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Review

Control of intracellular heme levels: Heme transporters and heme oxygenases

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ABSTRACT

Heme serves as a co-factor in proteins involved in fundamental biological processes including oxidative metabolism, oxygen storage and transport, signal transduction and drug metabolism. In addition, heme is important for systemic iron homeostasis in mammals. Heme has important regulatory roles in cell biology, yet excessive levels of intracellular heme are toxic; thus, mechanisms have evolved to control the acquisition, synthesis, catabolism and expulsion of cellular heme. Recently, a number of transporters of heme and heme synthesis intermediates have been described. Here we review aspects of heme metabolism and discuss our current understanding of heme transporters, with emphasis on the function of the cell-surface heme exporter, FLVCR. Knockdown of *Flvcr* in mice leads to both defective erythropoiesis and disturbed systemic iron homeostasis, underscoring the critical role of heme transporters in mammalian physiology.

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1. Introduction

Porphyryns are heterocyclic organic rings composed of four pyrrole subunits that are usually linked by methine bridges; their conjugation to diverse divalent metal ions such as Mg^{2+} , Co^{+} and Fe^{2+} gives rise to the “pigments of life”, i.e., chlorophyll, vitamin B12 and heme, respectively. Heme, a complex of iron with protoporphyrin IX, is ubiquitous in aerobic cells and essential for cellular oxidation–reduction reactions. It serves as a critical component of hemoproteins, a large group of proteins that includes cytochromes (for mitochondrial respiratory chain electron transfer and drug metabolism), oxidases (e.g., NADPH oxidase) and peroxidases, catalases and synthases (e.g., nitric oxide synthase, NOS), as well as the oxygen storage and transport molecules, myoglobin and hemoglobin (Hb) (for reviews see [1,2]). In addition, two-thirds of Western dietary iron intake is derived from dietary heme [3], while the majority of iron in the body is present in the form of heme (as Hb, myoglobin and cytochromes [4]).

In single-cell organisms the primary importance of heme derives from its role as a source of iron and as a critical component of enzymes of the respiratory chain. Its regulated production and use appears to be intimately related to the presence of oxygen, providing the cell with the ability to exploit the benefits of oxidative metabolism [5,6]. Thus it is not surprising that heme (like Mg^{2+} -protoporphyrin in plants [7]) has itself

evolved to serve as a fundamental cell regulatory molecule in single-cell and more complex organisms. In mammals heme has pleiotropic functions, but its underlying relationship to oxygen and oxidative metabolism remains central. For example it is intimately involved in the regulation of erythropoiesis, regulating erythroid gene transcription—e.g., nuclear heme inhibits repression of the globin locus by the transcription factor Bach1, likely through direct binding [8]; erythroid gene translation, through inhibition of an erythroid-specific eIF-2 α kinase (HRI) that regulates protein, especially globin translation [9,10]; and heme synthesis protein targeting and stability [11,12]. Recently, the role of heme in other fundamental cell processes – e.g., the regulation of microRNA processing [13], circadian rhythm [14] and ion-channel functions [15] – has been described. In addition, heme appears to promote specific gene expression patterns in mammalian adipose, neuronal and erythroid cells, inducing their differentiation [16–20]. Finally, in addition to its redox ability in electron transfer (e.g., the respiratory chain hemoproteins), iron compartmentalization function and aforementioned regulatory roles, heme also binds NO, CO and O_2 , allowing hemoproteins to serve as gas sensors and signal transducers [21,22].

The observations outlined above would seem to argue for a physiological role for a “free” or “uncommitted” heme pool, i.e., intracellular heme that is not a component of hemoproteins. However, free heme is lipophilic and toxic to cells, promoting lipid peroxidation [2,23] and the production of reactive oxygen species (ROS) [24], resulting in membrane injury and cell apoptosis [2]. Therefore, both from the viewpoint of its toxicity and its regulatory function, the intracellular levels of free heme must be tightly controlled (estimated at $<0.1 \mu M$; reviewed in [2,25]). Like zinc in prokaryotes [26], “free heme” has been proposed to be chaperoned by

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proteins within cells [27]. Putative intracellular heme carrier proteins include glutathione S-transferase B (ligandin, [28]), Z-type-fatty acid-binding protein [29], heme-binding protein 22 (p22HBP, [30]) and HBP23, [31], and, more recently, biliverdin reductase (BVR) [32]. However, the specific proteins responsible for the safe carriage of heme within cells remain for the most part unidentified.

Control of intracellular heme levels was previously thought to occur through a balance among its mitochondrial biosynthesis, utilization by hemoproteins, and catabolism by heme oxygenases (HO) (predominantly by the heme-inducible HO-1) [1]. However, studies describing transporters of heme and heme synthesis intermediates indicate further layers of complexity in heme homeostasis. Being lipophilic, heme readily associates with cell and organelle membranes; yet, the presence of anionic carboxylate side chains, responsible for heme binding to heme carrier proteins, such as albumin and hemopexin (Hpx), limits transmembrane diffusion [33], indicating the need for specific transporters, as supported by a number of studies [34–38] (see also [39,40] for recent reviews).

In recent years a number of these heme transport proteins have been identified. The purpose of this review is to describe relevant aspects of heme synthesis and catabolism and then outline our current understanding of heme transporters. We discuss heme transporters that function at different sites within the cell, with emphasis on the role of the plasma membrane heme exporter FLVCR. Table 1 summarizes our current knowledge of these transporters.

2. Heme synthesis and transport of heme synthesis intermediates

The first of eight steps in heme synthesis (Fig. 1; for comprehensive reviews see [41–43]), mediated by the enzyme ALA synthase 2 (ALAS2) in erythroid cells or by the heme-repressible enzyme ALAS1 in non-erythroid cells, is the condensation of succinyl co-enzyme A and glycine to form 5-amino-levulinic acid (ALA), which occurs within the mitochondrial matrix. ALA is then transported across both mitochondrial inner and outer membranes (IM and OM) into the cytosol. Patients with mutations of ALAS2 develop sideroblastic anemia, a hematological disorder characterized by mitochondrial iron overload and impaired heme synthesis [44], while accumulation of substrates related to defects in the remaining pathway enzymes results in porphyrias, disorders characterized by either acute neurovisceral attacks or photosensitivity or sometimes both (reviews, [43,45]). Intriguingly, a recent study identified mutations of the solute carrier SLC25A38, a mitochondrial IM transporter, as a frequent cause of inherited sideroblastic anemia [46]. Deletion of the yeast ortholog decreases heme synthesis, which is rescued by supplementing the culture media with glycine or ALA. Yeast cytosolic levels of ALA were reduced 6.7-fold compared to controls, leading the authors to propose that SLC25A38 functions to facilitate ALA production by transporting glycine into the matrix or exchanging glycine for ALA across the IM.

In the cytosol the cyclic tetrapyrrole, coproporphyrinogen III (CPgenIII), is formed from ALA in a series of four enzymatic reactions.

Table 1

Transmembrane proteins implicated in the transport or biosynthesis of heme.

Protein	Description	Function	Localization	Disorders	Knockout	Reference
FLVCR	MFS transporter. FeLV-C viral receptor. Virus cause anemia in cats.	Exports heme (and PPIX). Does not transport bilirubin.	Plasma membrane.	Diamond Blackfan anemia? Posterior column ataxia and retinitis pigmentosa	Mouse KO is embryonic lethal. Early erythroid progenitor arrest and systemic iron overload in conditional KO.	[36,37,189]
ABCG2/BCRP	ABC family member. Confers multidrug resistance in breast cancer cell line.	Potentially exports heme. Exports (conjugated) PPIX, pheophorbide A, urate.	Plasma membrane.	Increased serum urate level, resulting in gout.	Mouse KO have 10-fold elevated PPIX in RBCs and in plasma. No hematologic abnormality, no evidence of iron overload.	[229,235,236,238]
ABCB6	ABC family member. Functionally complements yeast <i>Atm1p</i> mutant which accumulates iron in mitochondria.	Unknown, suggested may transport mitochondrial CPgen III.	Mitochondrial OM, plasma membrane, golgi, endosome/lysosome.		Mouse KO generated by Schuetz group (unpublished).	[47,58]
ABCB10	ABC family member. GATA-1 regulated.	Transport substrate unknown. Interacts with <i>Fech</i> and <i>Mfrn1</i> . Stabilizes <i>Mfrn1</i> to allow increased mitochondrial iron uptake during erythropoiesis.	Mitochondrial IM.		Mouse KO embryonic lethal. Arrested erythroid differentiation, increased mitochondrial oxidative stress.	[67,70,71]
ABCB7	ABC family member. Human ortholog of yeast <i>Atm1p</i> which accumulates iron in mitochondria.	Interacts with <i>Fech</i> . Cytosolic Fe-S protein maturation, iron homeostasis.	Mitochondrial IM.	Hereditary X-linked sideroblastic anemia and cerebellar ataxia	Mouse conditional KO has defective hematopoiesis, sideroblastic-like anemia, ataxia and hepatocellular iron deposits.	[75–77,79]
PCFT/HCP1	MFS transporter. Proton coupled folate transporter/ Heme carrier protein isolated from hypotransferrinemic mouse duodenal tissue.	Primarily a folate transporter. Physiological role in duodenal heme import unclear	Plasma membrane. Endosome?	Hereditary familial folate malabsorption, folate-responsive anemia in newborns	Mouse KO has increased Nhomocysteine protein levels.	[139,241,249]
HRG-1	Transporter with 4 predicted transmembrane domains. Heme regulated gene in <i>C. elegans</i> . Ortholog present in humans.	Exports heme from lysosome/endosome into cytoplasm.	Endosome/ Lysosome.		Transient knockdown of zebrafish ortholog results in hydrocephalus, yolk tube malformations and a profound defect in erythropoiesis.	[150,248]
HRG-4	Transporter with either 3 or 5 predicted transmembrane domains. Heme regulated gene in <i>C. elegans</i> , no mammalian orthologs.	Imports heme.	Plasma membrane.			[150,248]

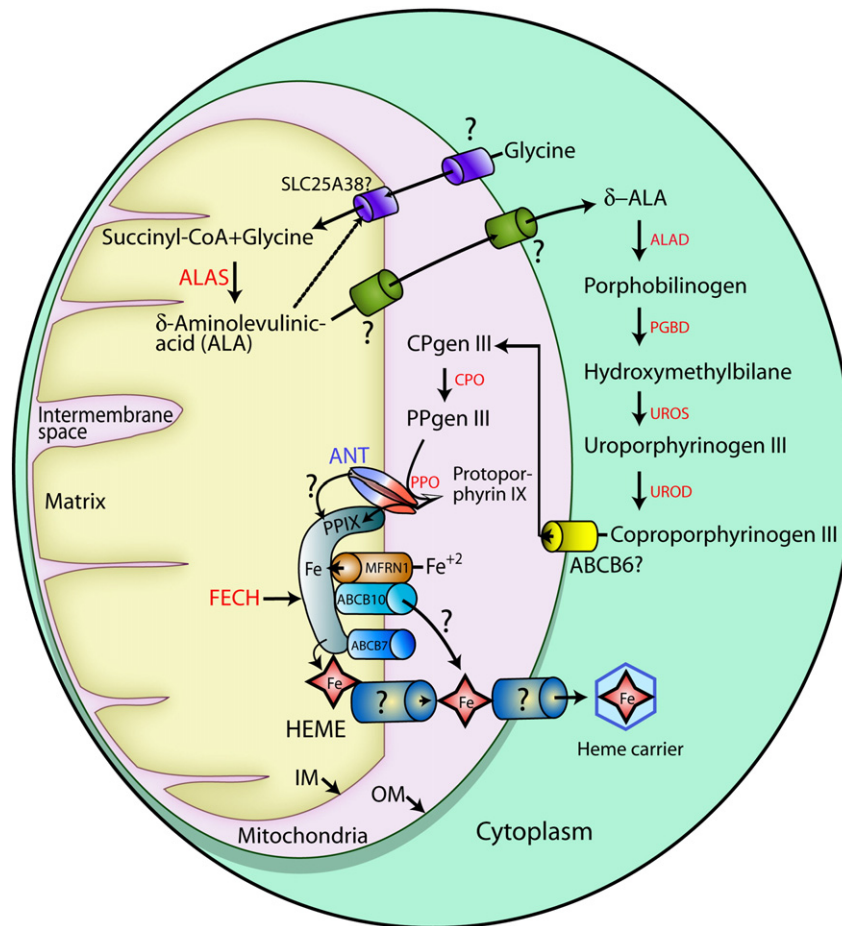


Fig. 1. The heme biosynthesis pathway and putative mitochondrial transporters. The first step of heme synthesis occurs in the mitochondrial matrix with the condensation of succinyl-CoA and glycine by ALAS to generate ALA. ALA may be exported in exchange for glycine by an IM transporter SLC25A38. The mechanism of ALA export across the OM into the cytoplasm is unknown. ALA is converted to CPgenIII by four enzymatic reactions. CPgenIII is then transported back into the mitochondrial intermembrane space (IMS), possibly via ABCB6, where it is converted to PPgenIX by CPO. The conversion of PPgenIX to PPIX by PPO, its transport into the matrix and the addition of Fe^{+2} by ferrochelatase (FECH) to generate heme are coupled processes, with transport of PPIX likely occurring via a proposed direct channeling through a complex of PPO–FECH (likely part of a larger complex with the mitochondrial iron importer, Mitoferrin, ABCB7 and ABCB10). The transfer of heme from the matrix to the IMS, for respiratory chain cytochrome assembly, may be mediated by ABCB10; the transporter responsible for its transfer across the outer mitochondrial membrane remains unidentified. See text for details.

How the heme synthesis intermediates are transferred from one cytosolic enzyme to the next in the pathway is at present unknown, but a macrocomplex comprising all four cytosolic enzymes, as has been proposed for the terminal pathway enzymes (see below), may occur. CPgenIII is then transported back across the OM into the mitochondrial intermembrane space (IMS). Krishnamurthy et al. suggest that an ATP-binding cassette transporter ABCB6 is involved in porphyrin transport across the OM [47] (reviewed in [48]).

Cell surface ABC transporters serve to efflux a variety of compounds, including xenobiotics in eukaryotic cells [49,50]. In addition, family members are involved in mitochondrial transport [51]. These transporters have a characteristic structure consisting of two transmembrane domains (TMD) and two intracellular nucleotide-binding domains (NBD). Energy derived from hydrolysis of ATP by the NBD provides energy for the uphill movement of substrates across membranes. Approximately 50 ABC transporters have been identified in the human genome and classified into seven subfamilies (A–G) based on the structure of their ATP-binding domains.

ABCB6 was initially identified as a mitochondrial protein that functionally complements the *Saccharomyces cerevisiae atm1-1* mutant [52] that encodes Atm1p, a yeast mitochondrial IM protein believed to be involved in the biogenesis of cytosolic Fe–S clusters [53]. Fe–S clusters are modular protein cofactors consisting of iron and sulfur, which are usually linked by bonds joining the cysteine sulfur atoms of a polypeptide protein to iron atoms of the cluster. Fe–S

clusters function as part of enzyme catalytic centers (e.g., aconitase, succinate dehydrogenase), in chemical sensing, electron transfer and generation of radicals [54–56]. Similar to mutations of other genes involved in eukaryotic Fe–S cluster biosynthesis (e.g., human *fxn* and *glrx5*; see [56] for review) *Atm1* mutants accumulate free iron in the mitochondria and have depressed mitochondrial respiratory function, which is almost completely reversed by expression of human ABCB6 [52,57]. However, unlike its mitochondrial IM paralog ABCB7, ABCB6 is localized to the OM in the human erythroid cell line K562 [47,58] (although exclusive mitochondrial localization of ABCB6 in other cell types has been disputed; see [59–61]), and studies demonstrate conclusively that ABCB7 is the true *Atm1* ortholog [62]. Nonetheless, ABCB6 is upregulated by hemin or DMSO-induced differentiation of the murine erythroid cell line MEL cells [47] and a recent large-scale expression analysis of genes associated with heme biosynthesis also identified ABCB6 [63]. ATP- and temperature-dependent inward transport of ^{55}Fe -hemin into mitochondria isolated from K562 cells overexpressing ABCB6 has been demonstrated [47]; inward transport (although non-physiological) is consistent with the presence of the nucleotide-binding domain in the cytosol. The oxidized form of the physiological heme synthesis intermediate CPgenIII, coproporphyrin III (CPIII) competes with mitochondrial ^{55}Fe -hemin uptake and dissociates ABCB6 from hemin-agarose [47]. However CPIII is not a heme synthesis pathway intermediate and it remains to be established whether ABCB6 functions in CPgenIII uptake.

A further two oxygen-dependent enzymatic steps, mediated by the mitochondrial IMS enzyme, coproporphyrinogen oxidase (CPO) and protoporphyrinogen oxidase (PPO), are required to convert CPgenIII into protoporphyrin IX (PPIX); the latter was proposed to be transported across the IM into the matrix by adenine nucleotide translocator (ANT), without requiring ATP energy [64]. However, because the enzymes PPO and ferrochelatase (Fech), catalyzing respectively the penultimate and final steps of heme synthesis, are both localized at the mitochondrial IM–PPO is an IMS-facing protein while Fech is exposed to the matrix—a channeling of PPIX and PPIX through a complex formed by PPO and Fech within the IM is more likely, based on biochemical studies [65] and the crystal structure of the two enzymes [66]. The final step, the addition of one atom of Fe^{2+} to PPIX by Fech that results in the formation of protoheme or heme *b* occurs on the matrix side of the IM, necessitating its transfer, by as yet unidentified transporters, across both the IM and OM again in order to reach the cytosol.

Recent studies indicate that Fech interacts with mitoferrin-1 (Mfrn1)—a mitochondrial IM protein involved in mitochondrial iron uptake that is upregulated during erythroid differentiation [67]. Like ALAS, there are two forms of Mfrn in zebrafish and mammals, Mfrn1 and Mfrn2, respectively, with Mfrn1 uniquely important for iron import in developing erythroid cells [68]. Elegant studies subsequently showed that Mfrn1 but not Mfrn2 was stabilized in a post-translational manner during erythroid differentiation [69]. Chen et al. then isolated an IM ABC transporter, ABCB10 (formerly ABC-me; [70]), among a number of proteins that were precipitated with Mfrn1 isolated from mitochondria derived from MEL cells undergoing erythroid differentiation. Their studies demonstrate that ABCB10 stabilizes Mfrn1 during erythroid differentiation, to allow for high efficiency iron uptake into mitochondria for optimal heme synthesis [71]. Notably, in undifferentiated MEL cells, forced expression of *Abcb10* and *Mfrn1* enhances Mfrn1-mediated ^{59}Fe uptake by mitochondria approximately 2-fold, but has no effect on mitochondrial ^{59}Fe -heme levels. It was proposed that this lack of heme synthesis was due to an inadequate supply of protoporphyrin precursors [71]; however an accompanying commentary speculated that Fech levels may be limiting and that the enzyme formed part of a macromolecular complex with Mfrn1 and ABCB10 [72]. In fact, further studies have shown that the expression of all three proteins is co-induced during MEL cell erythroid differentiation, whereas analysis of the higher order protein complexes of Mfrn1 during differentiation indicates that Mfrn1 and ABCB10 transiently interact with Fech [73]. Previous studies had shown that MEL cells transfected with *Abcb10* undergoing erythroid differentiation had enhanced hemoglobin synthesis (2–4-fold; [70,74]). Yet overexpression of *ABCB10* alone does not initiate erythroid differentiation or alter the timing of hemoglobin synthesis upon differentiation. It would be interesting to discern whether the structural role of ABCB10 in stabilizing Mfrn1 is sufficient to produce enhanced Hb levels or whether ABCB10 transport function is required. The potential substrate of ABCB10 has not been identified, but heme is a possible candidate. Mfrn1 is found in higher-order protein complexes than those predicted from a macromolecular complex of Mfrn1–ABCB10–Fech suggesting that yet unidentified proteins are also part of a multi-protein complex at the IM. Chen et al. speculate that PPO and perhaps CPO are also present in this complex to allow coordinated supply of PPIX and Fe^{2+} to Fech for heme synthesis [73] (and perhaps coordinated heme transport by ABCB10).

In addition to its use for heme synthesis, mitochondrial iron is also utilized for mitochondrial Fe–S cluster biogenesis. Fech possesses a [2Fe–2 S] cluster that is not required for catalytic function, but the enzyme is sensitive to the availability of Fe–S clusters. Rouault's group has recently shown that when Fe–S cluster synthesis is impaired, due to deficiency of proteins required for Fe–S cluster biosynthesis or iron deficiency, apo-Fech is rapidly degraded in the mitochondria, potentially providing a direct link between Fe–S cluster and heme

synthesis [75]. As mentioned, the mammalian IM transporter ABCB7 is the yeast *Atm1* ortholog and appears to be important for cytosolic but not mitochondrial Fe–S cluster biogenesis [76]. Mutations in the human *Abcb7* gene result in defective cytosolic Fe–S-containing protein maturation and mitochondrial iron overload, leading to an X-linked congenital sideroblastic anemia with ataxia [77,78]. Prior to the identification of cytosolic Fe–S cluster biogenesis (review; [79]) it was believed that ABCB7 was involved in mitochondrial Fe–S cluster export. More recent studies suggest that it exports a substrate important for cytosolic iron homeostasis or a signal of mitochondrial iron status, with deficiency of this signal indicating depletion of mitochondrial iron and resulting in diversion of cytosolic iron to the mitochondria [76]. The specific reason for the impaired heme synthesis observed in *Abcb7* knockout mice remains unclear. The physical interaction of ABCB7 with the C-terminal region (Fe–S ligation site) of Fech has been proposed to function in mitochondrial iron homeostasis [80]. The knockout mouse model shows elevated mitochondrial iron levels without an increase in PPIX levels, yet zinc PPIX levels are increased (which requires functional Fech) suggesting that ABCB7 plays a role in the availability of iron destined for incorporation into PPIX by Fech [78].

Nilsson and colleagues recently performed a large-scale gene expression analysis to identify potential mitochondrial transporters of heme and heme synthesis intermediates, seeking genes co-expressed consistently and specifically with genes encoding enzymes of the heme synthesis pathway [63]. Reassuringly, ABCB6, ABCB10 and Mfrn-1 were identified, as expected. The transporters SLC25A39, TMEM14C, and SLC22A4 and the Fe–S cluster biogenesis proteins ISCA1 and C1orf69 were identified as potential candidates involved in mitochondrial heme/iron metabolism. Silencing of each candidate gene in zebrafish results in an anemic phenotype. SLC25A39 was the only gene characterized in detail. Silencing of this gene in differentiating MEL cells results in a 4-fold decrease in the ^{59}Fe -heme content of mitochondria, without the accumulation of ^{59}Fe . The authors predict a role for SLC25A39 in Fe–S assembly that affects heme synthesis.

3. Heme catabolism and the need for heme transport

Although chemical degradation of heme occurs in mammals through the action of H_2O_2 or ascorbic acid [81,82], the HO system is the major physiologically relevant mechanism of heme catabolism (for review see [83]). In humans, HO is present in two functionally active isoforms, HO-1 and HO-2, encoded by different genes and, apart from their heme degradative function, dissimilar in molecular and biochemical properties. A pseudogene variant of HO-2, HO-3 has also been described in the rat brain [84–86].

HO is a mixed function oxidase, i.e., it utilizes molecular oxygen, generating oxidized products and H_2O . The oxidase catalyzes the regio-specific cleavage of the α -methine bridge of heme-producing iron, CO, and biliverdin IX α (BV)—and is the rate-limiting step in the degradation sequence. The concerted activity of NADPH-cytochrome P450 reductase is also necessary, both to supply electrons and to activate oxygen. BV, a physiological inhibitor of HO function, is rapidly converted to bilirubin IX α (BR) by BVR. HO and NADPH-cytochrome P450 reductase are located in the endoplasmic reticulum (ER), forming a complex with each other and, at least *in vitro*, with cytosolic BVR [87]. Incidentally, HO-1 has also been localized to plasma membrane caveolae (with NOS and BVR [88]), mitochondria, and the nucleus [89,90]. BR is, in turn, conjugated in the liver by UDP-glucuronyl transferase and eventually excreted into the bile. The daily production of BR in man is ~400 mg, of which ~300 mg is derived from Hb breakdown [91].

In bacteria, breakdown of imported heme serves primarily as a source of iron, and early studies in mammals likewise focused for the most part on the importance of HO for heme catabolism during Hb breakdown. However, it is now well recognized that HO-mediated

heme degradation has multiple roles in mammals, including anti-oxidative and iron reutilization functions.

3.1. Antioxidative function of HO-1 and HO-2

HO-1 is activated by a greater number of stimuli than that of any other gene [83]. It is inducible by increased heme levels; various metals (including sodium arsenite, cobalt and selenium); hypoxia in certain cell types; hyperoxia; environmental chemicals; H₂O₂; NO; depletion of intracellular glutathione; UV light; and heat shock (and thus known as HSP32). Notably, basic leucine zipper (bzip) “stress-responsive” transcription factors, including members of the ATF family, c-Fos, c-Jun and Nrf2, maf family members and Bach-1, all regulate HO-1 expression. These transcription factors function as homo-dimeric transcriptional complexes or, with other bzip members, as hetero-dimeric transcriptional complexes, vastly expanding the scope of HO-1 regulation [92,93]. More recently, BVR has been identified as a key regulator of HO-1 expression. The enzyme has kinase, transcription factor and intracellular heme transport activities. Thus BVR can serve as a dimeric bzip transcription factor; or through its kinase function, directly phosphorylate and regulate other bzip members; or via transport of heme into the nucleus enable derepression of Bach-1-mediated repression of HO-1 [92–94]. An overriding principle of HO-1 expression appears to be its rapid induction in response to an increase in cellular oxidative stress and then subsequent repression to low levels.

In contrast, HO-2, the predominant form of HO protein in the brain and testis, is constitutively expressed in all cells, with the promoter responding solely to glucocorticoids (for review see [83]). The N-terminus of HO-2 protein contains heme-binding Heme Regulatory Motifs (HRMs) consisting of CP-based short amino acid sequences [95], an ancient motif found in proteins that function as heme–oxygen sensors in bacteria [27], yeast (HAP-1 [41]) and mammals (ALAS2, Bach-1 and HRI [8,9,11]). The presence of HRMs thus suggested a further function for this enzyme—that of an intracellular sink, binding and regulating (or “sensing”) the heme gaseous ligands NO, CO and O₂. Subsequently, HO-2 has been shown to co-localize with NOS, binding the vasodilator NO with high affinity [96] and, with less affinity, the potential vasodilator CO, or O₂. Furthermore, HO-2 interacts with and, through proximal production of CO, stimulates the calcium-sensitive potassium (BK) channels that mediate the excitatory responses of the carotid body to hypoxia [97]. HO-2^{-/-} mice have mild hypoxemia and their carotid bodies are enlarged (as also seen in patients with chronic hypoxemia [98]); however, their carotid bodies respond normally to acute hypoxia [99], and the mice have only partially impaired acute ventilatory responses and (proposed) chronic pulmonary ventilation/perfusion mismatches, perhaps related to changes in CO signaling [100]. Thus it is postulated that redundant mechanisms, including CO, NADPH-oxidase and AMP:ATP ratios, regulate the acute response of both central and peripheral chemoreceptors to hypoxia through inhibition of ion channels [101]. In addition, HO-1 appears to be upregulated in the lungs of HO-2^{-/-} mice, perhaps functioning as an alternate source of CO production. Recent studies indicate an additional role for the HRMs of HRM-containing proteins like HO-2; that of a thiol/disulfide redox switch that responds to the cellular redox state. Thus, during oxidative stress, heme binds with a much higher affinity to the disulfide cysteine residues, increasing its function as a sink for NO and derivatives, CO and O₂, while, under reducing conditions, the cysteines would become thiols and thus have a lower affinity for heme [102].

The reason for the reduction of the heme catabolite BV to BR has been questioned, as BV is water soluble and thus easily excreted, whereas BR is lipid soluble and believed to be toxic. However, studies by Stocker et al. [103], extended by Snyder's laboratory [104–106] and others, have clearly shown that BR at intracellular nanomolar (i.e., physiological) concentrations is a potent antioxidant that protects

cells from up to a 10,000-fold excess of H₂O₂; in so doing, it is itself oxidized to BV, which is then recycled back to the reducing agent BR through the action of PKC-dependent signaling that activates BVR [105]. In neurons this reaction depends on the substrate BV being supplied by HO-2, rather than by HO-1 [106]. Recent studies indicate that BR serves mostly to defend lipid membranes from ROS, whereas the other major intracellular antioxidant, the water-soluble glutathione system (GSH/GSSG), protects water-soluble proteins [104].

In closing this section on the antioxidant function of HO, we should note that there have been reports suggesting that HO-1 induction could be harmful to the cell under certain circumstances (see [92]). In particular, the release of heme–chelated ferrous iron that could potentially interact with H₂O₂, producing the highly toxic hydroxide radical (the Fenton reaction), is seen as problematic. Yet, provided the iron released is either sequestered by storage within ferritin or exported out of the cell by the cell membrane iron exporter ferroportin (FPN1) [107,108], then an increase in HO-1 activity should be beneficial to the cell. One group found that transfection of human cells with catalytically inactive rat HO-1 (mHO-1) provided greater protection from (very high levels of) H₂O₂ than transfection with wild-type HO-1, an effect reversed by treatment of the cells with a HO inhibitor (zinc protoporphyrin) or iron chelators. They also noted an inverse correlation between the levels of glutathione and catalase and HO-1 activity [109]. One could argue that these are not physiologic studies; HO-1 is never induced in cells for long periods of time, as heme levels may fall precipitously, perhaps leading to decreases in catalase and other heme-containing antioxidant proteins (see [58]). Ferris et al. noted equivalent levels of ferritin in HO-1^{-/-} cells and cells overexpressing HO-1 [110]. Similarly, a study published recently demonstrates a lack of ferritin synthesis, despite HO-1 induction, in a cell-line in response to oxidizing reagents (as opposed to an appropriate response to extracellular heme) [111]. As the known intracellular level of free heme is very low, it was suggested that there is insufficient substrate for HO-1, or that it perhaps acts through other, non-enzymatic mechanisms [111]. However, one could also speculate that under oxidative stress all available iron is shunted directly to the mitochondria for heme synthesis for heme-containing antioxidant proteins, without a concomitant increase in ferritin synthesis. Moreover, as these investigators acknowledge, they may be unable to measure the small quantity of heme degradation that occurs during exposure to oxidative stress. In sum, there is a large body of literature that supports a seminal role for the HO system, in particular the inducible HO-1 enzyme, in protecting cells from oxidative stress, provided the result is limited heme degradation, which might otherwise limit the function of other critical cell hemoproteins.

3.2. Iron reutilization function of HO-1 and HO-2

Under normal physiological conditions, ~25 mg of iron is consumed daily by immature erythrocytes in the bone marrow (BM) for heme biosynthesis. As the daily dietary iron intake is 1–2 mg, recycling of heme–iron from senescent erythrocytes constitutes the main source of iron for erythropoiesis. Erythrophagocytic macrophages in the liver, spleen and BM are responsible for this recycling. Hb-derived heme is catabolized by HO-1 to release iron, which is subsequently exported into circulation by the sole cell iron exporter FPN1 (reviews [112,113]). Hemoglobin also appears in the plasma under physiological conditions due to intra-vascular hemolysis of senescent erythrocytes (~10% [114]) and during enucleation of erythroblasts in the BM. Hemoglobin is bound by haptoglobin for delivery to CD163-receptor-bearing macrophages, whereas any free heme is rapidly bound to albumin and lipoproteins before being passed on to the high-affinity heme binding plasma protein Hpx (review [115]). Hpx–heme complexes undergo (LRP1/CD91) receptor-mediated endocytosis by hepatic parenchymal cells; however,

they can also be taken up by macrophages, neurons and syncytiotrophoblasts [116].

In approaches to determining the relative contributions of HO-1 and HO-2 to erythrocyte heme–iron recycling, both HO-1^{-/-} and HO-2^{-/-} mice were generated [117,118]. In contrast to HO-1^{-/-} mice, HO-2^{-/-} mice have no evidence of disturbed iron homeostasis, apart from increased pulmonary iron in the setting of hyperoxia [119]. As mentioned, it is likely that the inducible enzyme HO-1 is upregulated in HO-2^{-/-} mice. A patient with homozygous HO-1 deficiency has been described [120]; the child, who died at age 6 years, had evidence of severe hemolytic anemia with high serum heme levels, low serum bilirubin, absent Hpx, increased haptoglobin, and hypoferrremia. Very high von Willebrand Factor and thrombo-modulin levels and disseminated intravascular coagulation were observed, indicating endothelial injury. The child also had hyperlipidemia, and, at autopsy, aortic atheromas were noted. There was iron overload of the liver (involving both the hepatic reticulo-endothelial system [RES] Kupffer cells and parenchymal cells) and kidney (proximal tubule epithelium). The findings are consistent with an important role for heme catabolism both in protection of the vascular endothelium from potentially high levels of exposure to heme and for heme–iron reutilization. HO-1^{-/-} mice develop a similar iron overload phenotype [110,117] that becomes evident between 20 and 40 weeks, with twice the amount of iron in the liver at 10 weeks, when compared with controls. The erythrocytrophagocytes of the liver (Kupffer cells) showed evidence of iron overload, but no excess iron deposition was observed within splenic erythrocytrophagic macrophages; the spleen however, was enlarged due to increased hematopoiesis (the child had asplenia). More recently, the murine model of HO-1 deficiency has been re-examined at various ages between 1.5 and 22 months [121]. The animals develop asplenia over time, due to fibrosis and loss of the red pulp. Notably, there is a marked loss of erythrocytrophagocytes in the liver spleen and BM, which is shown to be due to the toxicity of heme released within macrophages during ingestion of senescent erythrocytes. The authors suggest that the high levels of haptoglobin observed in the human subject with HO-1 deficiency are explained by the specific loss of erythrocytrophagic macrophages (which also bear the haptoglobin receptor CD163). Iron loading of the proximal tubules of the mouse kidney was seen as previously, and splenic tissue iron levels were low (due to a lack of macrophages). Notably, liver tissue non-heme iron levels were similar to controls, however the distribution was altered, with iron found in hepatocytes, perhaps from Hpx–heme delivery, rather than in (the reduced numbers of) Kupffer cells. Although HO-2 knockdown in mice is not associated with disordered systemic iron homeostasis, it has been postulated to contribute to iron homeostasis in liver parenchymal cells [122]. Finally, when HO-1^{-/-} BM cells are transplanted into irradiated HO-1^{+/+} mice, the animals show no evidence of hepatic or renal iron overload at 20 weeks, suggesting a role for endothelial, hepatic and renal parenchymal HO-1 in heme–iron recycling [117]. In sum, the analysis by Kovtunovych et al. indicates that the loss of erythrocytrophagic macrophages from heme toxicity, resulting in a large increase in circulatory heme and Hb is the key to the pathogenesis of HO-1 deficiency [121].

Hepcidin is the hormone that regulates systemic iron homeostasis through binding to and thus decreasing the cell-surface expression of FPN1 [123]. Hence, it regulates both enterocyte iron absorption and the release of iron from erythrocytrophagic macrophages. Hepcidin is itself regulated by iron, erythropoiesis, tissue hypoxia and inflammation (review [124]). Of interest, FPN1 and HO-1 are co-induced in macrophages upon ingesting erythrocytes, whereas macrophages from HO-1^{-/-} mice have chronically high levels of FPN1 expression [121], likely related to the presence of a heme-inducible MARE/ARE element in the enhancer region of the gene [125]. Recent studies have shown decreased hepcidin levels in a HO-1-deficient patient [126], despite evidence of chronic inflammation, which normally

increases hepcidin levels, whereas studies of the murine HO-1^{-/-} animal demonstrate normal hepcidin levels [121]. These findings reiterate the predominance of the proposed erythroid iron regulator [127] and serum hypoferrremia in suppressing hepcidin to allow for the flow of iron from the RES to the BM for erythropoiesis.

As expected, human and murine HO-1^{-/-} cells rapidly undergo apoptosis upon exposure to heme [120], and HO-1^{-/-} mice also have evidence of increased oxidative stress, with increased free radical expression in response to heme, H₂O₂, paraquat or CdCl₂ [128]. The mice are extremely sensitive to endotoxemia, dying upon exposure to low doses of lipopolysaccharide. Notably, these deaths are due to massive parenchymal hepatocyte iron loading, resulting in liver necrosis. A similar hepatocyte injury pattern is seen when Hpx-null mice are given moderate intravenous doses of hemin; that is, the majority of intravascular heme ends up in the liver parenchyma (presumably bound to albumin and LDL [115]), resulting in hepatic inflammation and congestion, likely secondary to sinusoidal injury and thrombosis. This inflammation is prevented by prior induction of HO [129]. Hershko et al. reported hepatic and RES iron loading in a rat model of inflammation more than three decades ago [130]. After injection of heme in the form of damaged erythrocytes or Hb, there was increased catabolism of heme to ferritin, as well as a decrease in ferritin secretion. It is now acknowledged that the iron loading of RES and hepatic parenchymal cells in response to inflammation is, for the most part, secondary to a lack of release of iron from these cells due to cytokine-mediated upregulation of hepcidin, decreasing FPN1-mediated iron export [131]. Presumably HO-1 is upregulated to cope with the heme load. It is unknown why or how mobilization of iron and heme to the liver occurs with inflammation/infection. It may be a protective mechanism to decrease the levels of both iron and heme–iron in the systemic circulation, thus preventing bacterial access to iron (iron in ferritin is likely inaccessible to bacteria) or, perhaps, as Poss and Tonegawa propose, to reduce oxidative stress in the tissues [128].

In summary, both HO-1 and, to a lesser extent, HO-2 are important for heme–iron re-utilization. What is striking, however, is the longevity of the HO-1 knockout animals, given the low-level expression of HO-2 (e.g., there appears to be no upregulation of HO-2 in PBMCs isolated from the patient with HO-1 deficiency [120]), and the enormous iron requirement for continued high level erythrocyte production in the face of severe hemolysis. This observation would suggest that there are other methods of recycling heme–iron. Poss and Tonegawa suggest contributions from a microsomal cytochrome P450 reductase and a cytosolic xanthine oxidase [117]. In the following sections we will discuss the potential importance of FLVCR-mediated heme export.

4. Heme absorption

Ingested heme serves as the most important source of dietary iron in meat-eating animals [3,132], comprising one-third of ingested iron in Western diets but up to two-thirds of absorbed body iron. This is likely related to the increased bioavailability of heme relative to iron, especially when bound to proteins [133–135]. Unlike expression of the duodenal iron importer (DMT1, [136]), which increases approximately 10-fold during iron deficiency, intestinal heme uptake increases 3-fold, suggesting a separate, carrier-mediated process [137]. Although the molecule(s) mediating duodenal brush-border enterocyte heme uptake has not been identified, considerable evidence suggests uptake occurs via a receptor-mediated endocytotic pathway (see [138] and [134] and references therein) or by heme importer(s) (possibly HCP1 [139]; see below). Early studies demonstrated the presence of a mammalian enterocyte heme-binding protein that binds heme with high affinity (K_{diss} up to 10^{-9} M [140]), with binding subsequently lost upon treatment of the cells with trypsin. More recent studies indicate heme uptake is temperature dependent and

saturable, decreased by inhibition of oxidative phosphorylation, but unaffected by ATP depletion [141–143]. Similar findings in mammalian erythroid and hepatic tissue cell lines suggest heme import is a generalized cell process [141,144]. Two separate electron microscopy studies of closed mammalian duodenal loop tissues indicate that heme is taken up intact by enterocytes—subsequently appearing first in apical tubulo-vesicular structures within the cytosol and then in secondary lysosomes [145,146]. Notably, heme disappeared from these vesicles within 3 h and was not present in the basolateral regions of the cells, the extracellular space or the portal vein, indicating intracellular catabolism, consistent with findings in mammals, including humans, where the majority of ingested radioactive Hb iron is detected in the portal vein as non-heme iron within 2–3 h [133,147,148]. In this scenario, either the endosome/lysosome possesses HO activity and an iron transporter for ferrous iron, such as DMT1 [136] or TRPML1 (see review; [149]), or perhaps the lysosomal heme transporter HRG-1 delivers heme to the cytosol ([150]; discussed below). HO is upregulated 2.5-fold in rat enterocytes in response to iron deficiency, and enzyme activity is highest in the proximal small intestine, the site of maximal heme absorption [151–153]. Studies in dogs confirm that HO activity is limiting for heme absorption [154], while HO inhibitors – e.g., Sn mesoporphyrin (po or iv) – inhibit intestinal heme absorption and result in increased biliary excretion of heme [155]. Surprisingly, recent confocal microscopy studies of rat enterocytes after heme dosing show that, while HO-1 is distributed throughout the cell, HO-2 is the enzyme found exclusively in the subapical region, where it co-localizes extensively with endosomes [156].

In contrast to these studies on the importance of HO-mediated heme catabolism for heme–iron absorption, an older study of Hb absorption in guinea pigs suggested the absorption of intact heme [157]. In addition, in the human enterocyte–like cell line, Caco2, which has very high expression of the heme exporter FLVCR, heme appears to be exported across both the basolateral and apical surfaces [158]. In addition, haptoglobin gene knockout in mice causes fpn1 upregulation in intestinal cells, which may also suggest the uptake of heme from the circulation into intestinal cells [159].

In summary, in most mammals, dietary heme is transported intact into intestinal mucosal cells, where it is mostly catabolized, with the subsequent appearance of inorganic iron in the portal vein. Systemic heme may also be taken up by hepatic (or perhaps intestinal) cells and transferred, if necessary, into the gastrointestinal lumen [160].

5. Heme trafficking and transporters

Both cell surface and organelle-associated heme transporters are likely involved in the sequestering and trafficking of intracellular heme (Fig. 2; see also reviews [39,48,138,161–164]). Because the last step of endogenous heme synthesis occurs in the mitochondrial matrix, heme must traverse both mitochondrial membranes to enter the cytoplasm (Fig. 1). Although the identity of the mitochondrial heme exporter is unknown, ABC transporter family members may channel heme synthesis intermediates across both mitochondrial and plasma membranes (see Sections 2 and 5.1.2) [47,165]. Heme is also known to function as a transcriptional regulator; therefore, it must enter the nucleus, probably through binding to a carrier protein such as BVR [32]. Approximately 85% of synthesized heme is utilized for Hb production in the BM. The other 15% is produced in the liver, primarily for cytochrome (especially P450) synthesis in the ER and for catalase synthesis in peroxisomes, suggesting the presence of heme transporters in these organelle membranes [39,166]. Thus, heme needs to be transferred to various compartments within the cell, presumably chaperoned by cytosolic heme-binding proteins (reviewed in [39]). However, the identity of the intracellular heme-binding proteins and many of the heme transporters has not yet been identified. In the next section, the current understanding of various established and potential heme transporters is discussed.

5.1. Heme exporters

5.1.1. FLVCR, a cell surface heme exporter

Infection of cats with Feline Leukemia Virus subgroup C (FeLV-C) invariably causes a severe non-regenerative anemia (hematocrit 4%–15%), with an absence of reticulocytes in the circulation and hemoglobinized cells in the BM, despite high erythropoietin levels. Studies have subsequently shown evidence of an arrest in erythropoiesis at the colony-forming unit-erythroid progenitor (CFU-E)/pro-erythroblast stage [167,168], where heme synthesis begins [169,170]. The disease bears a resemblance to human pure red cell aplasia because, despite viral infection of all BM cells, there is normal development of the myeloid and megakaryocytic lineages [171]. FeLV subgroups (A, B and C) were defined by host cell range, neutralization and interference assays that indicate their viral envelopes exploit different cell receptors [172,173]. The nonpathogenic FeLV-A has a narrow host range, while both FeLV-B and FeLV-C have broad host ranges. The genomes of FeLV A, B and C are most divergent in the segment that encodes for variable region 1 (VR1) of their envelope proteins. In studies using chimeric envelope genes of FeLV-A and FeLV-C, an 11-aa sequence of VR1 of FeLV-C envelope protein was sufficient to transform FeLV-A into a pathogenic feline virus causing anemia [174]. This suggests that the anemia is caused by a dominant negative effect of the FeLV-C envelope protein interfering with the display and/or function of its receptor on CFU-E/pro-erythroblasts. As the function of the receptor appeared to be critical for the development of erythroid progenitors we and others functionally cloned the gene, termed *Flvcr*, Feline Leukemia Virus subgroup C Receptor [175,176].

5.1.1.1. Gene, protein structure and expression. FLVCR is highly conserved throughout evolution with orthologs present in animals, plants and bacteria, suggesting an important role in cell biology [177]. The human gene (also known as *Mfsd7b*) is located at chromosome 1q32, contains 10 exons and spans approximately 40 Kb. The promoter region contains consensus GATA-1, GATA-2 and STAT5a binding sites, providing potential mechanisms for upregulation of the gene during early erythroid development [36]. Of interest, both *FLVCR* and one of its 3 paralogs (on chromosome 3q, GI:74732717) have an adjacent gene upstream that shares the same promoter; LQK-1, 177 bps upstream of *FLVCR*, is of unknown function, whereas the 3q gene product HSPBAP1, binds HSP27, an antioxidant protein [178]. In addition, there is a marked homology between a group of genes that includes *FLVCR* on chromosome 1q and a paralogous cluster that includes *FLVCR2* on chromosome 14q, a duplication that occurred over 500 Ma ago [177]. Such clustering of genes has been previously associated with functional linkage—e.g., the murine surfeit locus [179].

The major mature *FLVCR* transcript encodes a protein of 555 aa with a predicted molecular weight of 60 kDa and a pI of 5.72 (Fig. 3). The protein belongs to the major facilitator superfamily (MFS) of secondary permeases, which transport small solutes such as glucose (e.g., GLUT-1), amino acids, and ions across cell membranes in response to chemico-osmotic gradients. Topology predictions indicate that FLVCR is a 12-transmembrane domain protein with both N- and C-termini inside the cell. Both the N- and C-termini of MFS transporters generally contain domains important for transporter trafficking and proper localization—e.g., in the polarized epithelial cells of the intestine and kidney [180]. FLVCR has both a proximal tyrosine-based motif YXXØ and a more distal di-leucine motif in the N-terminal intracytosolic region. These signals in mammalian proteins mediate rapid internalization and targeting to endosomal–lysosomal compartments, suggesting that FLVCR may be targeted to these locales. Sequences conforming to this motif have also been implicated in baso-lateral targeting in polarized epithelial cells [181,182]. In addition, the C-terminus contains a PDZ domain-binding motif that likely promotes protein–protein interactions (see below and reviews [183,184]). Both the putative human heme importer PCFT/HCP1 and the *C. elegans* lysosomal heme transporter HRG-1 (see

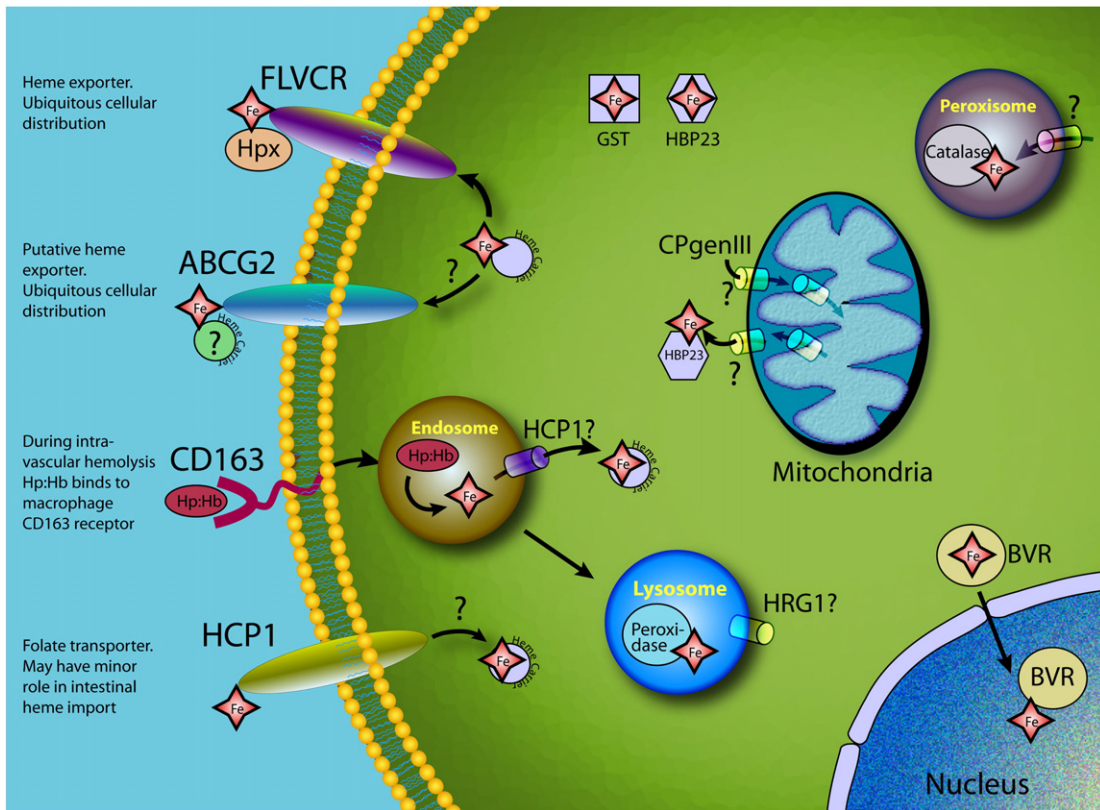


Fig. 2. A generalized depiction of known and putative cell-surface and organelle-associated heme transporters. FLVCR is a cell-surface heme exporter. ABCG2 is a potential cell-surface heme exporter; HCP1 is a putative heme importer. CD163 receptor-mediated endocytosis of the Hp:Hb complex suggests the existence of an endosomal/lysosomal heme exporter; HCP1 or HRG1 may fulfill these role(s). The transporters that mediate import of heme into peroxisomes and lysosomes for assembly of catalases and peroxidases are unknown (it is also unclear at present where final assembly of hemoproteins occurs). Heme is transferred into the nucleus in association with BVR, while cytoplasmic heme appears to be chaperoned by proteins such as glutathione S-transferase B and HBP23. See text for further details. Putative transporters of mitochondrial heme and heme biosynthesis intermediates are shown in detail in Fig. 1.

below) have di-leucine motifs, whereas FPN has a predicted PDZ domain-binding motif.

Human *FLVCR* mRNA is expressed ubiquitously with the highest expression observed in the liver, duodenum, kidney, lung, spleen, brain, placenta and BM (including CD34⁺ stem/progenitor cells) [36,37].

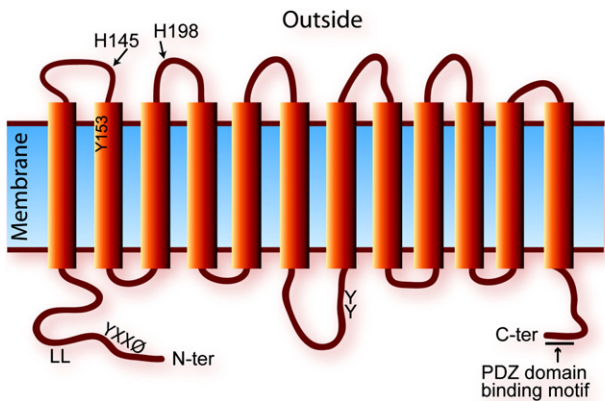


Fig. 3. *FLVCR* membrane topology. *FLVCR* is predicted to contain 12-transmembrane domains, with both N- and C-termini within the cell. It has di-leucine (LL) and YXXØ and motifs (where Ø denotes a hydrophobic amino acid) in the N-terminal region that may allow proper membrane domain targeting and recycling. His and Tyr residues in the extracellular loops are predicted to be important for heme binding/transfer, whereas Tyr residues in the large intracellular loop may serve as targets of kinases and phosphatases. The 4-aa PDZ domain-binding motif at the C-terminal region is important for its interaction with the PDZ domain of the nonreceptor tyrosine phosphatase PTPN3.

Notably, in studies of human erythropoiesis, specific upregulation of *FLVCR* protein expression is observed at the CFU-E stage; *FLVCR* is subsequently downregulated with further differentiation [36]. There are wide disparities between mRNA levels and cell surface protein expression (see [36,37]) suggesting that posttranslational control mechanisms, such as protein trafficking, regulate *FLVCR* expression. This is not unexpected as regulation of transporter cycling is a control mechanism important in the cell surface expression of other MFS members, such as glucose and iron transporters (e.g., GLUT-4 [185]; Arn1p [186]). More recently, alternative splicing of *FLVCR* mRNA has been described, with enhanced alternative splicing resulting in reduced levels of the normal protein observed in erythroid cells from patients with a congenital form of red cell aplasia, Diamond-Blackfan anemia (DBA) [187]. In earlier studies we found no evidence of *FLVCR* gene mutations in a subset of DBA patients with linkage to the *FLVCR* locus [188].

Of interest, a recent study describes homozygous missense mutations in *FLVCR* in affected individuals from three families with a syndrome of posterior column ataxia and retinitis pigmentosa [189]. Analysis of murine posterior column neurons and retinal tissues shows high expression of *FLVCR*. The authors hypothesize that *FLVCR* mutations abrogate the known neuro-protective effects of a hemoglobin-related protein, neuroglobin. However, *FLVCR* may also serve to protect these tissues from heme toxicity related to capillary leaks.

5.1.1.2. *FLVCR* trafficking and localization. Interacting proteins regulate trafficking of MFS members like *FLVCR* to and from the plasma membrane. For example, the glucose transporter-4 (GLUT4) resides in intracellular storage compartments, but rapidly translocates to the plasma membrane in response to a signaling cascade initiated by

insulin [190,191]. Studies of FLVCR expression demonstrate a marked discordance between protein expression and mRNA levels [36,37], while analysis of mammalian FLVCR protein sequences suggests they contain a PDZ domain-binding motif at the extreme C-terminus, suggesting PDZ domain-containing proteins may regulate FLVCR trafficking and localization. A PDZ domain is a protein structural motif of 80–90 aa that helps anchor transmembrane proteins to the cytoskeleton [192,193]. Using a yeast-two-hybrid screen, we recently identified a PDZ domain-containing protein, the nonreceptor protein tyrosine phosphatase, PTPN3 that interacts with FLVCR. Both proteins co-immunoprecipitate in cell expression and pull-down assays. Importantly, their interaction is abolished when either the PDZ domain of PTPN3 or the 4-aa PDZ-domain binding motif of FLVCR is deleted [194]. A role for PTPN3 in anchoring FLVCR to the plasma membrane may thus have important implications for trafficking and/or membrane retention of FLVCR in for example, the polarized epithelial cells of the intestine and kidney.

5.1.1.3. FLVCR-mediated heme export. Heme synthesis (for Hb production) initiates at the CFU-E/pro-erythroblast stage of erythroid development [169,170]; thus, a potential role for FLVCR in heme transport was examined. Using rat kidney epithelial (NRK) cells overexpressing FLVCR, we demonstrated that FLVCR exports ⁵⁵Fe-hemin or zinc mesoporphyrin (ZnMP, a heme analog that also inhibits HO) over a 90 min washout period, after initial loading [36]. Approximately 50% of the heme taken up by the cells is exported. Using HPLC methodology, the export rate of exogenously supplied heme by NRK/FLVCR cells was quantified as approximately 2-fold that of control NRK cells (which presumably express the rat ortholog), provided that HO function is first blocked by a short incubation with ZnMP. In K562 cells, interference with endogenous FLVCR expression by FeLV-C envelope protein dramatically reduces heme export, to 3% versus 54% in naive K562 cells. FLVCR-mediated heme export in K562 cells can also be blocked using a polyclonal antibody specific to FLVCR (10% export versus 54% in control cells), confirming that cell-surface FLVCR mediates heme export. The heme export activity of FLVCR has also been demonstrated using macrophages from wild-type and *Flvcr*-deleted mice [37]. There is no evidence that FLVCR imports heme [36]. Recently, endogenous or exogenously supplied heme was demonstrated to be the primary physiological substrate for FLVCR. In addition, under (non-physiological) conditions that increase intracellular PPIX or coproporphyrin levels, FLVCR exports these porphyrins [38]. However FLVCR does not export unconjugated bilirubin.

Heme export by FLVCR requires an extracellular heme-binding protein such as albumin or Hpx, as export is not observed in the absence of a carrier protein [38]. Thus heme may be channeled through FLVCR, docked at extracellular amino acid residues, and then released to a heme carrier protein. By analogy to the bacterial hemophore HasA [195,196], CsbA [197], and other heme-binding proteins, the residues His 145, Tyr 153 and His 198 of FLVCR are likely to be involved in the heme transport route and/or heme transfer to extracellular heme-binding proteins (the common predicted motif is: H-x(7)-Y-x(44)-H, using the Prosite system [198] pattern syntax; see http://ca.expasy.org/tools/scanprosite/scanprosite-doc.html#pattern_syntax). The histidine residues, conserved in mammalian FLVCR proteins, but absent in the closely related paralogs that do not export heme, are predicted to be on extracellular loops 1 and 2, and thus are optimally located to coordinate heme docking and export. Recent studies suggest that Hpx – with a similar arrangement of His residues – functions physiologically to bind heme that is bound to FLVCR, therefore facilitating for example, the uptake of heme via FLVCR from macrophages [38]. Export of heme by NRK/FLVCR cells is 100-fold more efficient when the media contains Hpx rather than albumin [38], in keeping with the relative affinities of Hpx and albumin for heme (Kd <1 pM versus Kd = 5 nM; [199,200]). The model is also supported by the finding that apo-Hpx directly binds to a 70 aa FLVCR peptide that encompasses His 145, Tyr 153, and His 198. The *in vitro*

binding of Hpx to the peptide can be inhibited by incubation of the (heme-binding) peptide with heme: presumably Hpx removes heme from the peptide and is then released into the media [38]. Notably, Hpx is present in high concentration in human serum (6.7–25 μM [201]), a concentration similar to that of the iron transport protein transferrin (22–31 μM [202]).

5.1.1.4. FLVCR heme export function in erythropoiesis. As discussed, cats infected with FeLV-C lack reticulocytes in the circulation and hemoglobinized cells in the marrow, indicating a block in erythroid differentiation. Similarly, inducible deletion of its murine receptor, *Flvcr*, in one-week-old mice rapidly results in a severe hyperchromic macrocytic anemia, reticulocytopenia, splenomegaly and a lack of hemoglobinized cells, again caused by a block in erythropoiesis at the CFU-E/pro-erythroblast stage, as demonstrated by colony assays or FACS analysis [37]. In contrast to this purported role for FLVCR in heme export during early erythropoiesis, previous studies have shown that exogenous heme promotes erythroid differentiation of K562 cells and of the early erythroid progenitor cells, burst-forming units—erythroid [203–205]. In addition, increasing levels of heme in the nucleus directly repress a transcriptional repressor of globin genes, Bach1, during erythropoiesis [8], presumably to allow globin expression for maximal Hb production. Thus, the requirement for a heme exporter during erythropoiesis appears counterintuitive.

It is likely however that regulation of potentially toxic heme in erythroid cells is markedly different from that in non-erythroid cells. For example, the first step in heme synthesis is initiated by erythroid ALAS2, which is not repressed by increasing heme levels, but rather regulated by iron supply (see [1]), in contrast to heme repression of the ubiquitous enzyme ALAS1 in non-erythroid cells [161]. There is also no evidence to date that HO-1 is induced at this stage of erythropoiesis—studies, for example, of differentiation of a murine erythroid (MEL) cell line describe downregulation of HO-1 [206,207]. There is therefore a paucity of mechanisms in erythroid progenitors to cope with any excess free heme that may occur as heme and then globin synthesis ramps up. We hypothesize that FLVCR is specifically required during this initial phase of heme synthesis, serving as an overflow valve to protect CFU-E/pro-erythroblasts from fluctuating heme levels and potential heme toxicity until sufficient globin becomes available to bind heme. In accordance with this hypothesis, FLVCR expression is downregulated with further erythroid differentiation [36].

Inhibition of FLVCR function during erythroid differentiation of K562 cells leads to apoptosis, which suggests apoptotic pathway(s) may be stimulated by direct cellular heme toxicity or as a secondary effect of impaired erythroid differentiation [36]. It appears that FLVCR, through rapid modulation of its surface expression by trafficking or regulatory factors, is well suited to augment regulation of the heme content of erythroid progenitors, as opposed to the alternative: heme degradation by induction of HO-1, which, as an enzyme with a long half-life (~20 h) may prevent optimal erythroid hemoglobinization.

Of interest, a potential imbalance between heme and globin synthesis during erythropoiesis may be key to a human congenital form of red cell aplasia, DBA. Mutations of ribosomal proteins (notably RPS19, 24 and 17) are seen in approximately 45% of patients with DBA (reviews, [208,209]). Mutation of RPS19 results in defective maturation of the 40S (translation initiation) ribosomal subunit [210], whereas RPS24 and 17 are also associated with the 40S subunit [202]. These mutations may result in defects in ribosome biogenesis and, in developing erythroid cells thus delay or decrease initiation of protein, predominantly globin, translation. Therefore a transient excess in erythroid progenitor intracellular free heme may occur, analogous to that predicted for *Flvcr*-deleted mice where heme export is impaired. Although a clear link between ribosome biogenesis defects and the development of the erythroid and myriad non-erythroid tissue defects seen in DBA is still unclear, *Flvcr*-null mice embryos do exhibit

not only erythroid tissue but also morphologic abnormalities similar to those observed in patients with DBA—specifically, flattened faces, hypertelorism, upper limb and hand/digit abnormalities (see [211]), suggesting that FLVCR dysfunction and/or heme excess may be important in the pathogenesis of this disease.

5.1.1.5. FLVCR heme export function in macrophages and systemic iron homeostasis. Macrophage processing of senescent erythrocytes, ~360 billion per day [212], generates 25 mg or ~85% of our daily iron requirements. The iron derived from heme catabolism is either exported by the phagocytic cell-specific divalent metal transporter (Nramp1) [213] and FPN1, or stored as ferritin [214]. But, during erythrocytaphagocytosis not all heme appears to be broken down by HO-1 [215,216], and FLVCR provides an important heme (iron)-export function that impacts systemic iron homeostasis [37]. When compared to control macrophages, *Flvcr*-deleted murine macrophages have reduced heme export. Upon phagocytosis of opsonized erythrocytes they accumulate more ferritin (from erythroid heme catabolism) than controls; with their ferritin content increasing further when FPN1-mediated iron export is blocked by prior incubation with hepcidin [37]. These findings confirm studies by Knutson et al. indicating that approximately 25%–30% of heme is re-exported intact from macrophages following erythrocytaphagocytosis [215]. This fraction may increase during anemia of chronic disease, when hepcidin levels are high and FPN1 expression is downregulated.

Hpx, a high affinity heme-binding protein, is expressed in the liver, CNS, retina, skeletal muscle and kidney (review; [217]). It preferentially transports heme in the plasma to CD91-expressing hepatocytes, where it is subsequently catabolized by heme oxygenases. Hpx interacts with FLVCR in heme transfer studies and is present in the circulation at levels similar to that of the iron transporter transferrin (see above; [38]), suggesting that the systemic circulation of heme bound to Hpx (or the more abundant plasma protein albumin) may be of relevance to iron homeostasis. Although it is difficult to find measurements of circulating heme in the literature, a review [201] indicates the heme-binding capacity of Hpx is approximately 600 µg/dL (in agreement with an older study [218]), which would contain approximately 60 µg/dL of iron. In comparison, plasma transferrin-bound iron lies in a similar range, 50–180 µg/dL. Hpx knockout mice have been generated [219]; they show no evidence of anemia, with plasma iron levels and blood parameters (e.g., hematological parameters, BR, and albumin) comparable to those of control mice. In addition there was no evidence of oxidative damage to tissues or abnormal iron deposits. The lack of a phenotype under physiological conditions is likely related to transport of circulating heme by albumin, present in the plasma at a concentration approximately 100 times that of Hpx (30–55 mg/mL versus 0.5–1 mg/mL). However it may be possible to dissect the role of systemic heme circulation further in that the *Hpx* knockout mouse is available while the heme-binding sites on albumin are known; in particular, Tyr 138 and Tyr 161 appear critical [220].

FLVCR is also highly expressed at other potential sites of heme flux – e.g., in the liver, kidney, duodenum, placenta and uterus – suggesting a role for cell heme export at these sites. Furthermore, inducible deletion of *Flvcr* in one-week old mice results in iron overload in hepatocytes within 5 weeks and subsequently in enterocytes and splenic macrophages [37], while FLVCR appears to traffic to the apical membrane of both hepatic and duodenal cells *in vitro* when cellular iron levels are high. Notably, by 7 months there is swelling of the hepatocytes that line bile canaliculi and biliary stasis, suggesting that heme export from the liver into bile may serve as a mechanism to ameliorate systemic iron overload. This mechanism may be especially important for example, during episodes of hemolysis (likely occurring in *Flvcr*-deleted mice) when there is increased heme delivery to the liver. Kappas et al. found that there was little or no excretion of heme into the biliary system of rats under physiological circumstances, but infusion of heme or heat-damaged erythrocytes leads to excretion of small amounts of

biliary heme (e.g., 14% of the dose of injected heme) [160]. The amount excreted into the bile increased 3–4-fold when Sn-protoporphyrin, a HO inhibitor, was infused simultaneously with either heme or heat-damaged erythrocytes, this latter result suggesting that tissue macrophages can export heme to hepatocytes when HO function is impaired. Similar to studies where HO-1^{-/-} BM cells were transplanted into irradiated HO-1^{+/+} mice (see Section 3), when *Flvcr*-deleted BM cells were transplanted into irradiated *Flvcr*^{+/+} mice the transplanted mice showed no evidence of hepatic iron loading at 5–6 weeks, suggesting that liver excretion (or, more speculatively, enteroocyte secretion [158]) of heme may be important in conditions of heme overload.

A related question is why or under what circumstances cells would export rather than catabolize heme. We have no clear understanding of this at present. However, one could speculate that (i) when cells are overwhelmed by heme and/or iron, for example, when endothelial cells are exposed to large amounts of heme during intravascular hemolysis, both pathways are operative; (ii) under hypoxic conditions cells may preferentially export excess heme, rather than utilize molecular oxygen to degrade heme using HO; (iii) as intact heme may also serve as an intercellular signaling molecule, macrophages may export heme as a signal of increased erythroid cell turnover; and (iv) more speculatively, cells may export heme as a form of redox reservoir for adjacent cells.

5.1.1.6. Anopheles FLVCR-like proteins export heme—relevance to malaria. Hematophagous mosquitoes (females) ingest large amounts of blood (up to 3× their bodyweight). The responses triggered include activation of genes for blood digestion, peritrophic matrix formation, and egg development (vitellogenesis). The rewards are iron (98% from Hb-heme) and amino acid absorption (60% from Hb-globin), nutrients critical for vitellogenesis. Hb degradation, however, also releases large amounts of heme into the mosquito midgut lumen, with a resultant increase in epithelial exposure to toxic ROS [221–223]. Thus, multiple systems exist to detoxify heme, potentially including mosquito FLVCR-like transporters that may export heme from the apical surface of midgut epithelial cells back into the lumen and/or via the basolateral surfaces into the hemolymph. Using bioinformatics, orthologs of human *Flvcr* in the genomes of the most prevalent malaria transmission vectors, *Anopheles gambiae* and *A. stephensi* were identified and cloned. Similar to human FLVCR, overexpression of these proteins in NRK (or Sf9 (insect)) cells leads to increased export of ZnMP, which is decreased by treatment of the cells with specific siRNA. In addition, mosquito midgut expression of *Flvcr* mRNA and protein is upregulated within 24 h of hematophagy, as expected for a heme exporter during assimilation of a heme load, while confocal microscopy of midgut cryo-sections reveals epithelial cell-surface expression of AsFLVCR following a blood meal [224].

Notably, hematophagy represents the major bottleneck for *Plasmodium* transmission from mosquitoes to humans. Fertilization of *P. falciparum* gametocytes in infected ingested blood produces <100 ookinetes in the midgut lumen, with *susceptible* strains of *A. gambiae* killing >80% of them within the midgut [225]. Surviving ookinetes are susceptible to ROS, and mosquitoes with increased midgut oxidative stress are *resistant* to *Plasmodium* transmission [226]. Recent studies emphasize the importance of oxidative stress during *Plasmodium* ookinete invasion of the mosquito gut epithelium; *P. berghei* carrying a deletion of an antioxidant enzyme are arrested at the oocyst stage [227]. We hypothesize that dysregulation of midgut epithelium FLVCR-mediated heme export will increase epithelial cell oxidative stress, potentially impacting *Plasmodium* transmission at its weakest point.

5.1.2. The role of ABCG2 in cell heme export

The proposed role of the mitochondrial transporters ABCB6, ABCB7 and ABCB10 in transport of heme biosynthesis intermediates has been discussed. Here we review the role of ABCG2/BCRP, a potential cell surface heme exporter.

ABCG2 or Breast Cancer Resistance Protein (BCRP) is an ~70 kDa reverse (i.e., NBD is at the N-terminus) half transporter that requires dimerization to form a functional transporter [228]. It is expressed in multiple tissues including the intestine, kidney, liver, blood-brain barrier and placenta, where it can modulate the absorption and excretion of multiple xenobiotic compounds (reviewed in [229,230]). It is also expressed in stem cells, including hematopoietic stem cells [231]. ABCG2 expression is upregulated during erythroid differentiation and the transporter is present on the cell surface of erythrocytes [232]. Like *Flvcr*, the *Abcg2* promoter region contains consensus GATA-1 and GATA-2 sites, providing potential mechanisms for upregulation of the gene during erythroid development.

Knockout mice were generated to investigate the physiological role of *Abcg2* [233,234]. The mice are normal with no apparent phenotypic (including hematologic) abnormalities. However, knockout mice serendipitously housed close to a light source while feeding an alfalfa-containing chow, developed skin photosensitivity with lesions on their ears, tails, snouts and eye rims [233]. The photosensitivity was traced to an accumulation of pheophorbide a, a degradation product of chlorophyll structurally related to PPIX. Compared to wild type mice, knockout mice are 100-fold more photosensitive to orally administered pheophorbide a, and have 20-fold higher plasma pheophorbide a levels. Importantly, erythrocytes and the plasma of *Bcrp1*^{-/-} mice contain higher levels of PPIX relative to controls, regardless of their diet [233]. Control murine erythrocytes accumulate similar levels of PPIX when ABCG2 function is inhibited either by a general decrease in cell ATP synthesis (using 2-deoxy-D-glucose) or by a specific ABCG2 inhibitor, Ko143. Conversely, K562 cells engineered to overexpress ABCG2 have decreased retention of exogenously supplied PPIX and an 11-fold decrease in endogenous PPIX accumulation after ALA treatment. This increase in PPIX export is specifically blocked by Ko143 [232]. Jonker et al. observed significantly increased levels of conjugated PPIX in the porphyrin-producing harderian gland and liver of *Bcrp1*^{-/-} mice, with increased PPIX in the biliary system. They suggest that ABCG2 transports conjugated PPIX, and has a potential role in murine hepatic excretion of excess PPIX [235].

A recent report indicates that human ABCG2 exports the heme analog ZnMP when overexpressed in K562 cells [236], an effect abrogated by Ko143. Unfortunately only a single (60-minute export) time point was presented, precluding assessment of initial loading. In addition, examination of transport of ⁵⁵Fe-hemin or HPLC analysis of the cell media to rigorously demonstrate export [36] was not performed. Using biophysical methods, the binding of heme to ABCG2 or its large extracellular loop 3 was demonstrated (Kd 0.2 μM and Kd 0.5–1 μM, respectively). PPIX, ZnPPiX (present in iron-deficient maturing erythroid cells) and pheophorbide a also bind to ABCG2. As albumin can remove ABCG2-bound hemin, a heme export and transfer mechanism similar to the mechanism of transfer of heme from FLVCR to Hpx is proposed [236]. Based on these studies, ABCG2 appears to have functional redundancy with FLVCR.

Both FLVCR [36,237] and ABCG2 are expressed in hematopoietic stem cells and erythroid progenitors. However, the *Abcg2* knockout phenotype suggests that ABCG2 heme export function is not critical during erythropoiesis or for systemic iron homeostasis. ABCG2 has broad substrate specificity including xenobiotics, porphyrins, and, as a recent study indicates, urate [238]. A physiological role for ABCG2 in heme transport therefore requires further investigation.

5.2. Heme importers

Although older studies have suggested the presence of cell-surface heme importers (e.g., [140]), they have not been identified to date. Recently two groups of investigators have used novel approaches in attempts to identify these proteins in mice and the heme auxotroph, *C. elegans*.

5.2.1. Proton coupled folate transporter/heme carrier protein 1 (PCFT/HCP1)

Hypotransferrinemic mice have anemia related to low levels of the iron-binding protein transferrin, which results in low serum iron levels, repression of hepcidin and a marked increase in intestinal iron absorption. Since dietary heme represents the most abundant pool of bio-available iron, it was postulated that heme uptake would also be increased in this mouse model. Therefore, Shayeghi et al. used a subtractive RNA hybridization approach on duodenal (the site of heme import) versus ileal (“zero” heme import) tissues derived from hypotransferrinemic mice to isolate a putative duodenal heme importer, heme carrier protein 1 (HCP1, SLC46A1) [139]. The protein (~50 kDa), like FLVCR, is an MFS member, conserved among species and predicted to have 12 transmembrane domains [239]. The role of HCP1 in heme/iron biology [240] was later overshadowed by the finding that HCP1 primarily functions as an intestinal proton-coupled folate transporter (PCFT) [241]. Several patients with hereditary familial folate malabsorption have been identified as having mutations of the PCFT/HCP1 gene; one mutation, an exon 3 deletion, was shown to eliminate membrane localization of the protein and (folate) transport activity [241,242]. Although a consequence of the resultant folate deficiency in newborns is anemia, this is fully corrected by oral doses of 5-formyltetrahydrofolate. Furthermore, PCFT/HCP1 gene variants are infrequent in hemochromatosis and iron overload screening (HEIRS) study participants [243].

The heme import activity of PCFT/HCP1 was demonstrated in *Xenopus laevis* oocytes; injection of PCFT/HCP1 cRNA increases the uptake of L-arginine-conjugated ⁵⁵Fe-heme 2.5-fold in a temperature-dependent manner. In comparison, PCFT/HCP1-mediated ³H-folic acid uptake was 200-fold that of controls, demonstrating PCFT's preference for folate [241]. Competition studies with an antibody against PCFT/HCP1 inhibited uptake of both heme and folate, and each substrate competed with the other to some extent [244]. However, the greater affinity of PCFT/HCP1 for folate (Km 1 μM) as compared to hemin (Km >100 μM) suggests that folate is the primary and physiologically more relevant substrate for this transporter [244,245]. However it should be mentioned that the concentration of heme in the gastrointestinal tract following a meal is likely many times higher than that of folate.

Ectopically expressed PCFT/HCP1 in HEK293 cells co-localizes with Hb:Hp complexes and transferrin in early endosomes, and has thus been hypothesized to cycle back and forth to the plasma membrane, possibly due to its di-leucine motif [246]. These investigators speculate that PCFT/HCP1 functions in endosomes to export heme released from Hb:Hp into the cytosol for degradation by microsomal HO. A *bona fide* physiological role for PCFT/HCP1 in heme metabolism awaits further clarification.

5.2.2. Heme responsive genes in *Caenorhabditis elegans*

The nematode, *C. elegans* is a heme auxotroph, that is, it lacks endogenous heme synthesis, depending on environmental heme for survival, thus providing a “clean” genetic background to study intracellular heme trafficking pathways. *C. elegans* has been studied as a model organism for more than 40 years. The genome has been sequenced, and many human genes are conserved in *C. elegans*. In addition, the ease of genetic manipulation makes it a unique model system for the identification of heme transporters [162,247]. Heme uptake is a regulated process in *C. elegans*; when worms are grown in optimal-to-low heme conditions (<20 μM) and then exposed to ZnMP, upregulation of heme transporters allows rapid internalization of ZnMP, however ZnMP uptake is reduced significantly when worms are grown in medium containing 100 μM heme [150]. Hamza's group performed microarray analyses to identify heme-regulated genes (*hrg*) in *C. elegans*, during growth under low heme (4 μM) versus optimal (20 μM) versus high heme conditions (500 μM), identifying a total of 288 genes (now available, [248]). Initially focusing on genes upregulated in low heme conditions, they further sorted them based on the predicted presence of transmembrane domains, known transporter function and/or heme- or metal-binding domains. A heme-

responsive gene fulfilling these criteria, *hrg-4*, and three of its putative paralogs (*hrg-1*, *hrg-5* and *hrg-6*) were subjected to further studies [150]. The expression of both *hrg-4* and *hrg-1* is modulated by the heme concentration in the growth media in contrast to *hrg-5* and *hrg-6*, which are expressed constitutively. RNAi-based knockdown of CeHRG-4 abolishes uptake of ZnMP in worms grown in low heme, and their progeny are resistant to feeding with the cytotoxic heme analog gallium protoporphyrin IX (GaPP). In addition, in voltage clamp experiments on *Xenopus* oocytes in the presence of hemin, injection of the *Cehrg-4* cRNA into the oocytes resulted in significant inward currents. These findings combined with localization studies indicate that CeHRG-4 is a plasma membrane-associated heme importer, expressed in the worm intestine. Unfortunately, mammalian orthologs are lacking [150].

In contrast, *hrg-1* has orthologs in vertebrates. CeHRG-1 (19 kDa) has four predicted transmembrane domains and, like FLVCR, contains predicted YXXØ and distal di-leucine motifs. These motifs lie adjacent to a C-terminal heme-binding motif. CeHRG-1 knockdown paradoxically increases ZnMP uptake and does not protect *C. elegans* from GaPP toxicity. The human ortholog hHRG-1 is widely expressed, but not regulated by heme or iron, at least at the transcriptional level. Both CeHRG-1 and hHRG-1 extensively co-localize with acidic endosome-lysosome organelles in HEK293 cells, and their affinity for heme decreases with increasing pH. *Xenopus* oocyte studies (as above) were also consistent with heme transport across a membrane. Thus the available evidence indicates that these proteins transport heme across the endosome-lysosomes into the cytoplasm. Notably, knockdown of the zebrafish ortholog results in marked anemia and developmental defects suggesting the importance of lysosomal heme-recycling from organelle hemoproteins.

In a more recent study [248], Hamza's group has systematically knocked down all 288 worm genes in a transgenic worm that responds to changes in heme levels by altering GFP expression. Genes causing alterations of ± 2 -fold were further examined using a cytotoxicity assay (resistance to GaPP toxicity). In addition to identification of HRG-4, an ABC transporter, MRP-1, which appears to be a cell membrane permease that exports heme, and F22B5.4, a single TMD protein of unknown function, were identified. In sum, their studies vindicate the use of *C. elegans* to rapidly identify both cell surface and cell organelle heme transporters. Further analysis may uncover yet other heme transporters. Approximately 40% of the 288 worm genes have orthologs in the human genome and thus it is likely that vertebrate transporters will also be identified.

6. Conclusion

There is a large body of literature that supports a seminal role for the HO system, predominantly HO-1, in protecting cells from oxidative stress. Although both HO-1 and, to a lesser extent, HO-2 are important for heme-iron reutilization, the murine model of FLVCR deficiency emphasizes the importance of FLVCR-mediated heme export and heme recycling in systemic iron homeostasis. Future studies should delineate the importance of systemic heme recycling, especially in anemia of chronic disease where iron but not heme export is subject to suppression by hepcidin; the relative contributions of the HO system and heme export in maintaining intracellular heme levels; and the physiological conditions whereupon heme export rather than HO-mediated catabolism occurs.

FLVCR is required during erythropoiesis and likely functions as an overflow valve to mitigate fluctuations in heme levels that may occur prior to or at the onset of globin synthesis. Investigation of FLVCR trafficking, protein-protein interactions and internalization/degradation pathways will yield additional insights into the function and regulation of this protein.

Of the mammalian ABC transporters, ABCG2 may have functional redundancy with FLVCR. However, the distinctively different knockout mice phenotypes suggest their physiological roles do not overlap.

Notably, both proteins are expressed in K562 cells; thus, it would be of interest to inhibit the function of each in turn and determine their relative contributions to cell heme export.

PCFT/HCP1 was initially identified as a duodenal heme importer, yet subsequent studies indicate that it functions primarily as a folate transporter; thus, its physiological role in heme acquisition requires further clarification. The *Pcft* knockout mouse has recently been generated. Although it appears to have significant disorders in homocysteine metabolism related to folate deficiency, its potential role in heme/iron metabolism has not been addressed [249].

Both ABCB6 and ABCB10 are mitochondrial proteins consistently co-expressed with heme synthesis pathway enzymes, suggesting roles for these proteins in heme metabolism. A physiological substrate of ABCB6, relevant to heme synthesis, has yet to be identified. The last step of heme synthesis, iron insertion into PPIX by Fech, occurs on the matrix side of mitochondria, thus indicating the presence of unidentified heme exporters on both the mitochondrial IM and OM. As discussed, ABCB10 physically interacts with Fech and stabilizes Mfrn1, but it remains to be established whether it has a transport function (perhaps heme) in addition to its structural role on the mitochondrial IM.

A recent breakthrough in heme transport biology is the identification of HRG proteins in the heme auxotroph *C. elegans*. HRG-1 and its human ortholog co-localize to the endosome/lysosome. Further analysis of HRG proteins in *C. elegans* [248] and humans [63] will further our knowledge of heme transport and metabolism.

Increased understanding of cell-surface heme transport proteins will provide insights into disease states characterized by the release of free (toxic) heme, including both congenital and acquired hemolytic anemias, sickle cell disease, thalassemias, malaria and the myelodysplastic syndromes. Fundamental questions in heme physiology remain unanswered, including the identity of transporters involved in the transfer of heme and heme intermediates across mitochondrial membranes, the sites of heme transfer to apoproteins to form hemoproteins, the identity of intracellular heme chaperones, the comparative contributions of FLVCR and the HO system to cell protection from oxidative stress and/or heme excess, and the role of systemic circulation of heme in iron homeostasis and its other functions.

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