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One ring to bring them all and in the darkness bind them: The Trafficking of Heme without Deliverers.

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Abstract

Heme, as a hydrophobic iron-containing organic ring, is lipid soluble and can interact with biological membranes. The very same properties of heme that nature exploits to support life also renders heme potentially cytotoxic. In order to utilize heme, while also mitigating its toxicity, cells are challenged to tightly control the concentration and bioavailability of heme. On the bright side, it is reasonable to envision that, analogous to other transition metals, a combination of membrane-bound transporters, soluble carriers, and chaperones coordinate heme trafficking to subcellular compartments. However, given the dual properties exhibited by heme as a transition metal and lipid, it is compelling to consider the dark side: the potential role of non-proteinaceous biomolecules including lipids and nucleic acids that bind, sequester, and control heme trafficking and bioavailability. The emergence of inter-organellar membrane contact sites, as well as intracellular vesicles derived from various organelles, have raised the prospect that heme can be trafficked through hydrophobic channels. In this review, we aim to focus on heme delivery without deliverers - an alternate paradigm for the regulation of heme homeostasis through chaperone-less pathways for heme trafficking.

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Declaration of competing interest

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Keywords

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1. Introduction

Heme (iron protoporphyrin IX) is an essential but potentially cytotoxic iron-containing organic ring that functions as a cofactor, signaling molecule, and nutrient [1–9]. As a cofactor, heme endows proteins with the ability to mediate electron transfer, catalysis, and gas binding and transport [2, 7]. As a signaling molecule, heme binding to or dissociation from a target protein can alter its expression, activity, or both [4, 10–16]. For instance, heme regulation of transcription factors, translational activators, ion channels, kinases, and micro-RNA processing factors collectively act to control a multitude of pathways, including the antioxidant stress response, mitochondrial respiration and biogenesis, mitophagy, translation, apoptosis, circadian rhythms, and cell proliferation [5, 17–33]. As a nutrient, heme-iron is a major source of dietary iron for many microbes and animals, being more bioabsorbable than non-heme iron [34]. However, the very same properties of heme that nature exploits to support life, including its redox activity, Lewis acidity, and hydrophobicity, also renders heme potentially cytotoxic [11]. Indeed, if inappropriately handled by cells, heme can catalyze deleterious redox reactions and/or become misincorporated into proteins, nucleic acids, or lipid bilayers, thereby disrupting biomolecular structure. Unsurprisingly, defects in heme homeostasis are associated with numerous diseases, including cardiovascular, neurodegenerative, and hemolytic disorders, anemias, porphyrias, and certain cancers [6]. As a consequence of the duality of heme being an “essential toxin”, cells and organisms must safely mobilize heme from sites of synthesis or uptake to heme-dependent proteins and pathways throughout the cell. Here, we review current knowledge and discuss future paradigms in eukaryotic heme transport and trafficking.

In order to utilize heme, while also mitigating its toxicity, cells are challenged to tightly control the concentration and bioavailability of heme. Cellular heme concentration is governed by the relative rates of synthesis, degradation, import, and export. The synthesis and degradation of heme are well understood processes and have been reviewed extensively elsewhere [4, 5, 35–39]. Indeed, all eight eukaryotic heme biosynthetic enzymes and heme degrading heme oxygenases (HMOX)s are structurally characterized to atomic resolution and their molecular mechanisms have been studied in depth [36, 40–42]. By contrast, the cellular import and export of heme, as well as the factors governing heme trafficking, remain poorly understood and have been an area of active research over the past 15 years since the discovery of the first eukaryotic heme transporters [43–46]. Analogous to other transition metals, such as copper and iron, it is thought that a combination of membrane embedded transporters, soluble heme carriers, and chaperones coordinate the distribution and insertion of heme into hemoproteins residing in every subcellular compartment [6, 47–50]. Certainly, as discussed herein and summarized in Figure 1, this is borne out by the discovery of a number of proteinaceous factors that fall under each of the aforementioned categories. However, far less attention has been given to the potential role of non-proteinaceous

biomolecules in controlling heme trafficking and bioavailability, including lipids and nucleic acids [11].

Heme, as a hydrophobic molecule, is lipid soluble and can interact with biological membranes. The emergence of inter-organellar membrane contact sites (MCSs), as well as intracellular vesicles derived from various organelles, have raised the prospect that heme can be trafficked through hydrophobic channels present at MCSs or within vesicles [4, 11, 51]. Additionally, the π-conjugated electron system of the heme porphyrin can interact with other aromatics, including certain nucleobases, through π-stacking interactions. For instance, guanine-tracts in RNA and DNA, can stack to form guanine-quadruplexes (G4s) and bind to heme via π-π interactions, potentially regulating heme bioavailability *in vivo* [52]. In this review, we aim to focus on an alternate but emerging paradigm for the regulation of heme homeostatic pathways through "chaperone-less" pathways for heme trafficking.

2. Protein-based heme trafficking mechanisms.

2.1. Heme Import and Export Pathways.

Leading into the last decade, the canonical viewpoint of metazoan heme homeostasis was that every cell makes and utilizes its own mitochondrial-derived heme. Thus, the concept of heme transport was limited to intestinal heme absorption as a means of iron acquisition, and scavenging circulating heme and hemoglobin (Hb) released due to hemolysis via hemopexin and haptoglobin, respectively [53–60]. Internalized heme from hemolysis was largely thought to be degraded by heme oxygenases, releasing iron that can then be recycled for new heme synthesis, stored, or distributed to other cells and tissues. However, with the discovery of heme exporters and importers, new paradigms for cellular heme detoxification and new hypotheses involving the exchange of heme between cells for nutritional purposes or intercellular communication were born [4, 5, 8]. These concepts still remain controversial [61]. For instance, the need for a heme exporter to detoxify excessive heme clashes with current notions that heme degrading heme oxygenases are sufficient to protect cells from heme toxicity [61]. The need for a cell to import heme is obviated by the fact that heme is biosynthesized by most eukaryotic cells [61]. Nevertheless, life evolved heme transporters and in this section, we review heme import and export pathways.

The earliest examples of metazoan heme internalization involved pathways for scavenging cell-free heme and Hb. Hemolysis during diseases such as malaria, sickle cell anemia, autoimmune hemolytic anemia, and cancers causes rupturing of red blood cells which releases toxic heme and Hb into circulation [62–69]. The proteins required for scavenging heme and Hb include hemopexin and haptoglobin [70, 71]. Circulating heme is bound by hemopexin (HPX) and the complex is endocytosed via the LRP1 (CD91) receptor at many different tissues including macrophages, hepatocytes, placental syncytiotrophoblasts and neurons where the hemopexin is recycled and the heme is degraded [72–76]. Likewise, cell-free Hb is bound by haptoglobin and subsequently endocytosed, via binding to the CD163 receptors on macrophages, and degraded for iron recycling [77–81]. Additionally, other serum proteins such as human serum albumin (HSA) have been shown to participate in heme binding and scavenging, albeit with much lower affinities. For reference, the HPX-heme K_d

is 5 fM, whereas the HSA-heme K_d values have been measured to be as low as 20 pM or as high as 40 μM [82–85]. Alpha 1 microglobulin (A1M) and high density lipoproteins can also requisition heme in circulation and are thought to serve as additional scavengers [86–90]. Though not the focus of this review, these examples highlight the importance of proteins that bind and limit circulating heme to prevent cellular and tissue damage [57, 91].

Another pathway for cellular heme uptake is through phagocytosis of red blood cells (RBCs) by reticuloendothelial system (RES) macrophages [92–95]. Erythrophagocytosis (EP) eliminates RBCs from the circulation as they become damaged or senescent, and serves as the primary method for recycling the iron within heme [96–99]. Hb accounts for > 90% of a mature red cell protein, which translates to a staggering one billion heme molecules [92, 100–102]. After RBCs are broken down in the endophagolysosome, this bolus of potentially cytotoxic heme must be transported out of the lysosomal lumen and trafficked to the ER-tethered HMOX1 to be broken down for its iron [103–105]. The mechanism underlying how heme reaches HMOX1, particularly the trafficking to HMOX1's active site in the cytosol is not known [4, 93, 106, 107].

The notion that all free-living metazoans made their own heme was challenged by the discovery that the microscopic nematode, *Caenorhabditis elegans,* lacked all eight enzymes for heme biosynthesis [108]. However, this heme auxotroph still requires hemoproteins and acquires environmental heme through diet and then disseminates it to other cells throughout its body for survival [4, 108, 109]. Though clearly this mechanism is an outlier amongst eukaryotes, *C. elegans'* unique heme auxotrophy therefore allowed for the study of heme transport and trafficking without the confounding contributions from endogenous heme synthesis [8, 108, 110]. It was also possible that the pathways that enabled the distribution of heme to different tissues and cells in *C. elegans* may also be conserved in the vast majority of animals that can synthesize heme.

As has been reviewed elsewhere, genetic screens and subsequent characterization of heme-regulated genes have identified novel importers, exporters, chaperones and signaling molecules [4–6, 8, 9, 12, 39, 50, 110, 111]. HRG-1 and its homologs HRG-4, −5, −6 were the first *bona fide* eukaryotic heme transporters to be discovered, whose critical function of heme import into the cytosol is evolutionarily conserved [44]. The worm-specific HRG-4 localizes exclusively to the plasma membrane and helps import heme directly from the intestinal lumen, whereas HRG1 typically resides in endo-lysosomal recycling compartments and has orthologs from arthropods to vertebrates including humans [44]. HRG1's function has been demonstrated to be closely coupled with V-type ATPases and the lumenal pH of the organelle, as acidic conditions increase free heme solubility and a decrease in pH also raises HRG1's affinity for heme by transport from endosomes in cell lines [44, 112]. Early studies of this four transmembrane domain (TMD) importer showed transport by experimentation with yeast reporter assays, *Xenopus* oocytes, zebrafish embryos, fluorescent heme analogs and genetic manipulations in worms and cell lines but recent genetic knockouts in zebrafish and mouse models have now characterized HRG1's essential role in heme/iron homeostasis at the organismal level [44, 107, 113–115]. Upon EP, HRG1 is highly upregulated in RES macrophages where it is recruited to erythrophagosomal membranes and aids in recycling degraded RBCs by importing heme into the cytosol for

HMOX1 or subsequent trafficking [107]. In zebrafish this recycling occurs in the kidney marrow, the primary adult hematopoietic organ, where fish lacking HRG1 display aberrant histological and heme-iron metabolism gene expression [114, 116]. The loss of HRG1 in mice is even more striking, with a hyper accumulation of HMOX1 inaccessible heme in RES macrophages resulting in enlarged lysosomes and the endogenous production of hemozoin, a phenomenon never seen before in mammals [115]. Critically the organs where these macrophages reside turn visibly dark from the accrual of inert heme crystals which confer the mammalian cells tolerance to heme toxicity, the same mechanism employed by malarial parasites [58, 115, 117]. HRG1 is highly expressed in other non-RES tissues as well, including brain, kidney, intestine, lung, and smooth muscle though its role in these organs remains to be elucidated.

The reasoning that since mammalian cells are capable of synthesizing, utilizing and degrading their own heme calls into question as to why these cells would export heme across the plasma membrane in the first place [61]. This notion has been challenged with the discovery of heme exporters, including FLVCR and MRP5. The feline leukemia virus subgroup C receptor-related proteins (FLVCR) are members of the major facilitator superfamily proteins containing 12 TMDs and were first discovered for their role in non-regenerative anemia in cats resembling pure red cell aplasia in humans [118, 119]. Normally localized to the cell surface, these transporters act as receptors for feline leukemia retroviruses, forming the point of entry for infection and subsequent cellular disruption [119]. Later characterization of their endogenous function revealed their role in heme homeostasis, with FLCVR1 demonstrated to be a heme exporter while its closely related homolog FLVCR2 has been implicated as a heme importer into the cell [43, 45, 120]. Lines of evidence that support this role for FLVCR2 include: binding to hemin-agarose, expression in Xenopus oocytes and CHO cells resulted in accumulation of exogenous heme and heme sensitivity, and depletion by siRNA reduced the transport of the fluorescent heme analog zinc mesoporphyrin (ZnMP) [45]. On the other hand, mutations in the FLVCR2 gene in humans is the cause of the autosomal recessive Fowler syndrome, a proliferative vasculopathy of the brain, which despite associated defects in respiratory complexes, has no obvious dysfunction in heme homeostasis [45, 121]. Furthermore, ectopic expression of FLVCR2 in the yeast reporter system cannot rescue heme-deficient strain’s growth from exogenous heme [113]. For these reasons, FLVCR2 heme import function may need further investigation.

MRP5/ABCC5 was identified as an essential heme exporter in *C. elegans* and for regulating systemic heme homeostasis [122]. MRP5 belongs to the family of multidrug resistance-associated protein (MRP) within the type C subfamily of the ATP Binding Cassette (ABC) transporters, which have been investigated extensively over the past 20-plus years for their role in cancer drug resistance [123–126]. Closely related to MRPs 4, 8 and 9, MRP5 falls in the “short MRP clade” with 12 TMDs split into two membrane spanning domains each with a nucleotide binding cassette, and is typically localized to the plasma membrane and endosomal compartments [122, 126, 127]. Prior to the discovery in *C. elegans*, the role of MRP5 appeared to be that of cyclic nucleotide export from cells as demonstrated by transport of guanosine 3’,5’-cyclic monophosphate (cGMP) in membrane vesicles expressing human MRP5 *in vitro* [128, 129]. However, the generation of *Mrp5*$^{-/-}$ mice

demonstrated little to no contribution in cGMP efflux and no observable overt phenotypes, leaving the physiological relevance of this transporter unclear [130, 131]. However, genetic studies in *C. elegans* showed that intestinal heme acquired from the importers HRG-1/4 is transported by MRP-5 from the basolateral surface of the gut intestinal epithelium for utilization by extra-intestinal tissues [122]. Key lines of evidence for this model include functional heme transport assays in yeast, accumulation of fluorescent ZnMP in the intestine, survival in the presence of the toxic heme analog Ga protoporphyrin IX, high labile heme accumulation in the intestine with a concomitant reduction in extra-intestinal tissues, and embryonic lethality due to *mrp-5* deficiency that is rescued by excess heme supplementation [122, 132]. In vertebrates, knockdown of *mrp5* in zebrafish resulted in severe anemia, indicating a critical role in erythropoiesis for this transporter which is also highly expressed in human RBCs [122, 133]. Furthermore in mammalian cell culture experiments, MRP5 knockout mouse embryonic fibroblasts demonstrated reduced heme export into the secretory pathway, as measured by heme incorporation into a Golgi-targeted horseradish peroxidase reporter [122]. Once in the secretory pathway, the notion is that heme can then be trafficked for incorporation into hemoproteins or directed to other subcellular locations, including the plasma membrane / extracellular space [134].

ABCG2, also known as the breast cancer resistance protein or BRCP, is another family member of the ATP-binding cassette transporters that has been implicated in heme efflux from the cell. ABCG2 is one of the three well-known major multidrug resistance proteins to be discovered (along with MRP1 and P-gp) and consequently its mechanistic function in cancer cells and pathophysiology has been studied exhaustively for over two decades [135–138]. As is common amongst the ABC transporters, ABCG2 is quite promiscuous and has been shown to efflux myriads of drugs/xenobiotics, in particular mitoxantrone, estrone sulfate (E1S), dehydroepiandrosterone sulfate (DHEA), methotrexate, cGMP, topotecan, imatinib, irinotecan and 9-(2-phosphonyl-methoxyethyl) adenine (PMEA) [135–137, 139–144]. Although, a true consensus on ABCG2's endogenous substrate has yet to be identified, studies have shown that it can efflux heme, protoporphyrin IX, and other porphyrins from the cell [145–150]. ABCG2 is expressed in many different tissues, including the erythroid lineage like MRP5, and in particular ABCG2 has been shown to have a role in blood barrier tissues such as the placenta, mammary epithelium and the blood brain barrier [146, 147]. A key study demonstrated that ABCG2 requires an extracellular-binding loop to export heme to circulating albumin [148].

While FLVCR1 was initially described as a plasma membrane heme exporter in developing erythroid cells to efflux heme and reduce toxicity it was determined that FLVCR1 actually has two different isoforms: FLVCR1a and FLVCR1b with different subcellular localizations [46, 151, 152]. FLVCR1a, the full-length 12 TMD protein, which is targeted to the plasma membrane and transports heme from the cytosol, has an alternate start site in its first intron which generates the FLVCR1b isoform. This truncated six TMD protein localizes to the mitochondria and is thought to be responsible for heme transport after the completion of heme synthesis [46]. Strikingly, FLVCR1 null mice which lack both a and b isoforms die *in utero* during mid gestation due to a block in erythropoiesis [151]. In an effort to untangle the roles of each isoform, a mouse model still expressing the mitochondrial FLVCR1b but lacking the full length FLVCR1a was generated. This mutant mouse showed *in utero*

lethality due to hemorrhaging and skeletal deformations [46]. The restoration of erythroid maturation in these mice indicated that FLVCR1a is dispensable for definitive erythropoiesis, and the role for heme transport in the block of erythroid maturation was associated with the mitochondrial isoform [46]. The logical explanation is that rather than accumulation of excess cytosolic heme inducing toxicity, the cell is starved for heme that is trapped in the mitochondria [8, 153, 154].

These findings beg the question - what in fact is the role of FLVCR1a at the plasma membrane, and why does heme need to be exported from the red cell to prevent cell toxicity? This subject is discussed at length in other reviews, and undoubtedly additional questions still remain regarding FLVCR1 as a heme transporter [39, 61, 134]. In particular, gaps in mechanistic biochemical details as to how it functions require further elucidation. It is known that FLVCR1a requires an extracellular heme carrier protein in the media for heme export to be detected [155]. Furthermore, in the presence of a dedicated carrier, hemopexin, this efflux rate is increased over 100-fold versus albumin, in keeping with their relative affinities [155]. Interestingly, this has led to the theory that heme may be channeled from the cytosol through FLVCR1a, docked at critical histidine residues, and subsequently released to the heme carrier protein [153, 155]. Furthermore, this logic implicates extracellular HPX as more than just a traditional scavenger, with a designated role for facilitating heme efflux from the cell for intercellular trafficking. This notion is of particular interest within the scope of this review, as the key residues which are implicated in the interaction of FLVCR1a with hemopexin for facilitating heme transport and loading are missing from FLCVR1b [153]. How then is the heme exported from the mitochondria without a dedicated chaperone? This is a question that has been brought up repeatedly in recent reviews [8, 39, 50, 111, 134].

Long awaited biochemical characterizations of efflux notwithstanding, lacking a traditional chaperone-based export mechanism should not be viewed as a detractor for mitochondrial export, as conceivably heme transported by FLVCR1b or another yet to be identified mitochondrial exporter may be directly pumped into another organelle and subsequently trafficked to other subcellular compartments. Additional questioning lies in where FLVCR1b localizes within the mitochondria, as this has implications for how it acquires and subsequently transports heme. Is the transporter in the inner or outer mitochondrial membrane? Although it is possible that FLVCR1b could interact with the heme biosynthetic machinery clustered around ferrochelatase (FECH) on the inner mitochondrial membrane (IMM), data from affinity purification studies of the complex do not identify FLVCR1b in the complex [156]. If FLVCR1b is in fact on the IMM but not associated with the FECH complex, one possibility is that this may facilitate heme incorporation into certain types of mitochondrial-derived vesicles. Thus, FLVCR1b may constitute one way of contributing to labile heme in the cytosol, however it is likely not the only mechanism.

FLVCR1a makes up the third putative heme exporter described herein which has been shown to localize to the plasma membrane of the erythroid lineage and implicated in the efflux of heme to reduce toxicity [43]. In addition to MRP5 and ABCG2, eight other dedicated ABC transporters have now been shown to be expressed specifically on the plasma membrane of red cells, including the putative coproporphyrinogen III transporter, ABCB6 [157–159]. Originally thought to be yet another heme transporter, ABCB6 is normally

localized to the mitochondria but has an isoform which is directed to the plasma membrane [160, 161]. Critically, overexpression of this plasma membrane isoform was unable to efflux heme, but did reduce the cellular accumulations of another tetrapyrrole, pheophorbide A [161]. Though ABCB6 functions are still not well-understood, it recently was shown to be involved with cadmium detoxification and likely has affinity for multiple substrates [162].

Why are there so many transporters at the surface of RBCs, particularly those capable of effluxing porphyrins? This is perplexing, given the established cytotoxic nature of free heme in circulation and the concerted mechanisms for actively avoiding free heme by scavenging and recycling it. Indeed, it is hard to imagine that RBCs, which need to synthesize a billion heme molecules for incorporation into globins may contain multiple heme or porphyrin exporters. Part of the explanation however may lie with the compromise that is required for higher levels of specialization. In simplistic terms, mammalian red cells become specialized hemoglobin-producing cells to maximize oxygen delivery and systematically strip themselves of all intracellular organelles during differentiation [163]. Therefore a current model posits that FLVCR1a is essential during early stages of red cell differentiation to maintain stoichiometric amounts of heme and globin, and that excess heme causes cell death [164]. Thus, it is reasonable to assume that the exporters orchestrate a delicate balance between ridding the intracellular milieu of a toxic redox-active catalyst versus the physiologic disadvantage of exposing the extracellular milieu, which harbors heme scavenging proteins. Heme import and export may be highly dynamic, thereby mitigating the risk of both intracellular and extracellular heme toxicity. However, the physiological and pathological contexts in which various heme import and export pathways are activated and regulated remain to be determined. Moreover, it is unclear at this time if heme transporters work independently of each other or operate in a synergistic manner. The determination of a temporospatial hierarchy for heme transporters during cell and tissue development will be critical for helping to define heme transport dynamics in various contexts.

2.2. Intercellular heme trafficking.

The discovery that a wide range of cell types in metazoans can both import and export heme raises the possibility that cells and tissues can share their heme quota for a systemic control of heme homeostasis. In *C. elegans*, HRG-3 was discovered to be an essential 8 kDa intercellular heme chaperone protein which binds and delivers maternal heme to developing oocytes [165]. Originally secreted by the maternal intestine, HRG-3 binds heme with a stoichiometry of 2:1 respectively and is released into the interstitial fluid for transport to extra-intestinal cells, including oocytes [165, 166]. Loss of HRG-3 resulted either in death during embryogenesis or developmental arrest immediately post-hatching, which was completely rescued by maternal expression of HRG-3 [165]. Though there are no obvious homologs for HRG-3 in vertebrates, these results demonstrate that heme can be transferred between different tissues and cell types as a nutritional heme source [110, 165]. Another *C. elegans* protein, HRG-2, facilitates heme import and utilization in the hypodermis. HRG-2 is a predicted 32 kDa single-pass type I transmembrane protein and localizes to both the apical plasma membrane and the endoplasmic reticulum [167]. It is thought to function as a heme binding oxidoreductase, as it contains both a thioredoxin-like fold and a glutathione S-transferase (GST) domain, which have been recently demonstrated to bind heme in the

blood-feeding nematode *Haemonchus contortus* [168]. Based on these dual functions and localizations, HRG-2 likely reduces heme at the cell surface to facilitate its entry into the cell, and assists with sequestration or redistribution of intracellular heme at the ER, possibly transferring bound heme to membrane-bound or maturing luminal hemoproteins [110, 167].

The fact that heme can be transported between cells and distal tissues imply that systemic signals may be able to communicate and regulate its trafficking and usage accordingly [110, 165]. Such systems have already been demonstrated for other critical metallo-nutrients, such as hepcidin's regulation of systemic iron homeostasis and the yet to be identified factor that communicates copper deficiency from the heart to copper storage organs [169, 170]. Indeed, in *C. elegans*, a secreted signaling factor termed HRG-7 communicates intestinal heme status to extra-intestinal tissues [171]. HRG-7 belongs to the family of A1 aspartic proteases and is released from the intestine during heme starvation and signals to sensory neurons, which in-turn regulates *hrg-7* expression in the intestine through DBL-1, a worm homolog of bone morphogenetic protein 5 (BMP5), via the "small" worm phenotype "Mothers Against Decapentaplegic" family (SMAD) homolog SMA-9 [171]. Loss of HRG-7 causes a heme-dependent growth defect and a striking upregulation of heme deficiency signal, as the worms are unable to cell non-autonomously regulate intestinal heme import [171]. It remains to be determined whether similar pathways modulate systemic heme homeostasis in mammals as homologs for HRG-7, DBL-1, and SMA-9 exist in mammals. These long-range inter-organ communication systems allow for a concerted physiologic response by the whole organism in response to nutrient fluctuations.

2.3. Intracellular heme trafficking.

Once heme is synthesized on the matrix side of the mitochondrial inner-membrane, or transported into the cell, it must be trafficked to hemoproteins located throughout the cell [4, 5, 8, 39, 48, 173, 174]. To mitigate the potential for heme toxicity, there must be proteins that bind and buffer heme, deliver and insert it into downstream hemoproteins, sequester it into a chemically inert form until it is needed, and transport it into and out of various subcellular compartments [4, 5, 8, 12, 39, 134]. Here, we discuss proteins and processes implicated in these delivery systems.

Due to the high concentration of biomolecules in cells, there is likely no free unbound heme. Intracellular heme can be partitioned between exchange inert and labile heme binding sites. The former comprises proteins that have high affinity heme binding sites with considerable kinetic barriers for exchange. Labile heme (LH) comprises proteins with relatively modest heme binding affinities and sites that are accessible for facile heme exchange between various biomolecules. The network of proteins that bind and buffer LH, which are largely unknown, represent the source of heme for heme-requiring proteins and pathways. Recently, tools for measuring LH using fluorescence and activity-based sensors in yeast and non-erythroid mammalian cell lines have found that LH is in the nM regime, spanning ~20 – 430 nM [12, 132, 175–177]. This implies that the proteins that buffer LH exhibit heme dissociation constants of similar magnitude, providing a thermodynamic signature for proteins that comprise the LH pool.

Overall, while little is known about the speciation of the LH pool, certain proteins have been implicated in buffering heme, including glutathione S-transferases (GSTs), fatty acid binding proteins (FABPs), various heme binding proteins (HBPs), and glyceraldehyde phosphate dehydrogenase (GAPDH). Glutathione S-transferases are a family of Phase II detoxification enzymes that have been shown to bind heme in the cell and play a role in modulating heme homeostasis [178–180]. Highly abundant in the cytosol, the canonical function of GST is to catalyze the conjugation of glutathione (GSH) to electrophilic compounds in order to facilitate their clearance [181]. Though the scope of GST's impact on heme binding and trafficking in mammalian cells is still poorly understood, it is clearly critical in blood-feeding parasites which have redundant means to buffer cytotoxic heme from their diet [182–184]. FABP, whose function (as the name would suggest) involves the trafficking of fatty acids and lipids, can also bind heme, albeit its exact physiological role in maintaining heme homeostasis has yet to be determined [185, 186]. The FABP affinity for heme has been shown to be 10-fold greater than that of some fatty acids such as oleic acid, prompting speculation that it could traffic heme [4, 8, 185]. The heme binding proteins p22HBP and HBP23 also known as heme binding protein 1 (HEBP1) and peroxiredoxin I (PRDX1) respectively, are highly expressed in the liver and have been comparatively well-studied [187, 188]. Originally named for their molecular weights, HBP heme binding affinities (p22HBP/HEBP1: K_d = 26 nM, HBP23/PRDXI: K_d = 55 nM) are greater than those of GSTs and FABPs (K_d = 100–200 nM) [187, 189]. Additionally, their expression has been shown to be inducible by heme-related stimuli; p22HBP by erythroid differentiation in mouse erythroleukemia cells, and HBP23 by heme, protoporphyrin IX, and other metalloporphyrins in hepatocytes [187, 189, 190]. p22HBP bound to both heme and its precursor's crystal structures show tetrapyrrole binding [187, 189, 191]. In summary GSTs, FABPs, and HBPs all bind heme, though evidence for their specific roles in heme homeostasis is lacking. While all these factors exhibit heme binding affinities within the estimated concentration range of the LH pool, suggesting that these proteins may be components of a network of heme buffering proteins, experiments probing their individual and cumulative roles in heme homeostasis are currently lacking.

GAPDH is a canonical glycolytic enzyme that was recently found to play key roles in heme homeostasis as both a heme buffering molecule and a chaperone [192–194]. Of note, GAPDH is able to regulate heme delivery and insertion into hemoproteins such as nitric oxide synthase (NOS) and guanylate cyclase [193, 195, 196]. The stoichiometry and mechanisms of binding for GAPDH have been elucidated, with one heme bound per GAPDH tetramer and coordinated by a highly-conserved histidine, His51 [197, 198]. His51, which is conserved from yeast to human, appears to be essential for heme binding and trafficking to NOS and other hemoproteins, but also for heme delivery to transcription factors in the nucleus [175, 197, 198]. Furthermore, GAPDH has been shown to be an important component of the heme buffering system, with a heme binding affinity of 24 nM similar to estimates of labile heme in the cytosol [175, 199].

The progesterone receptor membrane component (PGRMC) proteins were recently classified as intracellular heme chaperones. Although PGRMCs have been known to be capable of heme binding, they now have been discovered to have essential roles in the distribution of heme from the mitochondria [156, 200–203]. Indeed, both PGRMC1 and

PGRMC2 can be localized to the mitochondria, where they were confirmed to be interacting with the heme biosynthetic machinery clustered around FECH, the final step in heme production [156]. In probing this relationship further, it was determined that PGRMC1 is in fact capable of regulating heme synthesis via interactions with FECH and was suggested to be a chaperone for efflux from the mitochondria [202]. Key supporting lines of evidence for this included: inhibition resulted in observed decrease in hemoglobinization in erythroid differentiation model; purified PGRMC1 was able to donate heme to apo-cytochrome b5; and *in vitro* measured FECH activity decreased in a PGRMC1 dose-dependent manner [202]. The ER-based PGRMC2 was required for heme delivery to transcription factors in the nucleus [203]. Specifically, deletion of PGMRC2 in brown fat adipocytes reduced LH in the nucleus and altered heme regulated gene expression resulting in severe mitochondrial defects and a failure to activate adaptive thermogenesis, while obese-diabetic mice treated with a small-molecule activator of PGRMC2 showed substantial improvement of diabetic features [203]. Though additional studies are required to determine their exact roles in trafficking, these PGRMCs serve as an outlet for newly synthesized heme to reach distinct compartments, such as hemylating ER-localized cytochromes or distributing to nuclear transcription factors [39, 134].

Unlike iron that is encased in ferritin for storage, there are no known heme storage proteins that sequester heme until it is needed. However, recently, transient absorption imaging of heme in *C. elegans* and mammalian cell lines revealed the presence of heme containing granules [204]. These compartments of heme appear to be highly dynamic and can be broken down and mobilized within the cell [204]. While the physiological function and physical composition of these granules are unknown, it is tempting to speculate that they represent novel heme storage sites. Though these heme granules were initially attributed to membrane-enclosed puncta-like structures, it is also possible that they may be maintained in distinct biocondensates, or “membrane-less” compartments akin to p-granules in the cell cytosol [205]. This liquid-liquid phase separation may facilitate its accumulation and rapid dissemination as a heme store for hemoproteins and signaling in an aqueous environment [205, 206]. Biocondensate formation may be driven by scaffolding or by heme binding / chaperoning proteins. Elucidation of these heme granules will be an exciting new avenue for future research in understanding cellular heme homeostasis and trafficking.

3. Membrane centered heme trafficking mechanisms.

3.1. Heme trafficking through membrane contact sites.

Inter-organelle membrane contact sites have emerged as being critical for signaling and metabolite exchange, including the transfer of various phospholipids [207–220]. MCSs are physical contacts between two membrane-bound organelles and have key characteristics, including structural proteins that physically tether two membranes, functional proteins that fulfill specific role(s) for a given MCS, and a defined lipid composition that supports MCS function [51]. While the type, frequency, function, and composition of MCSs are still under active investigation and a matter of debate, virtually all MCSs identified to date involve the ER, including ER-Mitochondria, ER-Plasma Membrane, ER-Golgi, ER-Peroxisome, ER-Lysosome/Vacuole, and ER-Lipid Droplet contact sites [190] (Table 2 and Figure 2). More

recently, mitochondrial-nuclear contact sites have been purported to be identified [221]. MCSs are also highly dynamic and remodeled in response to various physiologic stimuli and perturbations to other contact sites [222, 223]. Given that the ER forms contact sites with the mitochondria, where heme is synthesized, and many other subcellular compartments that house hemoproteins, we previously proposed that the ER network may serve as a highway for intracellular heme distribution [4, 11]. Indeed, we recently found that mitochondrial-ER contact sites, the first junction in this proposed heme distribution network, is important for regulating heme trafficking to the nucleus [224].

ER-mitochondrial contact sites are arguably the most well understood in *Saccharomyces cerevisiae*. In Baker's yeast, two types of ER-mitochondrial contact sites have been identified, the ER-Mitochondria Encounter Structure (ERMES) and ER Membrane Protein Complexes (EMCs) [51, 239, 252]. In humans, ER-mitochondrial contact sites are less well defined and are referred to as Mitochondria-Associated Membranes (MAMs). In yeast, ERMES facilitates calcium signaling, phospholipid exchange, protein import, and mitochondrial dynamics [204, 205]. In order to identify factors that regulate heme distribution, including the role of ER-mitochondrial contact sites, heme distribution dynamics were probed in yeast using genetically-encoded heme sensors targeted to different cellular locales, including the mitochondrial matrix, cytosol, and nucleus. Interestingly, upon initiation of heme synthesis, heme is trafficked from the matrix side of the IMM, where the last step of heme synthesis occurs, to the nucleus ~25% faster than to the cytosol or mitochondrial matrix [224]. The faster rate of heme trafficking to the nucleus suggested there is a pathway that bypasses export into the cytosol prior to import into the nucleus and raised the prospects of a more direct mechanism for heme transfer between the mitochondria and nucleus. One possibility is that there are mitochondrial-nuclear contact sites, which has only recently been proposed [221]. Another possibility is that heme is transferred via the ER network which forms contact sites with the mitochondria and is contiguous with the nuclear envelope [132].

In order to test the role of ERMES in nuclear heme distribution, *Saccharomyces cerevisiae* deletion mutants with defects in ERMES were screened for alterations in nuclear heme trafficking rates [224]. As indicated in Figure 3 and Table 2, there are four structural proteins, Mdm12, Mmm1, Mdm34, and Mdm10, that tether the ER and mitochondrial outer membrane in the ERMES complex [239, 240, 253, 254]. Moreover, ERMES is highly dynamic and responsive to mitochondrial dynamics. ERMES is required for the ER-mediated constriction of mitochondrial tubules to help facilitate mitochondrial division by the GTPase, Dnm1. After division, the GTPase Gem1 disengages ERMES, thereby releasing the ER from the mitochondrial tubule [240, 255]. There is an increased prevalence of ERMES if mitochondrial division cannot occur or occurs at a lower rate than fusion since the completion of a division cycle initiates the release of the ER from the mitochondria. Conversely, there is a decreased prevalence in ERMES if mitochondrial fusion cannot occur or occurs at a faster rate than division. Thus, deletion of core components of ERMES or mitochondrial dynamics factors are expected to alter the frequency of ERMES, thereby providing an excellent test bed to probe the role of ER-mitochondrial contact sites in heme trafficking. Consistent with a role for ERMES in mediating mitochondrial-nuclear heme transfer, deletion of *GEM1* and *DNM1*, which increase ERMES when deleted, increase

mitochondrial-nuclear heme trafficking rates [224]. Moreover, deletion of *MGM1*, a GTPase required for mitochondrial fusion that decreases ERMES when deleted, decreases mitochondrial-nuclear heme trafficking rates [224]. However, deletion of core ERMES components, Mdm10 and Mdm34, did not affect heme trafficking rates [224]. The lack of a phenotype in ERMES tethers might suggest that other mitochondrial-ER contact sites provide alternative routes for heme trafficking. Indeed, other studies have also reported that phosphatidylserine transfer is not affected in cells lacking ERMES [239, 256, 257]. A major experimental challenge to contend with is the fact that perturbation of one ER-mitochondrial contact site alters the distribution of other types of mitochondrial contact sites, thereby masking heme trafficking phenotypes. Future work should involve multi-genic knockouts of multiple contact sites to address how these pathways act to mobilize heme to distinct cellular locations or if these pathways are uni- or bi-directional. In humans, mitochondrial-ER contact sites have composition that is distinct from yeast and is less well understood [258]. Future studies will be needed to determine if ER-mitochondrial contact sites in mammalian cells influence heme trafficking and mobilization.

Mitochondria-lysosomal/vacuolar contact sites are a particularly intriguing axis with respect to heme homeostasis [250, 259–263]. Lysosomes are critical for mitochondrial recycling, fusing with autophagosomes to engulf, and degrade entire mitochondria in a process called mitophagy [264–267]. Furthermore, mitochondrial derived vesicles (MDVs) bud off from mitochondria and can fuse with distant lysosomes, likely facilitating more specific mitochondrial protein turnover [268]. We envision heme may be recycled in such processes, *i.e.* stored in the lysosome/vacuole upon degradation of mitochondrial material and subsequently re-routed to the mitochondria when needed via mitochondria-lysosome/vacuolar contact sites. Alternatively, a mitochondrial-lysosome/vacuolar contact site may serve as an exit valve if heme builds up in the mitochondria to excessive levels. Given the compendium of other MCSs (Table 2), systemic perturbation of these contact sites along with measurements of bioavailable heme distribution will reveal novel heme trafficking networks.

3.2. Heme trafficking through membrane vesicles.

Another mode of heme transport and trafficking involves the use of membrane vesicles. Heme can be exported, imported, and trafficked intracellularly using such vesicles. For instance, RBCs are known to shed heme-loaded microparticles, especially during pathophysiological conditions such as sickle cell disease and acute lung injuries / respiratory distress syndromes [269, 270]. These submicron vesicles have also been described in great detail as a challenge associated with the erythrocyte storage lesion, a term used to describe the structural, biochemical, and metabolic changes associated with prolonged aging of stored red blood cell units for transfusions [271]. RBCs suffer from a loss of structural integrity in accelerated senescence and as a result have increased shedding of exosomes loaded with hemoglobin and heme [272–274]. These microvesicles can be highly inflammatory to the vasculature, particularly to pulmonary endothelial cells which have been shown to endocytose them in Rab5-dependent manner [274]. Heme transport via microparticles or exosomes in circulation is a relatively new field of study and warrants additional investigation, particularly to determine if this mechanism of heme uptake by cells is really

only relevant in pathophysiologic contexts and if other cell types are capable of releasing heme in exosomes, microparticles, and other membranous components.

Heme can also be imported into the cell via vesicular endocytosis / phagocytosis. As discussed earlier, Hb and HPX receptors in vertebrates have well established mechanisms to bind their extracellular heme sources and internalize them via traditional endocytosis [70, 71]. Likewise, eukaryotic microbes such as *S. pombe* can also bind extracellular heme via cell surface heme receptors, *e.g.* Shu1, for import by endocytosis and subsequent trafficking to the vacuole for storage [275–277]. Intracellular movement of heme-loaded membrane vesicles already inside the cell could be facilitated via a similar process, with endosomal sorting driving mobilization to other organelles. Aligned with this, prior studies have found that iron can be transferred from the endosome to the mitochondria through a “kiss and run” mechanism [278, 279]. Heme could be trafficked to the mitochondria in a similar endosome-dependent manner. Moreover, the endosomal-based vesicular transport would allow for direct connection to the ER, as ER-endosome contact sites have been reported and function in mediating cholesterol transport [280, 281]. Heme trafficking to the ER highway via the endosome would allow heme to be mobilized throughout the entire cell.

An additional mechanism that may compliment this endosomal vesicle heme trafficking is the intracellular distribution of mitochondrial-sourced heme within MDVs, which are single- or double-membrane bound vesicles approximately 70 – 100 nm in diameter that are released from the mitochondria [282]. Initially, these MDVs were characterized as a specific form of vesicular transport to the peroxisome, as some vesicles that contained the mitochondrial marker mitochondria-anchored protein ligase (MAPL) were shown to be able to fuse with peroxisomes; likewise a subset of peroxisomes also possessed matching MDV markers [282]. Furthermore, this novel vesicular transport was determined to be cargo-specific as MDVs that were positive for MAPL lacked another mitochondrial outer-membrane marker, TOM20, while the MDVs that contained TOM20 excluded MAPL from their membranes [282]. This distinct vesicle population labeled with TOM20 did not fuse with peroxisomes, but rather was later determined to be selectively transported to the lysosome [283]. Critically, MDV formation targeted to the lysosome is distinct from mitophagy-dependent markers and results in the transport of proteins from healthy, non-fragmented mitochondria [283]. Taken together, these findings indicated that MDVs are able to discriminate between types of cargo loaded within them and likely the incorporation of specific cargo is a primary factor for their requisite trafficking [282–284]. Transfer of heme via such vesicles would not only minimize the release of cytotoxic heme, but could also facilitate regulated movement of sequestered heme from its “depot” for rapid availability [4]. Additionally, if it is determined that these MDVs can be targeted to additional organelles, it is easy to visualize mitochondria effectively becoming their own mini-secretory pathway for the distribution of heme as well as other mitochondrial components and products by cargo vesicles [285]. The trafficking of heme via MDVs may not even require heme crossing the IMM, as a subset of the MDVs are stainable by membrane-potential dyes (MitoTracker Red and tetramethylrhodamine, ethyl ester TMRE), indicating both their membranes and electrochemical potential are intact [282, 283]. Additionally, subsets of MDVs were shown to contain matrix protein markers such as pyruvate dehydrogenase (PDH) as well as

complexes II, III, and IV of the IMM but would not incorporate complexes I and V or nucleoids, further demonstrating their specificity of cargo incorporation [283, 286].

What are the major cargo for MDVs? It has been proposed that the primary function of MDVs is the selective removal of oxidized proteins, complexes and lipids, as oxidized species were found to be highly enriched in their cargo [286]. Ultimately, the contents being sequestered by these MDVs and their purpose still remain to be fully elucidated, and may very well include heme [286, 287]. What is further reinforced by these studies however is the longstanding intimacy already understood between peroxisomes and the mitochondria [288, 289]. With shared major roles in fatty acid metabolism, reactive oxygen species (ROS) production / scavenging, and now clear physical and metabolic interactions, it has long been postulated that these organelles are evolutionarily intertwined [287, 288, 290, 291]. Indeed, they are more than just distant relatives, as recent studies have now demonstrated the essential role for mitochondria in the *de novo* generation of peroxisomes in mammalian cells via the budding of vesicles coined pre-peroxisomes [292]. As there is a critical need for heme to be trafficked between these two organelles (incorporation into key antioxidant heme binding protein catalase), it may make both physiological and evolutionary sense for heme to be trafficking via this highly specific and conserved vesicular route.

4. Nucleic Acids and Heme

Nucleic acid polymers have been shown to form various secondary structures. DNA forms double stranded helices and guanine quadruplexes (G4), while RNA has a more diverse repertoire consisting of hairpins, internal loops, pseudoknots, bulges, multiloops, and G4s. One common distinct structure between DNA and RNA is G4s, formed at guanine-rich sequences via Hoogsteen hydrogen bonding of four guanine bases to create a square planar guanine tetrad (G-quartet or G-tetrad). Two or more of these G-quartets stacked together form a G4, which are typically folded in parallel, antiparallel, or hybrid structures. This nucleic acid folding results in various topologies as G4 formation is primarily determined by strand polarities and loop orientation. Whether these different topologies influence G4 function is yet to be elucidated.

4.1. Heme association with guanine quadruplexes.

G4 structures are enriched at promoter and telomere regions and have been shown to affect transcription, reverse transcription, translation, and epigenetics [293–295]. For example, during transcription elongation DNA G4 formation in the template strand can inhibit RNA polymerases and impede transcription. The presence of G4s have also been shown to contribute to double-stranded breaks inducing DNA instability [296]. As a protective mechanism, helicases can unfold these G4s to limit DNA damage [297]. In fact, many cancer cells have mutated, dysfunctional helicases allowing for G4 formation, resulting in the progression of DNA damage and transcription stalling [298]. Apart from transcription, it was reported that RNA and DNA G4 interacts with porphyrins, including heme, enabling catalysis and protection against heme-related oxidative damage [299, 300]. Specifically, G4s associated with hemin exhibited peroxidase activity [301, 302]. These interesting findings

represent yet another unique heme interaction in the cell that may affect its bioavailability and mobilization.

The earliest evidence for porphyrin interactions with G4s was nearly 25 years ago, with the discovery of “DNAzymes” capable of catalyzing the metalation of mesoporphyrin IX [301]. Since then, heme itself has been shown to interact with nucleic acids, as its planar and hydrophobic characteristics enable stacking with the G4 structure, corroborated by specific docking and molecular dynamics studies [303, 304]. Recently, a new role for G4s regarding heme bioavailability was reported [300]. This study showed that G4s in mammalian cell culture sequester heme and, when G4s are disrupted, genes related to heme and iron homeostasis are dysregulated, including over a 30-fold induction of *HMOX1*. These results highlight G4s’ potential ability to regulate heme availability and gene expression. However, it is unclear at this point if the displaced heme from G4s directly or indirectly affect HMOX1 expression. Indeed, other genes found to be upregulated by heme and synthetic G4 ligands included ferritin heavy chain *FTH1*, Glutamate-Cysteine Ligase Modifier Subunit *GCLM*, and NAD(P)H Quinone Dehydrogenase 1 *NQO1*, which all share nuclear factor erythroid 2-related factor 2 (NRF2) as a common regulator [300]. Therefore, the release of heme could be indirectly activating HMOX1 through the stimulation of oxidative stress and subsequent stabilization of NRF2 [300].

G4 formation in rRNA and human ribosomes *in vivo* have also been reported [305]. rRNA is the most abundant RNA in cells and the greatest source of cytosolic G4s. Mestre-Fos et al. reported rRNA G4-hemin interactions *in vivo* and established their role in buffering cytosolic heme [305]. Inducing rRNA G4s using PhenDC3, a small molecule that stabilizes G4s, resulted in lower cytosolic heme as measured by fluorescent heme sensors [305]. These results suggest that, upon induction, rRNA G4s can bind and sequester heme, thereby limiting its availability in the cytosol. Overall, these results provide evidence for G4s in regulating cellular heme levels. However, the precise physiological roles and context of G4-heme binding remain to be determined. Are G4s simply a storage site for heme? Or are there specific heme-dependent reactions catalyzed by G4s that are physiologically important? For instance, could the catalase activity of G4-heme defend nucleic acids against peroxide stress? Could rRNA G4s participate in catalyzing hemylation of nascent hemoproteins? Much work still remains to be done to characterize the significance of G4-heme interactions.

5. Conclusion

We envision that the area of heme trafficking and homeostasis will flourish as more discoveries, either planned or serendipitous, will fill in the enormous gaps in our knowledge. Clearly, in the past 15 years new molecules and pathways have been identified through directed genetic screening in model systems, creating sensitive reporters and sensors for dynamic heme monitoring, and establishing new platforms for label-free imaging modalities. Emerging concepts from membrane trafficking, some that were considered radical just 30 years ago, have further contributed to our understanding of how heme that exhibits properties of both, transition metals and lipids, can move within organelles [212]. We envision that this movement will not be restricted to between organelles but extends to the myriad of cell types that constitute whole organs. It is now time to rethink heme beyond a

static macrocycle contained within a protein inside a cell to one that is dynamic and available on-demand at the intercellular and organismal level.

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Highlights

- Heme is an essential cofactor, signaling molecule and metallonutrient.
- The mechanisms underlying heme transport and trafficking are not well understood.
- Heme trafficking is often thought to be mediated by transporters and chaperones.
- The mobilization of heme may also be mediated by nucleic acids and lipid membranes.

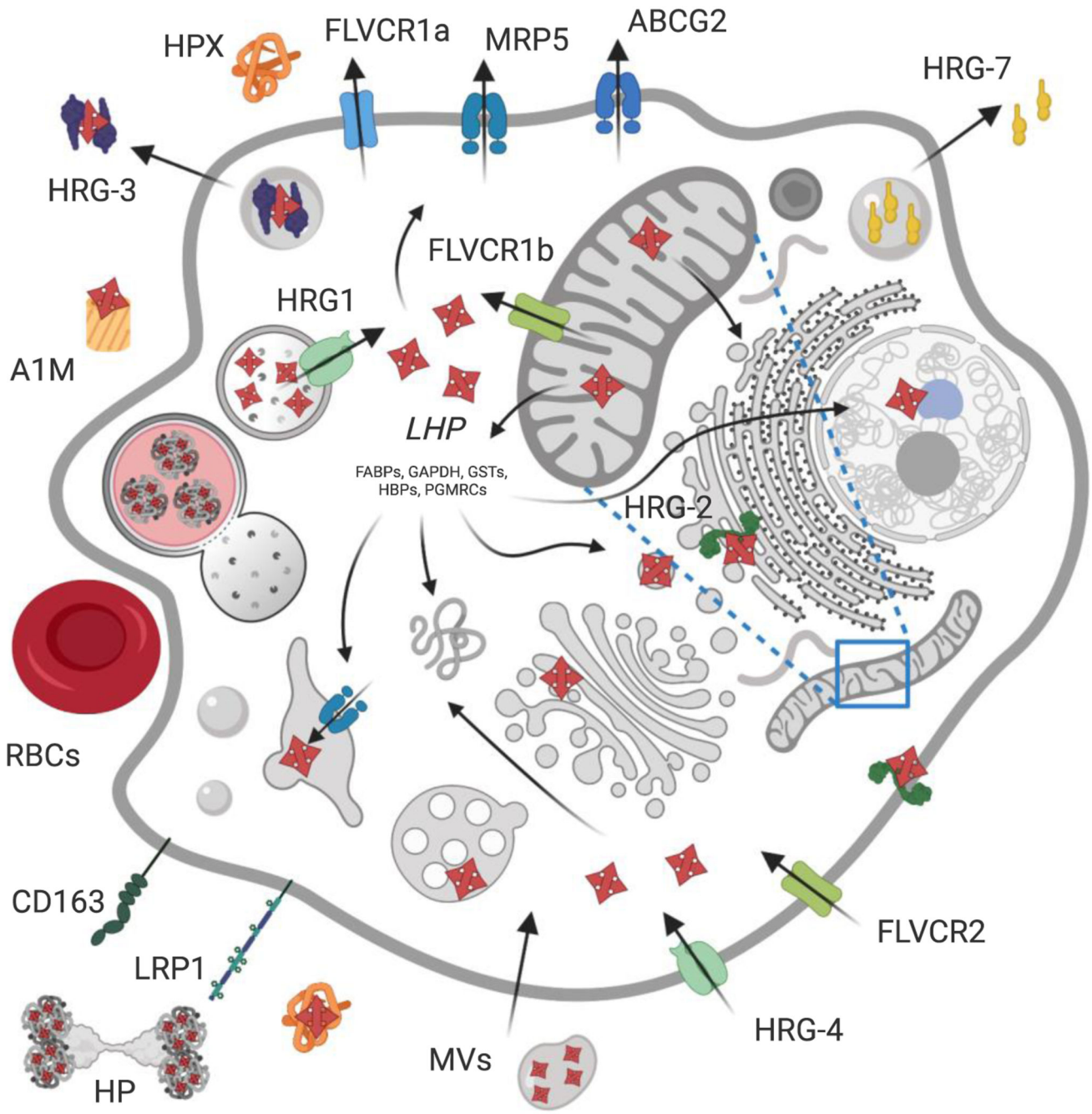

Figure 1.

Consensus model of known proteinaceous mechanisms for metazoan heme transport and trafficking within and between cells. Intracellular heme is either produced via *de novo* synthesis in the mitochondria or imported via transporters or endocytosis (green components). The efflux of heme to exoplasmic spaces is mediated by transporters or exocytosis (blue components). The labile heme pool (LHP) consists of heme that is buffered by a network of proteins and facilitates its trafficking to other organelles throughout the cell. In mammals, FLVCR2 has been implicated as a plasma membrane heme importer, whereas

LRP1 and CD163 are cell surface scavenger receptors for hemopexin (HPX) and the haptoglobin-hemoglobin (HP) complex (or hemoglobin alone), respectively. Heme is also transported via endocytosis of senescent red blood cells (RBCs) and shed microvesicles (MVs) or exosomes in vertebrates. In worms, HRG-4 homologs are dedicated plasma membrane importers and HRG-2 aids in heme utilization by functioning as an oxidoreductase. Finally, heme is imported into the cytosol from exoplasmic compartments via HRG1 and FLVCR1b at the endolysosome and mitochondria, respectively. Conversely, heme efflux is primarily achieved via plasma membrane exporters FLVCR1a, ABCG2 and MRP5 in mammals, with MRP5 also having been localized to the secretory pathway for heme efflux from the cytosol across metazoans. In worms, heme additionally is secreted from intestinal cells for intercellular heme transport via the heme binding protein HRG-3, while interorgan heme homeostasis is modulated via secretion of HRG-7. Created with BioRender - see text for additional details and Table 1.

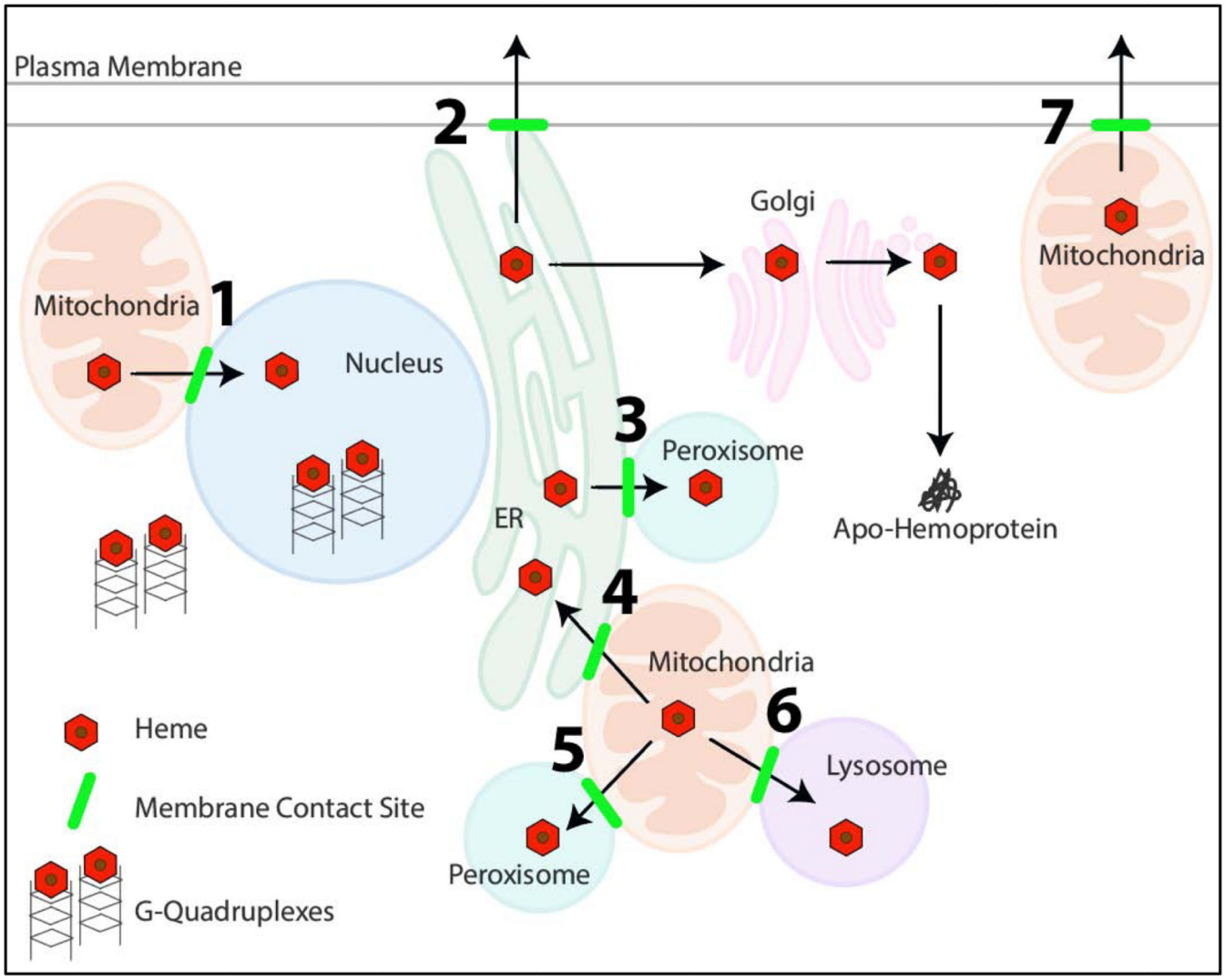

Figure 2.

Proposed model for “chaperone-less” heme trafficking via inter-organelle membrane contact sites (highlighted in green) and G-quadruplexes. The specific organellar contact sites, numbered 1–7, are described in Table 2. RNA and DNA G-quadruplexes are also hypothesized to regulate intracellular heme bioavailability.

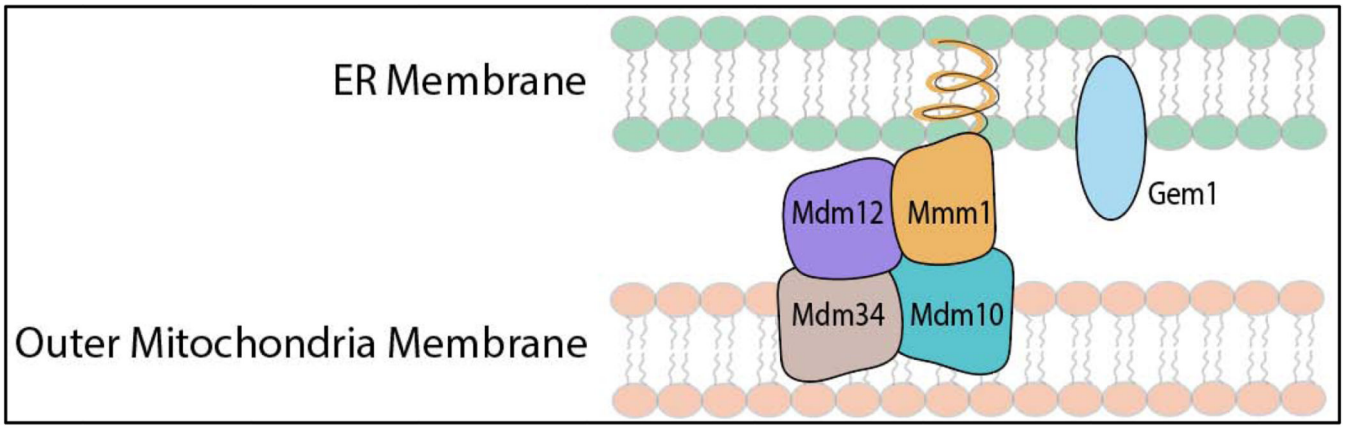

Figure 3.

Cartoon of the ER-mitochondrial encounter structure (ERMES) in yeast, which facilitates calcium and phospholipid exchange, as well as possibly heme trafficking. ERMES consists of four proteins, Mdm12, Mmm1, Mdm34, and Mdm10, that physically tether the ER and outer mitochondrial membranes. Gem1 is a GTPase that disengages ERMES after mitochondrial fission. Altering the frequency of ERMES, by deletion of Gem1, or GTPases that control mitochondrial fusion, *e.g.* Mgm1, and fission, *e.g.* Dnm1, has been shown to affect mitochondrial-nuclear heme trafficking rates. See text for details.

Table 1.

Metazoan Heme Transporters.

Directionality of Transport	Transporter	Localization	Species	Ref.
Import	HRG1	Endosome / Lysosomal	All metazoans	[115]
	FLVCR1b	Mitochondria	Vertebrates	[46]
	HRG-4	Plasma Membrane	Worms	[44]
	FLVCR2	Plasma Membrane	Mammals	[45]
Export	MRP5	Endosome / Plasma Membrane	All metazoans	[122]
	FLVCR1a	Plasma Membrane	Vertebrates	[172]
	ABCG2	Plasma Membrane	Vertebrates	[148]
	HRG-3	Secretory	Worms	[165]

Table 2.

Inter-organellar contact sites in yeast and mammals. Mito = Mitochondria; N/A = Not Available.

MCS		Mammals				Yeast			
		Complex	Protein	Locale	Ref.	Complex	Protein	Locale	Ref.
1	Mito-Nucleus		TSPO ACBD3 PKA AKAP95	Mito Cyto Nuc Nuc	[221]				
2	ER-Plasma Membrane		DHPRs RyRs	PM ER	[225]		Ist2 Scs2/22 Tcb1/2/3	ER ER ER	[229] [228] [229], [230]
			Orai STIM1	PM ER	[226]		Lam1/2/3/4 Osh2/3	ER PM	[231] [232]
			ORPs VAPs	PM ER	[227], [228]				
3	ER-Peroxisome		ACBD5 VAP A/B	Peroxisome ER	[233]		Pex3 Pex3 Inp1	ER Peroxisome Cyto	[234]
4	ER-Mito	Ca^{2+} Transport	IP3R VDAC GPR75	ER Mito Cyto	[235]	EMC	EMC1 EMC2 EMC3 EMC4 EMC5 EMC6 EMC7 EMC10 TOM5	ER ER ER ER ER ER ER ER Mito	[239]
		Unfolded Protein Response	Mfn1/2 Mfn2	ER Mito	[236]				
			Bap31 Fis1	ER Mito	[237]				
			VAPB PTPIP51	ER Mito	[238]	ERMES	Mmm1 Mdm12 Mdm10 Mdm34	ER Cytosolic Mito Mito	[240]
							Mmr1	Mito	[241]
							Lam5/6 TOM70/71	ER Mito	[242], [243]
5	Mito-Peroxisome		TOM20 ECI2	Mito Peroxisome	[244]		Pex11 Mdm34	Peroxisome Mito	[245]
							Pex34	Peroxisome	[246]
							Fzo1	Mito	[246]
6	Mito-Lysosome		Rab7 Mfn2	Lysosome Mito	[247], [248]		Ypt7 Vps39	Vacuole Vacuole	[250] [
			Rab7 VPS13A	Lysosome Mito	[247], [249]				
7	Mito-Plasma Membrane		N/A	N/A			Mdm36 Num1	Mito PM	[251]