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Regulation of heme biosynthesis and transport in metazoa

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Heme is an iron-containing tetrapyrrole that plays a critical role in regulating a variety of biological processes including oxygen and electron transport, gas sensing, signal transduction, biological clock, and microRNA processing. Most metazoan cells synthesize heme via a conserved pathway comprised of eight enzyme-catalyzed reactions. Heme can also be acquired from food or extracellular environment. Cellular heme homeostasis is maintained through the coordinated regulation of synthesis, transport, and degradation. This review presents the current knowledge of the synthesis and transport of heme in metazoans and highlights recent advances in the regulation of these pathways.

heme, iron, synthesis, transport, regulation

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Heme is an iron-containing tetrapyrrole that serves as a critical cofactor for numerous proteins. Globins and cytochromes require heme for their biological functions [1,2]. Heme is also the activity center of such enzymes as catalases, myeloperoxidases, thyroperoxidases, lactoperoxidases, cytochrome P450s, inducible nitric oxide synthases (iNOS), and soluble guanylate cyclases (sGC) [1,2].

Besides serving as a cofactor, heme is an essential regulator of several cellular processes. Heme can bind and regulate the transcriptional factors BACH1, Rev-erbs, CLOCK, neuronal PAS domain-containing protein 2 (NPAS2), and P53. BACH1 is a transcriptional repressor. Binding of heme relieves the transcriptional repression and up-regulates downstream genes, which are involved in hematopoiesis, macrophage development, and antioxidation [3–6]. Heme also regulates the circadian rhythm by physically interacting with the nuclear receptors Rev-erb α and β, CLOCK, and NPAS2 [7–10]. Additionally, the activity of tumor suppressor protein P53 is regulated by heme availability [11]. Heme inhibits the interaction between P53 and its target DNA element and facilitates the nuclear export and proteasomal degradation of P53 [11]. Heme is also required for the proper binding of the microprocessor subunit DGCR8 to primary microRNA transcripts (pri-miRNAs), and thus plays a critical role in the processing of pri-miRNAs [12–14].

Given the important biological role of heme in diverse cellular pathways, either insufficient or excess heme would have detrimental effects on cell metabolism and function. Further, free heme is a cytotoxic compound due to its intrinsic peroxidase activity. Thus, cells have developed exquisite mechanisms to regulate heme metabolism in order to maintain its homeostasis. This review presents the current knowledge of heme synthesis and trafficking in metazoans, and highlights recent advances in the regulation of these pathways. The regulation of heme degradation has been reviewed recently [15,16] and is not discussed here.

1 Regulation of heme biosynthesis

1.1 Uptake of iron for heme synthesis

In most metazoan species, heme is synthesized via an eight-step pathway from the substrates ferrous iron, glycine,

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and succinyl-CoA (Figure 1). Most cells, especially developing erythrocytes, take up iron through transferrin cycle [17,18]. Transferrin receptor (TFR) on the cell surface interacts with di-ferric transferrin, followed by clathrin-mediated internalization into early endosomes. During the maturation of endosomes, the associated iron is released. The rest of transferrin complex is recycled to the cell surface, where TFR and transferrin dissociate.

Regulatory molecules exist to ensure the unidirectional movement of the transferrin complex. Three studies unanimously reported that the phosphatidylinositol binding clathrin assembly protein (PICALM) plays an essential role in TFR endocytosis [19–21]. *Picalm*-deficient mice developed severe anemia, which is due to impaired uptake of iron in erythroid precursors. Further cell biological evidences suggest that PICALM facilitates the maturation of clathrin coat and the endocytosis of transferrin complex. Another trafficking protein required for the transferrin cycle is sorting nexin 3 (SNX3) [22]. SNX3 is highly expressed in the hematopoietic tissues of mice and zebrafish. Knockdown of *snx3* in zebrafish embryos caused profound anemia, a phenotype that can be rescued by supplementing non-transferrin bound iron. *Snx3*-deficient erythroid cells had accumulation of transferrin complex within early endosomes, indicating impaired trafficking from early to recycling endosomes. Once reaching recycling endosomes, the transferrin complex requires the exocyst complex component SEC15L1 for returning to the cell surface [23,24].

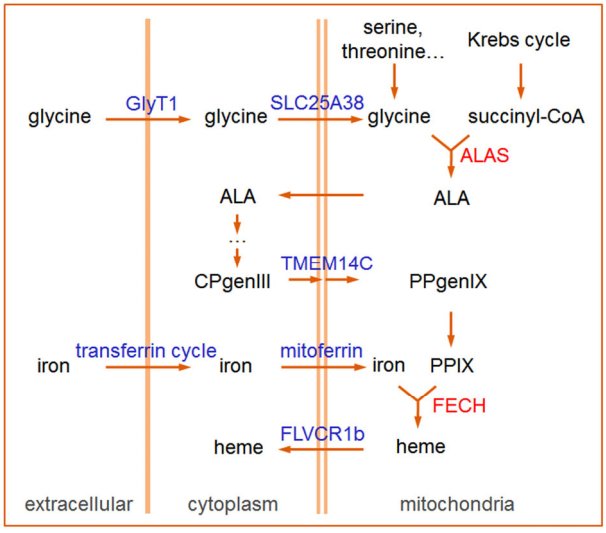

Figure 1 Regulation of heme synthesis in animals. Heme is synthesized via a conserved pathway comprised of eight enzyme-catalyzed reactions. Aminolevulinic acid synthase (ALAS) and ferrochelatase (FECH), the first and the last enzymes in the pathway, are regulated by the oxygen level and cellular iron status. Iron is imported into cells by the transferrin cycle, a process that is regulated by phosphatidylinositol binding clathrin assembly protein (PICALM), sorting nexin 3 (SNX3), and exocyst complex component 6 (SEC15L1). Mitoferrin mediates iron import into mitochondria. Glycine may be imported by glycine transporter 1 (GLYT1) and solute carrier family 25 member 38 (SLC25A38) or be generated within the mitochondria, and succinyl-CoA is provided by the Krebs cycle. Transmembrane protein 14C (TMEM14C) may translocate protoporphyrinogen IX (PPgenIX) across the inner mitochondrial membrane. The Feline Leukemia Virus subgroup C receptor 1b (FLVCR1b) may transport heme out of mitochondria.

The net outcome of transferrin cycle is the translocation of iron from the blood circulation to endosomes. How does the imported iron move out of the endosomes and get into the mitochondria for heme synthesis? First, six-transmembrane epithelial antigen of the prostate member 3 (STEAP3) and related proteins reduce the ferric iron to ferrous iron [25,26]. Second, the ferrous iron is exported out of the endosomes. The divalent metal transporter DMT1 is known to translocate iron across the endosomal membranes [27]. The cation channel protein mucolipin 1 was also reported to be an iron channel in endolysosomes [28]. However, the primary function of mucolipin 1 is likely to transport calcium [29]. Third, following endosomal release, iron is transported to the mitochondria. Cytosolic iron chaperones, such as poly (rC) binding protein 1 (PCBP1) and PCBP2, deliver iron to the iron storage protein ferritin and cytoplasmic iron-containing proteins [30–32]. The specific chaperone that mediates iron transport from endosomes to the mitochondria has yet to be identified. Next, iron is imported into the mitochondria by mitoferrins [33]. Mitoferrin 1 is highly expressed in vertebrate hematopoietic tissues and plays a primary role in supplying iron for heme synthesis in erythroid cells [33]. Its ubiquitously expressed paralog, mitoferrin 2, may be responsible for mitochondrial iron import in non-erythroid cells [34].

1.2 Supply of succinyl-CoA and glycine for heme synthesis

The first and rate-limiting reaction in heme biosynthesis pathway is the condensation of succinyl-CoA and glycine to form δ-aminolevulinic acid (ALA) (Figure 1). Succinyl-CoA is derived from the Krebs cycle (also called citric acid cycle). Over 50 years ago, it was proposed that the production of succinyl-CoA could be an important factor regulating heme production [35,36]. To date, however, it is still unclear whether succinyl-CoA is a limiting factor during heme synthesis and whether the rate of succinyl-CoA formation is increased to meet the high demand of heme production in such tissues as bone marrow and liver.

The amino acid glycine can be synthesized within the cell through several metabolic pathways [37,38]. For example, the serine hydroxymethyltransferase SHMT2 catalyzes the formation of glycine from serine and tetrahydrofolate within the mitochondria [39,40]. Glycine can also be produced from other sources such as choline and threonine [37,38]. Currently, the contribution of endogenously synthesized glycine to heme biosynthesis is unclear.

In addition to its biosynthesis, glycine can be imported into cells by transporters. Neuronal tissues use glycine transporters GLYT1 and GLYT2 to take up glycine [41–43].

A recent transcriptome analysis detected GLYT1 as one of the mRNAs enriched in developing erythrocytes [44]. The *Glyt1* knockout mice developed microcytic hypochromic anemia [45]. Glycine uptake and heme production were both reduced in the *Glyt1*-deficient erythroid cells [45]. These studies suggest that GLYT1 may be the primary transporter supplying glycine for heme synthesis in erythroid tissues. After entering the cytoplasm, glycine needs to be delivered to the mitochondria for heme synthesis. The solute carrier family protein SLC25A38 was proposed to transport glycine across the inner membrane of mitochondria [46]. Mutation of this gene caused nonsyndromic congenital sideroblastic anemia in humans [46,47]. Deletion of *SLC25A38* homolog in yeast led to reduced ALA production, indicating that this carrier protein regulates the first step of porphyrin synthesis [46].

1.3 Regulation of heme synthesis enzymes

While the regulatory mechanisms for most enzymes in heme biogenesis are unclear, the first and final enzymes have been shown to be regulated by iron status, oxygen levels, and other related factors.

Aminolevulinic acid synthase (ALAS) is the enzyme catalyzing the first step of heme biosynthesis. Two *ALAS* paralogs exist in vertebrate animals. The expression of *ALAS1*, a ubiquitously expressed gene, is stimulated by the circadian clock gene *NPAS2* and repressed by heme [7,48,49]. *ALAS2*, or *eALAS*, is specifically expressed in erythroid cells [50]. The expression of *ALAS2* is regulated post-transcriptionally by iron through the iron regulatory protein 1 (IRP1) [51,52]. When cellular iron is insufficient, IRP1 binds to the iron regulatory element in the 5′ untranslated region of *ALAS2* mRNA, leading to its reduced translation [51,52]. Besides, *ALAS2* expression is induced by hypoxia [53]. Hypoxia-inducible factor 1α (HIF1α) can activate the transcription of *ALAS2* [54].

Ferrochelatase (FECH) catalyzes the final step of heme synthesis—the incorporation of iron into protoporphyrin IX (PPIX). Similar to *ALAS2*, the expression of *FECH* is also regulated by oxygen levels through HIF1α [55]. Animal FECHs contain iron-sulfur [2Fe-2S] clusters. Both the stability and the enzymatic activity of metazoan FECH proteins are regulated by iron availability as well as the production of iron-sulfur clusters [56,57]. Because of the presence of [2Fe-2S] clusters, animal FECHs are also regulated by mitochondrial pH and redox potential. For example, alkalization of mitochondria due to deficiency of ATPase inhibitory factor 1 reduced the activity of FECH and the production of heme [57].

The insertion of iron into PPIX is facilitated by the ATP binding cassette protein ABCB10. ABCB10 physically interacts with both mitoferrin 1 and FECH [58]. This interaction ensures that the imported iron is preferentially utilized for heme production. Deletion of *Abcb10* in mice impaired heme biosynthesis, erythropoiesis, and caused embryonic lethality [59]. Tissue-specific knockout of *Abcb10* in mouse hematopoietic cells resulted in accumulation of iron and PPIX in reticulocytes, confirming its role in the final step of heme synthesis pathway [59].

1.4 Transport of heme synthesis intermediates

The biosynthesis of heme starts and ends in the mitochondria, while four intermediate reactions take place in the cytosol. Therefore, the products of several reactions need to be shuttled between the mitochondria and the cytosol in order to be used as substrates for the subsequent reactions (Figure 1). First, ALA needs to be transported across the mitochondrial membranes to the cytosol, where the subsequent four heme synthesis enzymes are located. A study suggested that ABCB10 might mediate this translocating event [60]. However, other biochemical and genetic studies indicate that ABCB10 is more likely to be involved in the final step of heme synthesis, as discussed above [59,61].

Coproporphyrinogen III (CPgenIII) is formed from ALA via four enzyme-catalyzed reactions in the cytosol. The enzyme receiving CPgenIII, coproporphyrinogen oxidase (CPOX), is anchored on the mitochondrial matrix with the active site in the intermembrane space [62]. ABCB6 was proposed to be responsible for transporting CPgenIII across the mitochondrial outer membrane [63]. However, a more recent study revealed that ABCB6 specifies the blood group Langereis and is not required for erythropoiesis [64]. Therefore, the real identity of CpgenIII transporter remains elusive.

The product of CPOX, protoporphyrinogen IX (PPgenIX), is then converted into PPIX by protoporphyrinogen oxidase (PPOX), which forms a complex with the final enzyme FECH in the mitochondrial matrix [62,65,66]. A recent report showed that PPgenIX may be transported to PPOX by transmembrane protein 14C (TMEM14C) [67]. TMEM14C was first identified by a large-scale bioinformatics analysis to co-express with heme synthesis genes [68]. Silencing of *tmem14c* with morpholinos resulted in profound anemia in zebrafish embryos [68]. *Tmem14c*-deficient mice die during embryonic development [67]. These mutant mice had CPgenIII accumulation accompanied by reduced level of PPIX in the fetal liver. This observation was further validated by the rescue of heme defects with a PPIX analog, deuteroporphyrin IX, in *Tmem14c*-deficient cells [67]. As a transmembrane protein localized to the inner mitochondrial membrane, TMEM14C may mediate the import of PPgenIX into the mitochondrial matrix [67].

2 Heme transport and regulation of heme homeostasis

Besides biosynthesis and degradation, cellular heme homeostasis is also maintained by trafficking. A number of mol-

ecules and pathways have been identified to transport heme within and between cells (Table 1).

2.1 Heme import

Heme is absorbed by human intestine at a much higher efficiency than inorganic iron, indicating the presence of specific heme uptake system. Heme carrier protein 1 (HCP1) was reported to be a heme importer in mammalian intestine [69]. However, HCP1 is actually a high-affinity, proton coupled folate transporter [82]. It remains to be tested whether the low affinity heme transport activity of this folate transporter has physiological relevance.

By using the model organism *Caenorhabditis elegans*, Rajagopal et al. [70] identified a high-affinity heme importer—heme responsive gene-1 (HRG-1). Because *C. elegans* and related nematodes are unable to synthesize heme, they completely rely on food for heme nutrition. In the worm intestine, HRG-1 is responsible for mobilizing heme out of the endosomal-lysosomal organelles, whereas its paralog HRG-4 imports dietary heme through the apical surface of intestinal cells. The heme transport activities of *C. elegans* HRG-1 and HRG-4 have been verified in worms, *Xenopus oocytes*, and yeast [70,83]. In a similar way, the homolog of HRG-4 in *Leishmania amazonensis*, *Leishmania* Heme Response-1 (LHR-1), mediates heme uptake in the parasites [84]. HRG-1, HRG-4, and LHR-1 are all transcriptionally up-regulated under heme-limiting conditions [70,84].

Another *C. elegans* gene, *hrg-2*, is also induced by heme deficiency [85]. HRG-2 localizes to the endoplasmic reticulum and apical plasma membrane of the hypodermal cells. It binds heme and facilitates heme utilization by the hypodermis of worms. The precise function of HRG-2 in heme homeostasis is currently unclear.

2.2 Heme export

The Feline Leukemia Virus subgroup C receptor (FLVCR or FLVCR1) is the first heme transporter identified in eukaryotes [71]. Two isoforms of FLVCR1, FLVCR1a and FLVCR1b, may play distinct roles in mammalian heme homeostasis. FLVCR1a is a heme exporter [71], whereas FLVCR1b may be an intracellular heme transporter [74]. In macrophages of the reticuloendothelial system, FLVCR1a exports heme that is derived from ingested senescent red blood cells [72]. The heme export activity of FLVCR1a was also reported in hepatocytes and T cells [86,87]. Hypoxia induces the expression of *FLVCR* through HIF2α and the HIF-dependent transcription factor v-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1) [88].

Korolnek et al. [73] identified the multidrug resistance protein MRP-5/ABCC5 as another heme exporter in animals. In *C. elegans*, MRP-5 localizes to the basolateral surface of the intestinal cells and mediates heme export out of the intestine. Deletion of *mrp-5* results in embryonic death, which is due to impaired heme transfer to extra-intestinal tissues [73]. In vertebrate cells, MRP-5 localizes to both plasma membrane and intracellular compartments [73]. Deficiency of *mrp-5* led to reduced heme loading into the secretory compartments in mouse embryonic fibroblasts and profound anemia in zebrafish embryos [73].

2.3 Intracellular heme transport

Within the cell, heme is produced in the mitochondria. However, many hemoproteins are present in other subcellular compartments. It was proposed that FLVCR1b, the mitochondrial isoform of FLVCR1a, is responsible for translocating heme out of the mitochondria [74]. Based on the observations that the *Flvcr1* (the gene expresses both *Flvcr1a* and *Flvcr1b*) knockout mice die during embryonic development and *Flvcr1a* knockout mice had normal erythropoiesis [72,74], Chiabrando et al. [74] concluded that the phenotypes observed in *Flvcr1* knockout mice were due to deficiency of *Flvcr1b*. Furthermore, silencing of *FLVCR1b* resulted in heme accumulation within the mitochondria. Therefore, it was proposed that FLVCR1b regulates heme export from the mitochondria while FLVCR1a regulates heme efflux through the plasma membrane [74].

Once inside the cytoplasm, heme needs to be delivered to target hemoproteins. Glyceraldehyde-3-phosphate dehy-

Table 1 Proteins involved in heme transport in animals

Category	Protein	Reported function	Reference
heme import	HCP1	import heme into intestinal cells	[69]
	HRG-4	import heme into intestinal cells	[70]
heme export	FLVCR	export heme out of macrophages	[71,72]
	MRP5	export heme out of intestinal cells	[73]
intracellular heme transport	FLVCR1b	export heme out of mitochondria	[74]
	HRG1	export heme out of endosomal-lysosomal organelles and phagolysosomes	[70,75]
	GAPDH	insert heme into iNOS	[76]
	HSP90	insert heme into sGC	[77]
intercellular heme transport	hemopexin	bind heme in the blood and deliver heme to hepatocytes and macrophages	[78–80]
	HRG-3	deliver heme from maternal intestine to developing oocytes	[81]

drogenase (GAPDH) and 90 kD heat-shock protein (HSP90) were reported to mediate heme insertion into iNOS and sGC, respectively. GAPDH is known to be an enzyme catalyzing the conversion of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate. Chakravarti et al. [76] found that GAPDH interacts with both heme and iNOS. Knockdown of *GAPDH* or mutation of the heme-binding site in GAPDH significantly reduced heme incorporation into iNOS. The product of iNOS, nitric oxide (NO), also inhibited heme insertion into iNOS through *S*-nitrosylation of GAPDH [76]. Heme insertion into sGC was found to be mediated by HSP90, which preferentially interacts with apo-sGC [77]. HSP90-mediated heme incorporation converts apo-sGC into a mature active form that is responsive to NO [77]. The insertion of heme into sGC is positively regulated by NO [89].

HRG-1 and its mammalian homologs also transport heme within the cell. As discussed above, *C. elegans* HRG-1 transports heme from the endosomal-lysosomal organelles to the cytosol within the intestinal cells [70]. Similarly, mammalian HRG1 transports heme from the phagolysosomes to the cytosol of the reticuloendothelial macrophages [75]. During erythrophagocytosis, macrophage phagolysosomes are responsible for breaking down ingested red blood cells to release heme. HRG1 specifically localizes to the phagolysosomal membranes during erythrophagocytosis. Silencing of HRG1 significantly reduced the heme levels in the cytosol of bone marrow-derive macrophages [75]. Knockdown of *hrg1* in zebrafish resulted in hypochromic anemia [70]. The expression of *HRG1* in reticuloendothelial macrophages is up-regulated by both erythrophagocytosis and heme [75]. Thus, vertebrate HRG1 plays a critical role in recycling heme and heme iron from the senescent red blood cells [75].

2.4 Intercellular heme transport

Hemopexin is a high-affinity heme binding protein in the blood [78]. It accepts heme from FLVCR through direct protein-protein interactions [90]. Binding of heme by hemopexin may be a critical step in the recycling of heme during erythrophagocytosis. Low-density lipoprotein receptor-related protein (LRP1) was found to be the receptor for hemopexin [79]. LRP1 is expressed in several cell types including hepatocytes, macrophages, neurons, and syncytiotrophoblasts. Hada et al. [80] confirmed that hepatocytes and macrophages are able to take up the hemopexin-heme complex. Uptake of hemopexin-bound heme but not free heme was inhibited by treating the cells with the inhibitor of clathrin-mediated endocytosis, validating that the hemopexin-heme complex enters cells through endocytosis [80].

The heme responsive gene 3 (HRG-3) is an intercellular heme trafficking protein in *C. elegans* [81]. Heme deficiency induces *hrg-3* expression in the worm intestine. HRG-3 protein binds stoichiometric amounts of heme and is secreted into the worm circulation pseudocoelom. HRG-3-heme can be taken up by tissues such as developing oocytes. During this process, heme is transferred from maternal intestine to the oocytes and embryos. The progeny of *hrg-3*-deficient worms are arrested during embryonic development or at early larval stages due to heme deficiency. Therefore, HRG-3 plays a critical physiological role in delivering heme to oocytes and related tissues in worms.

3 Conclusion

Sixty years ago, Shemin and other researchers [91,92] discovered the enzymes and substrates for the heme synthesis pathway. Since then, heme-related research has attracted a lot of interest. The recent years have witnessed a rapid expansion of knowledge in heme metabolism. A number of regulatory molecules and mechanisms have been identified in iron uptake and heme synthesis pathways. Importantly, discoveries of heme transporters such as FLVCR and HRG1 proved that heme does not move around freely in the cell. Several key gaps still remain in our knowledge of heme metabolism: (i) What is the real identity of the heme receptor in human intestine? (ii) What mechanisms regulate the intermediate steps of heme synthesis? (iii) How does heme enter nuclei, lysosomes, peroxisomes, and the secretory pathway? By using yeast, worm, zebrafish, and mouse as the model systems, researchers are actively trying to address these questions and to further our understanding of heme metabolism.

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