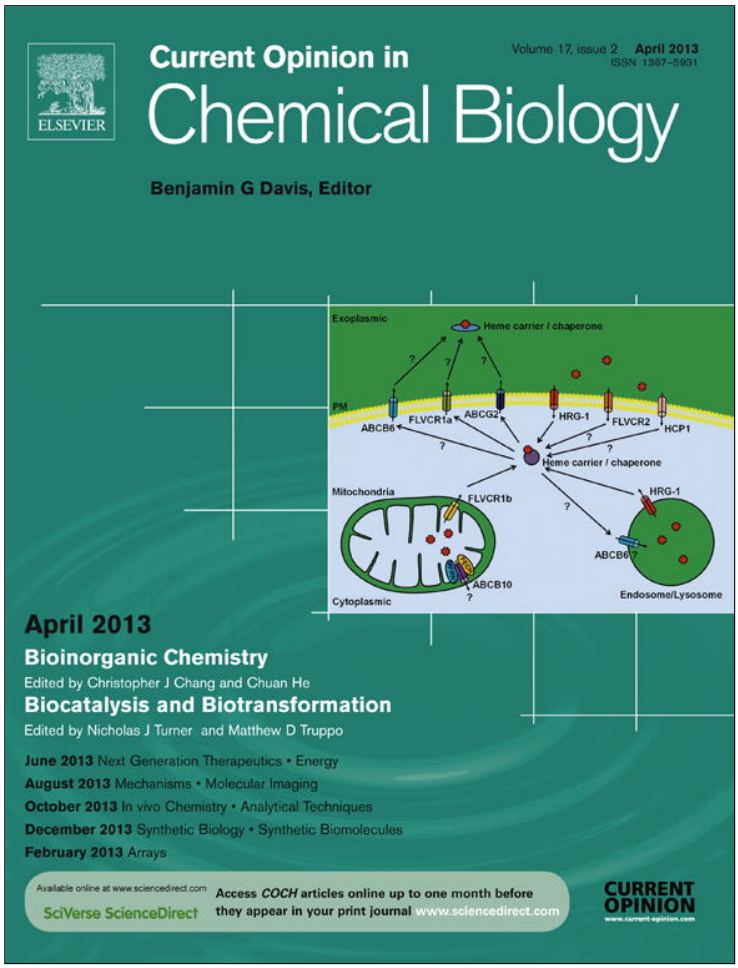

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Current Opinion in Chemical Biology

Heme transport and erythropoiesis

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In humans, systemic heme homeostasis is achieved via coordinated regulation of heme synthesis, transport and degradation. Although the heme biosynthesis and degradation pathways have been well characterized, the pathways for heme trafficking and incorporation into hemoproteins remain poorly understood. In the past few years, researchers have exploited genetic, cellular and biochemical tools, to identify heme transporters and, in the process, reveal unexpected functions for this elusive group of proteins. However, given the complexity of heme trafficking pathways, current knowledge of heme transporters is fragmented and sometimes contradictory. This review seeks to focus on recent studies on heme transporters with specific emphasis on their functions during erythropoiesis.

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Introduction

Heme homeostasis is a highly coordinated process during erythropoiesis, marked by a dramatic increase of heme synthesis which is essential for proper hemoglobinization of red blood cells (RBCs) [1,2]. Heme is also involved in transcriptional and translational regulation of erythroid specific gene expression, which is critical for coupling heme synthesis with protein production for erythroid cell differentiation [3,4]. In addition, a large amount of heme-iron is recycled for re-packing into hemoglobins by erythrophagocytosis (EP) in macrophages of the reticuloendothelial system (RES) [1,5••,6•]. Although heme biosynthesis and its regulation have been well characterized, the mechanisms for heme transport in eukaryotes remain poorly understood. Comprehensive reviews for generic heme trafficking and interorganellar transfer pathways have been covered elsewhere [5••,6•,7,8]. In this review we will seek to cover the following. How does newly synthesized heme exit the mitochondria for incorporation into hemoglobins and other hemoproteins? How does heme released from lysed RBCs cross the phagolysosomal membrane to be delivered to downstream effectors such as heme oxygenase-1 (HO-1) for degradation? Can heme be redistributed between different tissues through heme transporters and chaperones? Extensive efforts to identify heme trafficking pathways have been underway for over a decade and a number of heme transporters have been identified recently.

Heme import

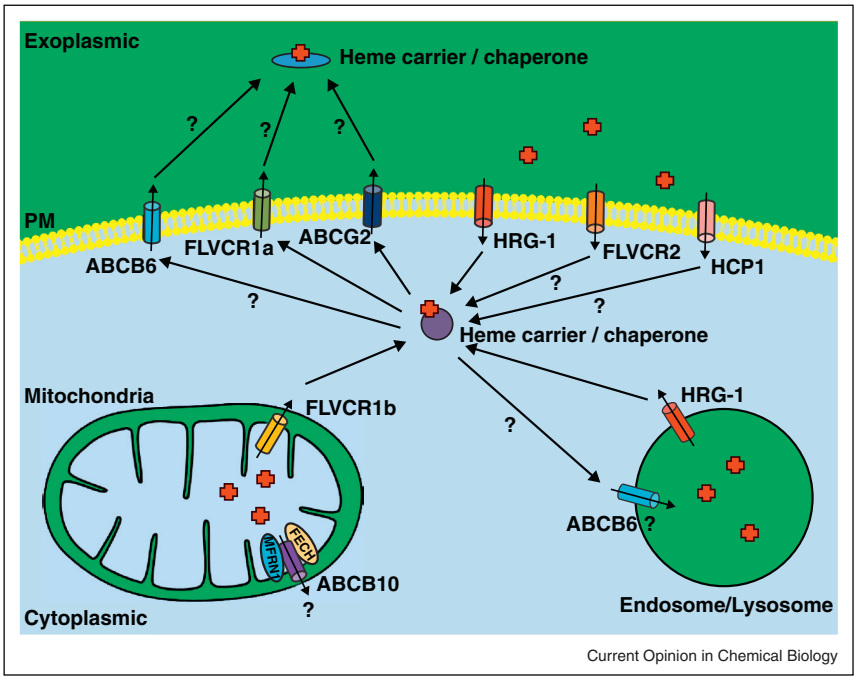
Heme is a more readily bioavailable iron source and contributes to two-third of body iron, even though heme constitutes only a third of total dietary iron [9,10]. In mammals, dietary heme is apparently taken up intact by enterocytes in the intestine. However, heme is a large amphipathic porphyrin and free heme can be cytotoxic. Thus, specific molecules and pathways are required for heme uptake and trafficking (Figure 1).

HRG-1

Rao *et al.* have demonstrated that the roundworm *Caenorhabditis elegans* is a unique model for heme trafficking studies because even though it is a heme auxotroph it acquires dietary heme via the intestine and subsequently disseminates heme throughout the organism for viability [11]. Genomic screens in *C. elegans* identified CeHRG-1 and CeHRG-4 as the first *bona fide* eukaryotic heme importers [12••]. CeHRG-1 has orthologs in vertebrates, while CeHRG-4 is worm-specific. Transient knockdown of *hrg-1* in zebrafish resulted in hydrocephalus, yolk tube malformations and severe anemia. Worm HRG-1 fully rescued all phenotypes observed due to knockdown of *hrg-1* in zebrafish [12••]. The phenotypes resulting from knockdown of zebrafish *hrg-1* were restricted specifically to the erythroid lineage and did not impact other hematopoietic cell lineages. Additionally, significant heme-induced inward currents were observed in *Xenopus* oocytes injected with cRNA for CeHRG-1, CeHRG-4, and the human homolog, hHRG-1, indicating heme-dependent transport across cell membranes [12••].

Human *HRG-1* (*SLC48A1*) mRNA was abundant in the brain, kidney, heart and skeletal muscle and in cell lines derived from duodenum, kidney, bone marrow and brain [12••]. hHRG-1 localized to acidic endosomal and lysosomal organelles in HEK293 cells, and its affinity for heme decreased with increasing pH. Additionally, tyrosine (YxxxØ) and acidic-dileucine (DxxIL) based sorting motifs were found in the C-terminus of both *C. elegans* and

Figure 1



A schematic description of known heme transporters. HRG-1 is a heme importer that localizes to endosomal/lysosomal compartments, but can traffic to the plasma membrane. HCP1 and FLVCR2 are two putative heme importers. The cell surface FLVCR1a and the ABC transporter ABCG2 have been implicated in heme export in erythroid cells, whereas the mitochondrial isoform FLVCR1b transports heme into the cytosol. ABCB6 was previously proposed to be a mitochondrial porphyrin/heme importer, but has recently been shown to localize to the plasma membrane and endosomal/lysosomal vesicles. ABCB10 forms a complex with MFRN1 and FECH, and stabilizes MFRN1. It is not clear whether ABCB10 transport heme. Heme carrier/chaperone that is responsible for intracellular and intercellular heme trafficking remains unknown. Question marks represent the presumptive heme trafficking pathways. PM, plasma membrane.

human HRG-1 [12••]. Yanatori and colleagues recently reported hHRG-1 localized to the plasma membrane and lysosomes in non-polarized HEp2 cells. In polarized MDCK cells, hHRG-1 was located to the basolateral membrane and a cytosolic organelle just under the apical membrane [13]. A recent study showed that hHRG-1 interacted with the c subunit of the vacuolar proton ATPase (V-ATPase) pump and enhanced endosomal acidification [14]. Together these studies suggest hHRG-1 plays a role in the transport of heme from the exoplasmic space or lumen of acidic endosome–lysosome compartments into the cytoplasm.

Interestingly, in addition to lysosomal localization in HEK293 cells, hHRG-1 is also recruited and colocalizes with Nramp1 at the erythrophagosomal membrane, surrounding ingested RBCs in bone marrow derived macrophages (BMDMs) [15]. However, the absence of HO-1 at this location indicates that during EP, at least a portion of heme released from degraded hemoglobin is mobilized by hHRG-1 to the cytoplasm [15]. The cytosolic heme can then undergo intracellular redistribution including degradation by HO-1 for iron recycling, or be exported by heme effluxers. Indeed, a recent study shows that HRG1 is essential for macrophage iron homeostasis and transports heme from the phagolysosome to the cytoplasm during EP [16••]. 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 suggesting that HRG1 is the heme transporter for heme-iron recycling in macrophages. The study proposes that genetic variation in *HRG1* may be an important genetic determinant in inherited iron disorders in humans [16••]. HRG-1, as observed for HO-1, was recently identified as a target of the heme-regulated transcription factor BACH1 in microarray expression analysis and ChIP-Seq experiments, further suggesting that HRG-1 may be an important player in erythropoiesis and the phagolysosomal heme transporter [17].

HCP1

Heme carrier protein 1 (HCP1/SLC46A1) is a membrane protein expressed by enterocytes in the duodenum implicated in the absorption of heme in the intestine [18]. Ectopically overexpressing *HCP1* in *Xenopus* oocytes revealed a 2–3-fold increase in heme uptake. Subsequent

studies, however, have shown that folate transport by this protein was at least 10-fold higher than that observed with heme, suggesting that folate might be the physiological ligand for HCP1. In addition, a missense mutation in *HCP1* in human that leads to the formation of a non-functional protein has been found to be associated with hereditary folate malabsorption. Thus, SLC46A1 is in fact a folate/proton symporter and was renamed as the Proton Coupled Folate Transporter (PCFT) [19•]. Interestingly, *HCP1* knockout mice displayed severe macrocytic normochromic anemia, which could be a secondary effect of folic acid deficiency. *HCP1* deficient erythroblasts failed to differentiate and had a higher apoptosis rate [20]. RNA interference assays of *HCP1* in CaCo-2 cells reduced both heme and folate uptake but increase HO-1 expression, suggesting *HCP1* could potentially function as a low affinity heme transporter [21].

FLVCR2

Feline leukemia virus subgroup C receptor 2 (FLVCR2), a member of the major facilitator superfamily, was recently reported to import heme in mammalian cells [22]. Knockdown of *FLVCR2* in human cells significantly decreased uptake of the fluorescent heme analog, zinc mesoporphyrin (ZnMP). However, unlike HRG-1, ectopically expressing FLVCR2 in yeast did not rescue heme dependent growth or import heme under the assay conditions [23]. Given the high degree of homology between FLVCR2 and the heme effluxer FLVCR1 [24,25,26••], it is possible that FLVCR2 may efflux heme. *FLVCR2* is expressed in a broad range of human tissues including the fetal liver, brain and kidney [22]. Recent studies have associated FLVCR2 with Fowler syndrome, a vascular disorder of the brain [27,28]. Currently, a direct physiological role for FLVCR2 in erythropoiesis is unclear.

Heme export

In humans, macrophages phagocytose over 360 billion senescent RBCs and recycle more than 25 mg of iron daily. The heme released from hemoglobin is either degraded by HO-1 to release iron for iron recycling or potentially exported as intact heme [29]. Heme export may serve as a potential detoxification strategy to prevent excess heme from accumulating, a phenomenon that could lead to tissue and cellular damage. Two heme exporters, FLVCR1 and ATP-binding cassette transporter G2 (ABCG2) have been implicated in heme export in humans.

FLVCR1

Quigley *et al.* identified FLVCR1 as a cell surface heme exporter, belonging to the major facilitator family of transmembrane transporters [26]. FLVCR1 is expressed in different hematopoietic cells and showed weak expression in the fetal liver, pancreas and kidney [30]. Ectopically expressing FLVCR1 can reduce intracellular heme levels and mediate efflux of ZnMP in rat renal epithelial and hematopoietic K562 cells [26]. FLVCR1-null mice fail to undergo erythropoiesis and die at midgestation [31]. These mice also exhibit cranio-facial and limb deformities reminiscent of patients with Diamond-Blackfan anemia (DBA), a severe but rare congenital erythroid anemia that presents in infancy. The current model postulates that FLVCR1 exports heme when macrophages phagocytose senescent RBCs. FLVCR1 interacts with the extracellular heme-binding protein hemopexin and exports heme at least 100-fold more efficiently in the presence of hemopexin [32]. FLVCR1 has a narrow substrate range including heme, protoporphyrin IX and coproporphyrin, but not bilirubin, the primary breakdown product of heme. Alves *et al.* recently showed that in nucleated RBC precursors (NRBC) from human bone marrow, FLVCR1 expression increased during erythropoiesis and reached maximal level at the intermediate stage of maturation, under condition in which the HO system was disrupted [33]. The authors suggest that FLVCR1 may export excess heme to prevent heme toxicity under conditions in which heme degradation (HO?) is not fully induced.

Interestingly, Tolosano *et al.* recently showed that in addition to full length FLVCR1 (FLVCR1a), there is another isoform, FLVCR1b, which is a smaller protein that localizes to the mitochondria [34•]. Overexpression of FLVCR1b increases cytosolic heme, whereas knockdown of FLVCR1b results in mitochondria heme accumulation, indicating that FLVCR1b is a mitochondrial heme exporter. In contrast with previous findings, targeted deletion of FLVCR1a resulted in skeletal defects and vascular abnormalities but did not affect erythropoiesis, whereas knockdown of FLVCR1b impaired erythroid differentiation *in vitro*. These results suggest that FLVCR1a is dispensable for definitive erythropoiesis, and the failure in erythropoiesis in FLVCR1-null mice may due to cytosolic heme deficiency rather than toxicity associated with excess heme in the cytosol [34•].

Recently, four missense mutations in FLVCR1 have been found in patients with the rare autosomal recessive disease posterior column ataxia and retinitis pigmentosa (PCARP) [35,36]. All of the four mutations diminish FLVCR1 heme export activity and mislocalize FLVCR1 to intracellular structures, including lysosomes [37]. Three of the four mutations occur in exon 1 of the gene and are FLVCR1a specific. However, none of the patients carrying these gene variations is anemic. The physiological function of FLVCR1a therefore remains unknown [36].

ABCG2

ABCG2, also known as breast cancer resistance protein (BCRP) has been identified as a second heme exporter in mammals [38]. ABCG2 is expressed in a wide range of tissues including hematopoietic stem cells and erythroid

progenitors. Whereas FLVCR1 is highly expressed during erythropoiesis, the expression level of ABCG2 is particularly high in the early stages of hematopoiesis [39,40]. ABCG2 binds to heme directly through the extracellular loop 3 (ECL3) which constitutes a porphyrin-binding domain [39]. Although there is no direct evidence demonstrating that ABCG2 exports heme, ectopically expressed ABCG2 exports ZnMP in K562 cells [39]. As observed in FLVCR1-mediated heme export, ABCG2 possibly exports and transfers heme to extracellular heme-binding proteins, such as albumin [41]. However, unlike FLVCR1, ABCG2 has a broad range of substrates including porphyrin and nonporphyrin substrates, suggesting that ABCG2 may not be a functional backup of FLVCR1. Human patients carrying null mutations in ABCG2 are defined as the Jr(a−) blood group with no apparent defects in erythropoiesis [42,43].

Since both FLVCR1a and ABCG2 appear to be dispensable for erythropoiesis, it raises the question of whether a third heme exporter exist that is essential during erythropoiesis. Severance *et al.* recently performed genome-wide analysis and identified two genes, *MRP-5* and *F22B5.4* that were involved in heme transport in *C. elegans* [44•]. As opposed to the heme importer *HRG-4*, knockdown of both *MRP-5* and *F22B5.4* resulted in ZnMP accumulation in the worm intestine [44]. *MRP-5* is the homolog of human ABC transporter *ABCC5*, and could possibly be a candidate heme exporter in humans.

Intracellular heme trafficking

Membrane proteins

ABCB6 is a mitochondrial ABC transporter that localizes to the mitochondrial outer membrane. ABCB6 was first identified as an iron transporter but later proposed to be a porphyrin/heme importer [45]. Recently, Polireddy *et al.* reported ABCB6 interacts directly with heme in hemin–agarose binding assays, and high-throughput screens identified compounds that competed ABCB6-mediated heme transport in isolated mitochondria [46]. In mice or humans, however, loss of ABCB6 function is not associated with blood defects, even though the expression profile of ABCB6 mimics that of other heme biosynthesis genes [47]. Indeed, recent studies have shown that ABCB6 is dispensable for erythropoiesis and instead of mitochondria, it is localized in the endosomal/lysosomal compartment and in the plasma membrane of mature erythrocytes [48,49]. Two missense mutations in the ABCB6 gene in humans have been associated with Familial Pseudohyperkalemia (FP), which increases leakage of potassium from RBCs [50]. In addition, an L811V mutation of ABCB6 causes ocular coloboma, a developmental defect in the closure of the optic fissure [47].

ABCB10 is another mitochondrial ABC transporter located in the inner mitochondrial membrane. ABCB10 interacts with mitoferrin1 (MFRN1) and ferrochelatase (FECH) and stabilizes the complex [51]. Hyde *et al.* recently reported that ABCB10 is essential for erythropoiesis *in vivo*. ABCB10 null mice displayed deficiency of primitive erythropoiesis and lack of hemoglobinized cells [52]. However, it is unclear whether heme is the *bona fide* substrate of ABCB10.

Cytosolic proteins

Free heme is a cytotoxic molecule that generates reactive oxygen species and disrupts lipid bilayers and organelles. As a potent hemolytic agent, free heme can alter the conformation of cytoskeletal proteins in RBCs. Consequently, cytosolic heme carriers or 'chaperones' that function to sequester or transport heme are an essential component of heme homeostasis — required for heme detoxification or incorporation into hemoproteins.

Currently, several intracellular heme-binding proteins have been found, including glutathione S-transferases (GSTs), heme-binding proteins (HBPs), and fatty acid binding proteins (FABPs) (Table 1). GSTs are abundant cytosolic proteins that have been shown to bind hemes and porphyrins. Although GSTs are important for heme detoxification in malaria parasites and helminths, little is known regarding their function in mammalian heme trafficking [53,54]. HBPs are proteins that have higher affinity to heme (p22HBP: K_d = 26 nM, HBP23: K_d = 55 nM) and tetrapyrroles than GSTs and FABPs (K_d = 100–200 nM) [55,56]. The expression of p22HBP is induced during erythroid differentiation in mouse erythroleukemia (MEL) cells, whereas the expression of HBP23 is induced by heme, PPIX, and other metalloporphyrins in rat primary hepatocytes or by oxidant stress in peritoneal macrophages [55–57]. Future studies are required to determine if HBPs act as genuine heme carriers/chaperones or they simply scavenge tetrapyrroles. Recently, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was reported to bind heme and with a preference for Fe(III) and Co(III)-protoporphyrin IX analogs, but does not bind Zn-free or the metal-free protoporphyrin IX [58]. It has been shown that GAPDH is required for heme insertion into a soluble hemoprotein — the inducible nitric oxide synthases (iNOS) [59]. Whether GAPDH plays a role as a heme chaperone for other proteins, in addition to its traditional function in glycolysis, needs further investigation. The molecular chaperone HSP90 is also reported to mediate heme insertion into NOS and another hemoprotein, soluble guanylyl cyclase (sGC) [60,61]. However, since HSP90 is a highly conserved molecular chaperone with a wide array of client proteins, its specific role in heme homeostasis is unclear [62].

Intercellular heme trafficking

To date, several extracellular heme binding proteins have been identified in mammals, including hemopexin, haptoglobin and human serum albumin (HSA). However,

Table 1

Putative heme transporters and heme binding proteins

Protein	Proposed function	Localization	Heme binding affinity	Blood disorders	Reference
HRG-1 (SLC48A1)	Import heme	PM, endosome/ lysosome		Inherited iron disorders?	[12]
PCFT/HCP1	Import heme?	PM	K_m = 125 μM (heme) K_m = 1.3–56 μM (folate)	Hereditary familial folate malabsorption, folate-responsive anemia in newborns	[18,19]
FLVCR2	Import heme?	PM		Fowler syndrome, lethal cerebral vasculopathy	[22,23]
FLVCR1a	Export heme	PM		Posterior column ataxia and retinitis pigmentosa?	[26]
FLVCR1b	Export heme	IM		Diamond-Blackfan anemia?	[34]
ABCG2/BCRP	Export heme	PM		Increased serum urate level, resulting in gout	[41]
ABCB6	Transport coproporphyrinogen III, heme?	OM? PM, endosome/ lysosome		Familial pseudohyperkalemia, ocular coloboma	[45,49]
ABCB10	Stabilize Mfrn1, transport heme?	IM		KO mice displayed deficiency of primitive erythropoiesis	[51,52]
p22HBP	Bind heme/tetrapyrroles	Cytosol	K_d = 26 nM		[56]
HBP23	Bind heme/tetrapyrroles	Cytosol	K_d = 55 nM		[55]
GST	Bind heme/tetrapyrroles	Cytosol	K_d = 100–200 nM		[66]
FABP	Bind heme/tetrapyrroles	Cytosol	K_d = 150 nM		[67]
GAPDH	Bind heme, heme insertion into iNOS	Cytosol	K_d = 24 nM		[58,59]
HSP90	Heme insertion into NOS, sGC	Cytosol			[60,61]
Hemopexin	Bind extracellular heme	Blood	K_d < 1 pM		[63]
Haptoglobin	Bind extracellular hemoglobin	Blood	K_d < 1 pM		[63]
Human serum albumin	Bind extracellular heme	Blood	K_d = 5 nM		[68]
HRG-3	Bind heme	Extracellular in *C. elegans*			[65••]

PM, plasma membrane; OM, mitochondrial outer membrane; IM, mitochondrial inner membrane; KO, knockout.

none of them has been shown to directly contribute to erythropoiesis [63]. Studies have shown that a portion of heme released from degraded RBCs in macrophage is exported as intact heme during EP, possibly through FLVCR1 or an unknown heme exporter [29,31]. This raises the possibility that heme maybe trafficked between cells and tissues. Mice that are mutant for heme synthesis are viable till embryonic day 8.5, indicating potential intercellular heme transport from a maternal source [64].

In *C. elegans*, maternal heme levels have a direct impact on embryonic development. Chen *et al.* recently demonstrated HRG-3, a secreted worm protein, is responsible for delivering heme from maternal intestine to the developing embryo [65••]. HRG-3 binds heme at a stoichiometry of two protomers to one heme. Although, deletion of HRG-3 had no visible effect on adult worms under low heme conditions, their progeny were either unable to hatch or growth arrested at the first larval stage. This phenotype could be rescued by ectopic expression of HRG-3 in maternal intestine, but not by embryonic specific expression [65••]. Even though a functional homolog of HRG-3 in mammals has not been discovered, it is reasonable to speculate that during development and erythropoiesis, a heme chaperone may be required to facilitate the targeted delivery and redistribution of heme between certain tissues and cell types.

Conclusion

In addition to the well characterized heme biosynthetic pathway, intracellular and intercellular heme trafficking pathways play an essential role in maintaining systemic heme homeostasis. Emerging studies have discovered several components in heme transport, yet their relationship with human blood disorders remains unclear and sometimes contradictory. Many issues remain unresolved including the function of HRG-1 in intestinal heme absorption and genetic iron disorders, the physiological function of FLVCR paralogs (FLVCR1a, 1b and FLVCR2), and the identity of intracellular and intercellular heme chaperones. Tackling these questions will undoubtedly provide a deeper understanding of the chemical role of heme and heme trafficking networks crucial for the genesis of RBCs.

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