing Golgi-HRP in the presence of the indicated concentrations of heme. Inset represents data for 0–5 μM heme in enlarged format.

(G and H) Golgi-HRP activity in BMDM treated with 0.5 μM heme:arginate (G) or fed RBCs at a 1:1 RBC:BMDM ratio (H). BMDM were cotransduced with adenovirus expressing Golgi HRP and either pShuttle-CMV vector, hHRG1-HA, or hHRG1 P36L-HA (see Figures S5E and S5F). Cell lysates were assessed for peroxidase activity (μU/ml). Values were normalized for total protein levels of corresponding lysates. Statistical analysis is as follows: HRP assay heme-treated samples, n = 9, vector versus hHRG1-HA $p < 0.0001$, vector versus P36L-HA $p > 0.05$; EP-treated samples, n = 9, vector versus hHRG1-HA $p < 0.0001$, vector versus P36L-HA $p > 0.05$; error bars represent the SEM (C, G, and H), values with different letter labels are significantly different ($p < 0.05$).

(I) Proposed model for HRG1-mediated heme transport in macrophages. Following phagocytosis of a senescent RBC by a macrophage cell, the RBC is sequestered in the phagolysosomal compartment and degraded via the activity of various enzymes, yielding the release of heme. During EP, HRG1 traffics from the endolysosomal compartment to the phagolysosomal membrane, where it functions to transport heme into the cytosol. Heme may be delivered to three separate pathways: (1) degradation by the HMOX1/2 enzymes, resulting in the release of iron, which may be exported back into the circulation via ferroportin (FPN1) or stored into ferritin (FTN); (2) incorporation in toto into hemoproteins; or (3) export via FLVCR. See also Figure S5.

demonstrated that the transcription of *Hmox1* and *Fpn1* is specifically regulated by heme following EP in BMDMs. Additionally, *Hmox1*$^{-/-}$ mice (Gottlieb et al., 2012) have little or no resident splenic and liver macrophages, possibly because of cytotoxic heme accumulation following EP (Kovtunovych et al., 2010).

We hypothesized that the human homolog of *C. elegans* HRG1 might be the phagolysosomal heme transporter based on the following rationale. First, both worm and human proteins bind and transport heme (Rajagopal et al., 2008). Second, HRG1 homologs have low primary amino acid sequence identity (~20%) but retain overall topological conservation and strategically placed heme-binding residues (Yuan et al., 2012). Third, worm and human HRG1 proteins colocalize with LAMP1 and the small GTPases Rab7 and Rab11 in HEK293 cells (Rajagopal et al., 2008), markers known to contribute to the mature phagolysosome (Desjardins et al., 1994; Tjelle et al., 2000; Vieira et al., 2003). Fourth, hemoglobinized RBCs are specified but cannot be maintained when *hrg1* is knocked down in zebrafish. Lastly, ectopic expression of HRG1 in Friend mouse erythroleukemia cells shows enhanced uptake of ZnMP, a fluorescent heme analog (Rajagopal et al., 2008). To test our hypothesis, we assessed the role of HRG1 in the RES during EP. We find that Hrg1 is abundantly expressed in the macrophages of the RES and localizes to the phagolysosomal membranes—a tissue and location highly relevant to heme-iron recycling, and consistent with the topological orientation of Hrg1 for heme transport from the lumen into the cytosol. These cell-biological studies are further supported by the concomitant increase in *Hrg1* mRNA and protein during EP, which parallels the regulation of *Hmox1* and *Fpn1* (Delaby et al., 2005, 2008).

Importantly, *Hrg1* depletion by siRNA results in a reduction in the regulatory heme pools, as demonstrated by the suppression of *Hmox1*, *Fpn1*, and ferritin inductions during EP (Delaby et al., 2008; Marro et al., 2010). Consistent with this finding, ectopic expression of HRG1 in BMDM results in enhanced cellular heme availability during EP as measured by a significant increase in HRP enzymatic activity, a synthetic Golgi-targeted hemoprotein reporter. These results are recapitulated in vivo by inducing a transient increase in heme by phenylhydrazine injection, or treatment with heme or damaged RBCs in mice. All three treatments cause a dramatic increase in Hrg1 levels in resident macrophages of the liver, spleen, and bone marrow. In line with these results, a recent study showed that *Hrg1* is upregulated in splenic macrophages derived from mice that have been chemically induced to mimic the anemia of inflammation and chronic disease (Prince et al., 2012). The significance of the Hrg1 response to conditions of increased physiological demand on the RES is further emphasized in the hypomorphic *Fech*^{m1Pas} mouse model of erythropoietic porphyria (Davies et al., 2005). Although the exact mechanism(s) regulating Hrg1 during hemolytic anemia is not well understood, recent studies have identified binding sites for the heme-dependent transcriptional repressor BACH1 in the *Hrg1*, *Hmox1*, and *Fpn1* gene promoters (Marro et al., 2010; Sun et al., 2002; Warnatz et al., 2011). Taken together, these studies establish HRG1 as a critical component of the heme-iron recycling pathway in the RES (Figure 5I).

To address the possibility that inherited mutations in *HRG1* could be associated with human disease, we screened DNA samples for rare variants and found a P36L variant in the AA population and three additional missense variants in EAs from the Exome Variant database. Results from our studies in yeast, zebrafish, and mouse macrophage show that the P36L variant is defective in heme transport. Previous studies in zebrafish have shown that HRG1 is critical for the maintenance of erythropoietic cells and the central nervous system (Rajagopal et al., 2008). Modest reduction of *hrg1* mRNA by antisense morpholinos in zebrafish embryos results in a hypomorphic morphant with only mild anemia, whereas a severe knockdown of *hrg1* causes developmental defects including hydrocephalus and a curved body with shortened yolk tube (B. Paw and I.H., unpublished data). These variations in *hrg1* phenotypes in zebrafish morphants may represent genetic alleles of *HRG1* hypomorphs in the human population. We speculate that polymorphisms in *HRG1* may be an important genetic determinant in inherited iron disorders in humans, including, for example, in the so-called "African Iron Overload" (AIO) or "Bantu Siderosis," (Gordeuk et al., 2003). Based on the results from our studies, we predict that *Hrg1* knockout in mammals will reveal iron retention in macrophages and secondary systemic iron overload phenotypes. Screening for mutations in *HRG1* in well-characterized cohorts of patients will provide further insight into the physiological significance of HRG1 in genetic disorders of iron metabolism (Adams et al., 2005; McLaren et al., 2003). Given the striking similarities between heme utilization from senescent RBC in macrophages and heme transport from a diet rich in red meat in enterocytes (Severance et al., 2011; West and Oates, 2008), it is conceivable that HRG1 may function as a heme transporter in the intestine.

EXPERIMENTAL PROCEDURES

Animal Strains, Cell Culture, and Reagents

All animal experiments were approved by the University of Maryland Institutional Animal Care and Use Committee and by the Animal Care and Use Committee at Children's Hospital (Boston, MA). C57BL/6J or 129/SvEvTac mice were purchased from Jackson Laboratories or Taconic, respectively.

Hrg1 protein expression in mice was induced by three methods. Mice were injected intraperitoneally with 3 mg/20 g weight phenylhydrazine hydrochloride (Aldrich) or equivalent volume PBS control. Furthermore, 1 mg total heme (Frontier Scientific) as heme:arginate (1 mM:10 mM [pH 7.5]) or arginine vehicle alone was injected retro-orbitally. Finally, whole blood was collected from C57BL/6J donor animals into Lithium-Heparin Microtainer tubes (Becton Dickinson), washed extensively with PBS, and treated with 0.2 mM $CuSO_4$ and 5 mM sodium-ascorbate (Sigma) for 60 min at 37°C. Cells were washed two times in excess 5 mM EDTA (PBS) and two times in excess PBS and brought up to 50% HCT in sterile PBS, and 200 ml damaged or untreated RBCs were injected retro-orbitally. Animals were euthanized 24 hr after injection and tissues harvested.

Isolation and culture of bone marrow from the femur of C57BL/6 mice was carried out as described elsewhere (Marim et al., 2010). Briefly, differentiation of fresh bone marrow cells was carried out in the presence of L929- conditioned media (LCM) as a source of granulocyte/macrophage colony-stimulating factor (Englen et al., 1995). Differentiation media composition is as follows: RPMI 1640 (Invitrogen) supplemented with 30% LCM, 20% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (Invitrogen). L-929 cells were purchased from the American Type Culture Collection (ATCC). For production of LCM, L-929 cells were grown in the presence of RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine for 30 days. Media was collected by sterile filtration and stored at −20°C for later use in BMDM differentiation

and culture media. In all experiments described below, BMDMs were cultured in the presence of heme-depleted serum (HD) media as untreated control. HD media was prepared similarly to differentiation media with the exception of substituting 20% heme-depleted FBS for FBS. Heme depletion was achieved by treating FBS with ascorbic acid for ~7–8 hr, followed by dialysis against PBS and filter sterilization. The depletion of heme from the serum was monitored by measurement of the optical absorbance at 405 nm. Depletion was considered successful when the absorbance of serum was reduced at least 50% following ascorbic acid treatment (Sassa and Nagai, 1996).

Unless otherwise noted, all chemicals and reagents were obtained from Sigma-Aldrich. Hemin and zinc protoporphyrin IX (ZnPPIX) were purchased from Frontier Scientific, Inc. Fresh 1 mM solutions of heme:arginate and ZnPPIX:arginate with 10 mM arginine were prepared by dilution in 0.2 M KOH/100% ethanol (1:1, v/v). Iron nitrolotriacetic acid (Fe:NTA) solution was prepared as a 1 mM stock with NTA (Sigma-Aldrich), and ferric chloride hexahydrate (molar ratio of 1:4). HRG1 antibody serum was produced in rabbit using the C-terminal 18 amino acid peptide sequence of human HRG1 as antigen (Epitomics, Inc.). Positive serum samples were selected based on high specificity to HRG1 as well as low unspecific background as tested by western blot analysis. Preimmune serum from the same rabbit was used as a negative control.

Statistical Analysis

Statistical significance was calculated by two-way analysis of variance with the Bonferroni multiple comparison test using Prism version 5.0 (GraphPad, San Diego, CA). Data values are presented as mean ± SEM. A p value < 0.05 was considered significant.

Additional material is available in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2013.01.005.

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