Regulation of intracellular heme trafficking revealed by subcellular reporters


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Heme is an essential prosthetic group in proteins that reside in virtually every subcellular compartment performing diverse biological functions. Irrespective of whether heme is synthesized in the mitochondria or imported from the environment, this hydrophobic and potentially toxic metalloporphyrin has to be trafficked across membrane barriers, a concept heretofore poorly understood. Here we show, using subcellular-targeted, genetically encoded hemoprotein peroxidase reporters, that both extracellular and endogenous heme contribute to cellular labile heme and that extracellular heme can be transported and used in toto by hemoproteins in all six subcellular compartments examined. The reporters are robust, show large signal-to-background ratio, and provide sufficient range to detect changes in intracellular labile heme. Restoration of reporter activity by heme is organelle-specific, with the Golgi and endoplasmic reticulum being important sites for both exogenous and endogenous heme trafficking. Expression of peroxidase reporters in Caenorhabditis elegans shows that environmental heme influences labile heme in a tissue-dependent manner; reporter activity in the intestine shows a linear increase compared with muscle or hypodermis, with the lowest heme threshold in neurons. Our results demonstrate that the trafficking pathways for exogenous and endogenous heme are distinct, with intrinsic preference for specific subcellular compartments. We anticipate our results will serve as a heuristic paradigm for more sophisticated studies on heme trafficking in cellular and whole-animal models.

Significance

The intracellular and extracellular trafficking of heme, a hydrophobic and potentially cytotoxic cofactor in proteins such as hemoglobin, remains an underexplored area. While cellular heme can be derived exogenously or from de novo synthesis, it is unclear if there is differential trafficking of heme from these two sources. To critically examine this possibility, we developed peroxidase-based enzymatic reporters for heme and deployed them in subcellular compartments in mammalian cell lines and in several tissues in the Caenorhabditis elegans animal model. Our results show that extracellular heme is used intact for incorporation into hemoproteins in virtually all intracellular compartments and that endogenous and exogenous heme trafficking is mediated by distinct cellular pathways. Furthermore, genetic studies in C. elegans.


Conflict of interest statement: I.H. is the president and founder of Rakta Therapeutics Inc. (College Park, MD), a company involved in the development of heme transporter-related diagnostics. I.H. declares no other competing financial interests.

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Heme is an essential but toxic macrocycle (1, 2) that serves as a protein prosthetic group and signaling molecule. As a cofactor, it enables diverse functions that include electron transfer, chemical catalysis, and gas binding/transport. As a signaling molecule, heme regulates the antioxidant response, circadian rhythms, microRNA processing, and cell differentiation and proliferation (3–9). All heme-dependent processes require that heme is trafficked from its site of synthesis in the mitochondria or its point of entry into the cell, to distinct hemoproteins located in numerous subcellular compartments. Further, heme transfer between cells and tissues is required for systemic heme homeostasis and organism development (10–15). Given that free heme is cytotoxic and hydrophobic (16, 17), the trafficking of heme for insertion and/or signaling is likely coordinated between heme transporters, chaperones, and carrier proteins (1).

Genetically engineered heme proteins have been developed in bacteria to study heme transport and coordination between heme proteins, i.e., cytochrome biosynthesis (18, 19). By using natural or engineered heme synthesis-deficient bacterial strains, researchers previously showed exogenous heme is directly incorporated into heme proteins (20, 21). However, such tools are not available to probe how mammalian cells handle heme, especially because hemoproteins reside in several subcellular membrane-enclosed compartments (1). Regardless of whether heme is synthesized in the mitochondria or imported from the environment, heme has to be translocated across membrane barriers (22, 23). It has been suggested that the majority of extracellular heme is degraded through the heme oxygenase pathway to extract iron from the porphyrin in mammalian cells (22, 24–28). Therefore, it is of significant interest to investigate whether exogenous heme is ever used in toto for cellular hemoproteins in animals and if there is a hierarchical preference by subcellular compartments to use one form of heme over another, i.e., from de novo synthesis or from exogenous sources.

Although the identity of heme transporters and trafficking factors has seen some headway due to genetic studies in model systems within the past decade (10, 13, 14, 29, 30), the lack of proper physical tools to probe heme availability and trafficking at the cell biological level has greatly hindered our progress in understanding the intricacies of cellular and organismal heme homeostasis. To overcome this obstacle, we developed peroxidase-based enzymatic reporters for heme and deployed them in subcellular compartments in mammalian cell lines and in several tissues in the Caenorhabditis elegans animal model. Our results show that extracellular heme is used intact for incorporation into hemoproteins in virtually all intracellular compartments and that endogenous and exogenous heme trafficking is mediated by distinct cellular pathways. Furthermore, genetic studies in C. elegans

heme | tetrapyrroles | iron | trafficking | subcellular
validate the utility of these hemoprotein reporters and demonstrate that interorgan heme transport is essential for organismal homeostasis.

Results

Subcellular Targeting of Peroxidase Reporters. To monitor labile heme that is trafficked to various subcellular organelles, we needed to first develop appropriate reporters that would measure heme levels uniformly, irrespective of the intracellular membrane compartment. More importantly, these reporters should have suitable affinity for heme. We used genetically encoded peroxidase-based enzymatic reporters because horseradish peroxidase (HRP) activity is dependent on a heme prosthetic group, and it can also be reconstituted into an active form in vitro using the apoprotein and heme (31) (Fig. 1A). The HRP–heme dissociation constant ($K_d$) was determined by difference absorption spectroscopy to be $270 \pm 40 \text{ nM}$, similar to previously reported $155 \pm 27 \text{ nM}$ using surface plasmon resonance technique (32) (SI Appendix, Fig. SI4A). This value is weaker than constituent heme proteins and enzymes (e.g., globins, cytochromes, and catalase) that have heme affinity ranges between $10^{-12}$ and $10^{-15}$ M (33), making HRP an ideal reporter for labile heme. Studies have used purified apoHRP to bind bioavailable heme in cell-free extracts as a way to measure regulatory heme (34–38). This in vitro HRP reconstitution method was found to be more sensitive than the traditional pyridine hemochromogen method (39), with minimal interference from fluorescent porphyrins (40). However, a major drawback for this method was that the biological material must first be disrupted and then mixed with apoHRP to measure the conversion of apo to holo, thereby preventing analysis of subcellular heme distribution (34, 35).

HRP variants were created by using specific subcellular targeting sequences to direct the expressed protein to the cytosol, mitochondrial matrix, peroxisome, endoplasmic reticulum (ER), Golgi complex, and plasma membrane (PM; GPI anchored) (SI Appendix, Table S1). To ensure the correct targeting of these genetically engineered probes, HRP variants were tagged with mCherry at the carboxyl-terminus (Fig. 1C and D and SI Appendix, Fig. S2A). However, only the ER, Golgi, and PM-targeted HRP was found to be active (SI Appendix, Fig. S2B). The non-active HRP fusions migrated faster than the active forms and were detectable by anti-mCherry antibodies but not with antibodies generated against holo-HRP (SI Appendix, Fig. S2C and D), suggesting that this class III peroxidase, which is glycosylated, requires the secretory pathway for proper folding (41). To generate reporters for the remaining compartments, we used the cytosolic ascorbate peroxidase (APX), a class I heme-containing homodimer (42, 43) (Fig. 1B and C). The APX–heme $K_d$ was determined to be $360 \pm 40 \text{ nM}$, a value comparable to HRP (SI Appendix, Fig. S1B).

Expression of both proteins in mammalian HEK293 cells found them to colocalize with the expected subcellular markers using confocal microscopy (Fig. 1D and E and SI Appendix, Fig. S3). In addition, targeting APX to the ER showed comparable expression levels to other APX probes, demonstrating that ER-HRP could be substituted by ER-APX in the secretory pathway (SI Appendix, Fig. S4). Thus, HRP and APX are both suitable as subcellular reporters for measuring labile heme.

Subcellular Labile Heme Dictates Reporter Activity. We tested the activity of our genetically targeted probes as a function of heme concentrations in HEK293 cells. Cells expressing engineered HRP exhibited 2,000- to 11,000-fold greater peroxidase activity (Fig. 2A). By contrast, APX reporters showed 50- to 60-fold greater activity. Activity was not detected in a monomeric version of APX (APEX) or a mutant in which the active site was mutated to mimic HRP (APXH; Fig. 2A (44)). In situ histochemical staining detected functional HRP and APX activities in all six subcellular compartments (Fig. 2B).

The large signal-to-background ratio observed for our peroxidase reporters could provide sufficient range to detect changes in intracellular labile heme. Because labile heme has been estimated by mixing purified apoHRP with cell lysate in vitro (38), it is possible that our genetically targeted peroxidase reporters may inadvertently bind heme released during cell lysis and not authentically reflect subcellular labile heme. To evaluate this possibility, we depleted intracellular heme levels by removing exogenous sources of heme from the media by using heme-depleted serum (HD) and treating the cells with succinylacetone (SA), an inhibitor of heme synthesis that blocks the formation of porphobilinogen (PBG). Consequently, apo-reporters formed by heme depletion in cells (ER-HRP and cyto-APX were chosen as representatives) were able to be reconstituted into holoenzymes by supplementation of the cell lysate with hemin (SI Appendix, Fig. S5 A and B) but not with lysates from HEK293 cells preloaded with heme (SI Appendix, Fig. S5 C–F). These results show that the genetically encoded reporters do not steal heme bound by other hemoproteins after cell lysis.

Native HRP is a monomeric glycoprotein that only dimerizes when expressed as a nonglycosylated catalytically active protein in *Escherichia coli*. The equilibrium between monomeric and dimeric recombinant HRP is affected by the peroxidase substrates (45, 46). By contrast, APX is not glycosylated and forms a noncovalent homodimer in a concentration-dependent manner (47, 48). Because the monomer–dimer equilibria of APX could interfere with our interpretation of changes in labile heme, we sought to characterize the active APX species in our cell-based assay conditions (48). We analyzed targeted APX reporters expressed in mammalian cells using an in-gel peroxidase activity assay and immunoblotting. APX reporters were active on native PAGE but not on...
Brightfield images were taken on a Leica DMIRE2 microscope using 63× objective lenses. PAGE gels (circles indicate inactive APX monomers; triangles indicate active APX activity) were used for the indicated heme concentrations for 18 h. Cell lysates were analyzed by SDS/PAGE (Fig. 2).

De Novo Synthesis and Extracellular Heme Influences Labile Heme.

SDS/PAGE (Fig. 2C, Left). APX reporters migrated as monomers and dimers on native PAGE gels and dissociated into monomers on SDS/PAGE (Fig. 2C, Right). Monomeric mutant APEX migrated exclusively as a monomer on both native and SDS/PAGE (Fig. 2C, lanes 4 and 9); by contrast, APXH migrated exclusively as a dimer on native PAGE and dissociated into monomers on SDS/PAGE (Fig. 2C, lanes 5 and 10). Furthermore, immuno blotting revealed that the active APX on native PAGE corresponded to dimers, whereas the monomers had no detectable activity (Fig. 2C).

To further investigate the effect of heme on the stability of APX dimers, we examined APX reporters expressed in HEK293 cells grown in different heme concentrations. In HD + SA conditions, APX migrated almost exclusively in its monomeric form (Fig. 2D, lane 4). Heme supplementation resulted in gradual conversion of APX from a monomer to a dimer on native PAGE. At 16 μM heme, the majority of APX in the cytoplasm, nucleus, and mitochondria was active and migrated as a dimer (Fig. 2D, lane 8). These results clearly demonstrate that APX can be converted from apo to holo by heme incorporation from extracellular sources.

De Novo Synthesis and Extracellular Heme Influences Labile Heme.

We sought to determine the source of labile heme by first creating a cellular heme deficiency condition using HD + SA. Total labile heme in HEK293 cells was assessed by mixing the cell lysates with commercially available apoHRP. The HD condition by itself only had a modest effect on intracellular labile heme, whereas cells treated with succinylacetone reduced labile heme by one-third (Fig. 3A). Furthermore, the combination of HD + SA decreased labile heme to 40% of basal values and could be fully restored by supplementation with 2 μM exogenous heme (Fig. 3A). Indeed, HPLC measurements of total cellular heme confirmed that HD + SA decreased total heme by 60–70% of basal levels (SI Appendix, Fig. S6A and Table S2). Moreover, heme supplementation resulted in a greater net change in total cellular heme in heme-depleted cells compared with cells grown in basal medium (SI Appendix, Fig. S6 B and C). The labile heme concentration in HEK293 cells as measured by apoHRP in basal condition was found to be 435 ± 125 nM (SI Appendix, Fig. S6D).

The incomplete depletion of intracellular heme by the HD + SA combination treatment could be because either cellular labile heme can be reduced only to a certain threshold or cells may require longer depletion periods (>40 h). Moreover, there is the possibility that an existing pool of endogenous labile heme persists even after HD + SA treatment, and imported exogenous heme triggers the release of an existing intracellular heme pool that gets incorporated into the reporter proteins. To address this question, we grew HEK293 cells in the presence of HD + SA and supplemented with metalloporphyrins as a surrogate for exogenous heme. Compared with other metalloporphyrins, swapping manganese protoporphyrin IX (MnPPIX) for heme caused APX to migrate as dimers, but these MnPPIX–APX dimers lacked any detectable enzymatic activity (SI Appendix, Fig. S7 A–C). Even though MnPPIX–APX dimers form stable complexes as heme–APX dimers (SI Appendix, Fig. S7D), increasing the concentrations of exogenous MnPPIX (2–16 μM) did not trigger heme release from a preexisting heme pool, because APX showed no activity (Fig. 3B).
activity at any of the MnPPIX concentrations (SI Appendix, Fig. S7 E and F). These results confirm that exogenous heme does not mobilize residual cellular heme stores for incorporation into APX.

We next determined the effect of intracellular heme perturbation on subcellular heme trafficking using our reporters. Unlike the in vitro reconstitution of apoHRP, genetically encoded HRP and APX activities were undetectable in HD + SA (Fig. 3B). The heme depletion was highly effective across all six intracellular compartments and persisted even in the presence of iron supplementation (SI Appendix, Fig. S8). PM-HRP activity was the only reporter that was significantly reduced by HD alone, whereas the remaining reporters showed greatly reduced activity only when heme synthesis was inhibited by succinylacetone (Fig. 3B). To determine whether the reporters interrogate subcellular labile heme, cells were first grown in the presence of HD + SA so they accumulated sufficient apo-peroxidase, followed by cycloheximide (CHX) treatment to inhibit new reporter synthesis. Supplementation with 4 μM heme to these cells fully restored peroxidase activity in all six compartments as assessed by in situ DAB staining (SI Appendix, Fig. S9). Moreover, restoration of reporter activity by exogenous heme appeared to be compartment-specific with the mitochondrial matrix requiring twice as much heme to fully restore reporter activity to basal conditions (Fig. 3C). A different preference was observed when PBG was supplemented to the grown medium to bypass the succinylacetone block. Under these conditions, 500 μM PBG was sufficient to restore the reporter activity to basal conditions in all compartments except the PM-HRP, which was engineered with a GPI anchor, and the active site faces the extracellular milieu (Fig. 3D). PBG supplementation restored reporter activity in the following order of ER > Golgi ≈ mitochondria > cytoplasm ≈ nucleus > PM, suggesting that the secretory pathway may play a significant role in the trafficking of mitochondria-derived heme. Together, these results indicate that both exogenous and endogenous heme contribute to cellular labile heme and that exogenous heme can be used intact by hemoproteins.

**Exogenous and Endogenous Heme Are Trafficked by Distinct Pathways.**

To differentiate how exogenous versus endogenous heme is transported and trafficked, we used Dynasore, a small molecule GTPase inhibitor that targets dynamin (49) (Fig. 4A). Treatment of Dynasore efficiently diminished uptake of fluorescent transferrin, a standard probe of clathrin-mediated endocytosis (50) (SI Appendix, Fig. S10A). Little or no effect was observed on HRP or APX activities when Dynasore was mixed with cells either after lysis or when cells were lysed immediately after Dynasore treatment (SI Appendix, Fig. S11 A and B). To restrict the source of intracellular heme, we grew the cells in either HD or HD + SA + 4 μM heme because both conditions have similar levels of intracellular heme (SI Appendix, Fig. S6). Total labile heme measured by in vitro reconstitution of apoHRP activity showed no difference between control and inhibitor treated cells under HD condition (Fig. 4B) but showed a significantly greater HRP activity in the presence of Dynasore and 4 μM heme (Fig. 4B).

We next examined the effects of these inhibitors on heme trafficking and compartmentalization using HRP/APX reporters. Even though the overall labile heme had not changed in the HD conditions, with the exception of PM-HRP, all other reporters showed a significant reduction in activity with Dynasore (Fig. 4C and SI Appendix, Fig. S11C). This reduction in endogenous heme compartmentalization could be because Dynasore also disrupts the mitochondrial dynamin Drp1 and consequently interferes with mitochondrial fission and formation of the ER–mitochondria encounter structure (ERMES) (49, 51). Surprisingly, cells supplemented with exogenous heme in the presence of Dynasore showed full restoration of cytoplasmic, nuclear, and mitochondrial reporter activities but not for the ER, Golgi, and PM reporters (Fig. 4D and SI Appendix, Fig. S11D). To differentiate the effect of Dynasore on endosomal dynamin from mitochondrial dynamin, we used Mdivi-1, a small molecule inhibitor that specifically blocks Drp1 function (52). Reporter activities in all compartments were significantly decreased in Mdivi-1 treated cells only in HD conditions (Fig. 4C), and exogenous heme supplementation reversed this effect (Fig. 4D). This result is consistent with the finding that Mdivi-1 does not impair clathrin-mediated endocytosis (SI Appendix, Fig. S10A). Because Dynasore has been reported to impair not only clathrin-mediated endocytosis, but also fluid-phase endocytosis (53), we used a dominant-negative dynamin mutant (K44A) that blocks clathrin-mediated endocytosis (54–56). Expression of dynamin K44A in HEK293 cells inhibited uptake of fluorescent transferrin (SI Appendix, Fig. S10F) and specifically reduced ER-HRP but not cyto-APX activity in the presence of exogenous heme (Fig. 4 E and F). These results further support our proposition that subcellular trafficking of endogenous and exogenous heme occurs via distinct pathways.

**Measuring Heme Homeostasis in Vivo.**

To measure subcellular heme in various organs within an intact animal, we exploited C. elegans because it is a heme auxotroph. Heme levels can be experimentally manipulated by either nutritional supplementation or genetically regulating the heme trafficking pathways mediated by HROGs (13, 14, 29, 30, 57). We generated transgenic worms expressing HRP and APX reporters under the control of tissue-specific promoters. Transgenic worms expressing the HRP reporters showed greater activity, so we characterized worms expressing ER-HRP driven from the intestinal (Pnva-6), hypodermal (Pdpy-7), muscle (Pmyo-3), and pan-neuronal (Punc-119) promoters (Fig. 5A and SI Appendix, Fig. S12). Wild-type (WT) worms were grown in mCeH2R2 liquid axenic culture supplemented with various concentrations of heme, and total worm homogenates were incubated with apoHRP to measure labile heme (57). Although there was a direct correlation between apoHRP reconstitution and increasing heme (R² = 0.996), at concentrations ≥100 μM the ratio of HRP activity to heme was almost 5:1 (slope = 4.9), indicating that a significant portion of heme was present as labile heme under these conditions (SI Appendix, Fig. S13A).

Transgenic worms expressing ER-HRP showed a heme-dependent increase in activity that was tissue dependent (intestine > hypodermis > neuron > muscle; SI Appendix, Fig. S13B). This variation in activity could be due to intrinsic differences in either tissue heme levels or transgene expression. We assessed the influence of variations in transgene expression by comparing two transgenic strains that express ER-HRP at different levels in the muscle (SI Appendix, Fig. S14 A and B). The high-expression strain showed greater than fivefold greater activity at 20 μM heme compared with the low-expression strain (SI Appendix, Fig. S14C). However, when HRP activity at various heme concentrations was normalized to activity at 1.5 μM, the lowest concentration of heme that permits worm growth, no discernable differences were observed in both transgenic strains in their response to environmental heme (SI Appendix, Fig. S14D). This result suggests that the effect of transgene expression levels can be minimized by appropriate baseline correction.

Worms expressing intestinal HRP showed a linear increase in activity at concentrations ≥50 μM heme, although the magnitude was not as great as apoHRP reconstitution by labile heme (Fig. 5B). Activity of HRP reporters expressed from extraintestinal tissues plateaued at much lower heme concentrations, typically around 20 μM. Neuronal HRP exhibited only modest changes in activity over a broad range of heme concentrations. Worms grown at 500 μM heme showed a 10-fold increase in neuronal HRP activity compared with worms grown at 1.5 μM (Fig. 5B), indicating that the neuronal threshold for heme is lower than nonneuronal tissues.
We next assessed whether heme incorporation into HRP could be regulated in a controlled manner by expressing ER-HRP-mCherry from the heat shock inducible promoter (Phsp16.2). HRP was transiently expressed by exposing the worms to Dynasore / Mdivi-1. Dynamin and Dynamin were indicated in the figure. The table shows the relative holo-HRP activity with different treatments. The graph shows the relative reporter activity with different treatments for ER-HRP, Golgi-HRP, PM-HRP, Cyto-APX, NLS-APX, and Mito-APX. The expression was normalized as in Fig. 3. Error bars represent SEM from three biological independent experiments. ns, nonsignificant; \( * P < 0.05 \), \( ** P < 0.01 \), ***\( P < 0.001 \).
37 °C for 30 min. Expression of HRP-mCherry reporter activity could be easily quantified within 1 h postinduction, and maximal activity was observed at 4 h in worms grown at the optimal heme concentration of 20 μM (Fig. 5C). These studies show that temporally controlled reporter strains could be ideal to evaluate the effects of environmental–gene interactions on organismal heme homeostasis.

**Regulation of Interorgan Heme Homeostasis by Intestinal Heme Export.** To directly measure tissue heme perturbations in genetically altered worms, we used the transgenic worm reporter strains. In *C. elegans*, knockdown of *mrp-5* causes embryonic lethality that is suppressed by heme supplementation supporting a role for MRP-5 in heme export from the intestine to extra-intestinal tissues (13). Depletion of the *hrp* transgene suppresses HRP activity in the intestine, hypodermis, and muscle but has less of an effect on the neurons because most neurons in *C. elegans* are resistant to RNAi (58). However, *mrp-5* depletion resulted in a striking increase in intestinal HRP activity concomitant with a significant decrease in HRP activity in the hypodermis, muscle, and neurons (Fig. 5D). Together, these results conclusively show that loss of *MRP-5*, the major intestinal heme exporter, causes heme accumulation in the intestine concomitant with heme deficiency in the extraintestinal tissues.

**Discussion**

Free heme is not readily soluble at neutral pH and is cytotoxic, causing tissue damage (16, 17, 59, 60). These chemical properties of heme necessitate that cellular heme levels are tightly regulated while retaining transitory pools of regulatory heme. Most of the evidence to date indicates that total heme pool within the cell comprises at least two components (15, 60). A portion of the total heme pool is committed to various housekeeping heme proteins and enzymes that are essential for the survival of the cell. For instance, c-type cytochromes bind heme irreversibly via covalent linkages to thiol groups on the heme vinyl substituents. Other heme proteins, such as b-type cytochromes and globins, bind heme noncovalently, but this interaction is still essentially irreversible under physiological conditions by virtue of strong coordinate bond(s) to axial heme ligands (usually His, Met, or Cys). These proteins have high affinity for heme, usually less than pM for the globins (61) and in the nM range for heme oxygenase (62, 63). The remaining portion is in the form of a regulatory heme pool, which is available for trafficking around the cell by as yet unspecified mechanisms. It is clear that this pool of exchangeable heme can be mobilized to regulate more complex biological events specified mechanisms. It is clear that this pool of exchangeable heme can be mobilized to regulate more complex biological events.
labile heme—a metabolically active form of heme associated with macromolecules or low-molecular-weight ligands and exchangeable with acceptor proteins. Intracellular labile heme has not been precisely estimated due to its relatively low abundance and lack of proper analytical tools applied at the subcellular level. Using anion-exchange chromatography, free heme was estimated to be 0.1–0.15 μM in normal human erythrocytes (72).

Given that the affinity of heme to intracellular and extracellular heme-binding proteins varies from 1 μM to <1 μM (3, 73), and the Km of heme oxygenase-1 is ~1 μM, cytosolic labile heme concentrations are likely to be less than 1 μM (33). Because heme can be transferred from one protein to another with a higher heme affinity (74, 75), in vitro reconstitution of purified apoHRP, which has a heme affinity lower than housekeeping proteins that bind protoheme or heme b, has been previously used to determine labile heme in IMR90 lung fibroblast cells (34, 35). This method estimated labile heme to be 614 ± 214 nM, in close approximation to our estimate of 432 ± 125 nM in HEK293 cells. It is noteworthy, however, that severe heme restriction imposed by HD + SA treatment for 40 h shows a 60% reduction in labile heme by the in vitro reconstitution method, whereas in cellular activity for subcellular-targeted HRP and APX reporters is virtually undetectable (Fig. 3A and B). One simple explanation for this discrepancy could be that there are unforeseen differences in the process for in vitro versus subcellular hemeylation i.e., conversion of apoHRP to holoHRP by heme. Another plausible explanation could be that labile heme is stored in an intracellular compartment and inaccessible to the subcellular HRP/APX reporters during heme deprivation.

Early heme absorption studies using dog intestines showed that heme is translocated intact across the membrane and collected in secondary endosomes in a subapical region colocalizing extensively with heme oxygenase-2 (HO-2) (76–80). Heme absorption in live digest cells of the cattle tick Boophilus microplus showed that ingested hemoglobin entered cells through endocytosis after being degraded in primary lysosomes; the released heme was transported into the cytosol while still bound to protein(s), and unbound heme finally accumulated in specialized membrane-bound organelles called hemosomes to prevent heme toxicity (81). We found that HEK293 cells supplied exclusively with exogenous heme accumulated 2.5-fold more labile heme when treated with Dynasore, resulting in attenuation of secretory pathway reporters without effect on nonsecretory reporters (Fig. 4A and D). These results suggest that a dynamin-dependent endocytic pathway may exist for heme compartmentalization in mammalian cells. Although we do not know the precise nature or composition of this compartment, developing additional heme sensors that can report labile heme changes in these heme storage compartments would be of great importance.

With the exception of heme-salvaging tissues such as the liver, extracellular heme is typically believed to be entirely degraded to liberate iron for endogenous heme synthesis in mammalian cells (15, 22, 24–28). Our results counter this generalized model and show that cells have the ability to transport and incorporate exogenous heme as an intact macrocycle; extracellular heme in the growth media restored reporter activity in all of the six subcellular compartments in the absence of endogenous heme synthesis (Fig. 3C). These findings may at least partially explain the phenomenon for why i.v. heme, administered as a therapeutic treatment to acute porphyria patients, increases heme-dependent enzyme activity in the liver because hepatocytes may import and use the injected heme in toto (82) in addition to its role in regulating transcription, translation, and import of ALAS into the mitochondria. Even yeast may have the capability to distinguish endogenously produced from exogenously acquired heme because overexpression of Pug1p, a heme transporter in Saccharomyces cerevisiae, selectively suppresses cell growth when exogenous heme is the sole heme source (83).

We found that ER appear to be an important locale for trafficking of both extracellular and de novo synthesized heme (Fig. 3C and D), implying that heme allocation to various subcellular compartments may be hierarchical, a concept recently reported for copper allocation in Chlamydomonas (84). Using a dynamic K44A mutant to inhibit chlorin-mediated endocytosis, we found that labile heme decreases in the secretory pathway without affecting the cytosolic or nuclear compartments when cells rely solely on extracellular heme (Fig. 4D). Given the contiguous relationship between the endocytic and secretory pathways, it is conceivable that exogenous heme is imported via endocytosis and enters the secretory pathway through dynamin-dependent vesicular trafficking, whereas cytosolic hemoproteins acquire heme through a dynamin-independent process. Although a role for the secretory pathway would be consistent for import of extracellular heme, why is it important for heme synthesized in the mitochondria? A potential explanation could be that heme transport requires specialized structures such as the mitochondrial-associated membranes or the ERMES (1, 85, 86). These structures facilitate calcium transport and lipid trafficking between the ER and mitochondria. Ferrochelatase, heme-binding protein 1 (HBPI), and HO-2 were all detected at these interorganelle contact points (87, 88). Thus, a presumptive heme trafficking route via the ER and bypassing the cytosol can partially explain our observations that Dynasore-treated cells under HD conditions showed less perturbation of secretory pathway targeted reporters than their counterparts in other compartments. Likewise, Mdivi-1–treated cells showed a greater heme reduction in the secretory pathway, presumably because inhibition of mitochondrial fission also impaired mitochondria heme export (Fig. 4C). Although a truncated isoform of the plasma membrane FLVCR1a heme exporter termed FLVCR1b was shown to perform this function (89, 90), organisms such as yeast lack FLVCR homologs, raising the possibility that interorganelle transport may serve as alternate modes of mitochondria heme transport.

Materials and Methods
All cell lines, culture conditions, reagents, and methods are described in SI Appendix, SI Materials and Methods. Heme-depleted FBS was prepared by treating FBS with ascorbic acid for ~7–8 h, followed by dialysis against PBS and filter sterilization. HEK293 cells were maintained in Dulbecco’s modified medium (DMEM) with 10% (vol/vol) FBS, 1% penicillin-streptomycin, and glutamine (PSG). HD medium was prepared as DMEM with heme-depleted 10% (vol/vol) FBS and 1% PSG. SA and HD + SA medium was prepared by adding 0.5 mM SA in basal or HD medium, respectively. C. elegans strains were maintained on nematode growth medium (NGM) agar plates seeded with OP50 bacteria or in axenic liquid mCehR-2 medium supplemented with 20 μM hemin at 20 °C.

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Supporting Information

Regulation of intracellular heme trafficking revealed by subcellular reporters

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Materials and Methods

Reagents preparation

Unless otherwise noted, all chemical and reagents were obtained from Sigma-Aldrich. Hemin, porphobilinogen (PBG) and metalloporphyrins were purchased from Frontier Scientific, Inc. Purified apoHRP was purchased from Calzyme. Fresh 10mM hemin stock solution was prepared in 0.3N NH₄OH, pH8.0. PBG was prepared as 50mM stock solution 0.3N NH₄OH, pH8.0 and stored at -80°C. Iron nitrolotriacetic acid (Fe:NTA) solution was prepared as a 1 mM stock with NTA (Sigma-Aldrich) and ferric chloride hexahydrate (molar ratio of 1:4). To eliminate residual HRP activity, apoHRP was extracted by acid acetone method as described elsewhere (1), dissolved in PBS as 50µM stock and stored at -20°C. Cycloheximide was prepared as 10mg/mL stock in water store at -20°C. Dynasore was prepared as 80mM stock in DMSO and store at -20°C. Mdivi-1 was prepared as 50mM stock in DMSO and store at -20°C.

Succinylacetone (SA) solution was prepared as 200mM stock and stored at -20°C. Heme depleted fetal bovine serum (FBS) was prepared by treating FBS with ascorbic acid for ~ 7–8 h, followed by dialysis against PBS and filter sterilization. The depletion of heme from the serum was monitored by measurement of the optical absorbance at 405 nm. Depletion was considered
successful when the absorbance of serum was reduced at least 50% following ascorbic acid treatment (2).

**Plasmid construction**

For mammalian cell expression, the open reading frame (ORF) of HRP and APX were amplified and fused with sorting signals by PCR, and cloned into pmCherry-N1 and pEGFP-C1 (Clontech) through 5’-HindIII and 3’-BamHI restriction sites, respectively. The signal peptides fused with HRP and APX for intracellular targeting are listed in Supplementary Table 1. Codon optimized APEX and APXH ORFs for mammalian expression were synthesized by Genscript and subcloned into pEGFP-C1 vector as described above.

To generate transgenic worm strains IQ9001 (Pvha-6::ER-HRP-mCherry::unc-54 3’utr), IQ9002 (Pdpy-7::ER-HRP-mCherry::unc-54 3’utr), IQ9003 (Pmyo-3::ER-HRP-mCherry::unc-54 3’utr), IQ9004 (Punc-119::ER-HRP-mCherry::unc-54 3’utr) and IQ9005 (Phsp-16.2::ER-HRP-mCherry::unc-54 3’utr), codon optimized ER-HRP with three artificial introns (gtaagtttaacatatatactacctgattatttaaattttcag) for worm expression was the synthesized by Genscript, fused with mCherry gene by PCR and cloned into the entry vector pDONR-221 by recombination using the Gateway BP Clonase kit (Invitrogen). Then the expression constructs were generated by recombining the promoter entry clones, fused hrp gene (ER-HRP-mCherry) and the 3’-UTR of unc-54 gene into a single destination vector, pDEST-R4-R3, using the Multisite Gateway system (Invitrogen). Constructs for RNAi were generated by cloning worm codon-optimized ER-HRP gene into L4440 vector through TA cloning, the mrp-5 knockdown construct has been generated previously (3). All the constructs have been confirmed by sequencing.
Mammalian cell culture

HEK293 cells were maintained in basal growth medium (Dulbecco’s modified medium (DMEM) with 10% FBS, 1% penicillin-streptomycin, and glutamine (PSG)). HD medium was prepared as DMEM with 10% FBS and 1% PSG. SA and HD + SA medium was prepared by adding 0.5mM SA in basal or HD medium, respectively.

For transfections, 1µg plasmid DNA / well was added with PolyJet (SignaGen) in 6-well plates according to manufacturer protocol. For peroxidase activity assay and immunoblotting, HEK293 cells were seeded on tissue culture plates and transfected with PolyJet the next day at a confluence of 90%. For fluorescent microscopy, HEK293 cells were seeded on poly-L-lysine coated glass coverslips and transfected with PolyJet the following day at a confluence of 50%.

Worm culture and generation of transgenic strains

*Caenorhabditis elegans* strains were maintained on nematode growth medium (NGM) agar plates seeded with OP50 bacteria or in axenic liquid mCeHR-2 medium supplemented with 20µM hemin at 20°C (4). Worm synchronization was performed as described elsewhere (5).

The transgenic worms were generated by microparticle bombardment (6). 10µg of the expression plasmids described above was mixed with 5µg of the *unc-119* rescue plasmid pDP#MM016B and co-bombarded into approximately 20,000 late L4 to young adult *unc-119(ed3)* worms using the PDS-1000 particle delivery system (Bio-Rad). The worms were then recovered on JM109 bacteria seeded NGM plates for two weeks at 20°C and screened by
genotyping and fluorescence signals. At least two transgenic lines of each construct were analyzed for each experiment.

**Fluorescence imaging and in situ DAB staining**

For mammalian cell fluorescent microscopy, 42 hours after transfection, HEK293 cells were fixed in 4% paraformaldehyde (PFA) for 1 hour at room temperature, stained with DAPI (4',6-diamidino-2-phenylindole) and mounted on coverslips with Prolong Antifade (Invitrogen). Images were taken on a Zeiss LSM710 confocal microscope using argon lasers and a 63x oil immersion lens. For co-localization, cells were stained with Alexa-Fluor-488-conjugated wheat germ agglutinin (WGA, Invitrogen) and MitoTracker Red CM-H2Xros (ThermoFisher) following the manuals, respectively.

For live worm imaging, young adult worms on OP50 bacteria seeded NGM plates were imaged directly under a Leica MZ16FA fluorescent stereomicroscope. For confocal microscopy, young adult worms were collected from axenic mCeHR-2 medium supplemented with 20μM hemin, washed with M9 buffer, immobilized with 10mM levamisole and imaged using a Zeiss LSM710 laser scanning confocal microscope with a 63x oil immersion objective.

The *in situ* DAB Staining was performed using the Pierce Peroxidase Detection Kit (ThermoFisher). Briefly, 42 hours after transfection, HEK293 cells were rinsed once with wash buffer (BupH Tris-buffered Saline) before adding 1x DAB-solution in stable peroxidase buffer. After 15 min incubation at room temperature the cells were rinse three times with wash buffer, follow by fixation with 4% PFA solution for 20 min. After removing PFA solution coverslips
were mounted on coverslides with Prolong Antifade (Invitrogen). Brightfield and fluorescence images were taken on a Leica DMIRE2 microscope using 63x oil immersion lens.

**Heme extraction from HRP and APX**

Heme extraction was a modification of the method of Teale (7). For extraction of heme from HRP, 20 mg of HRP (purchased from Sigma) was dissolved in 5 mL ice cold water at pH 1.9. An equivalent volume of ice-cold butanone was added and the solution mixed gently and stored on ice for 5 minutes. Two distinct layers were observed: the top (organic) layer containing the heme was removed by manual pipette, and fresh butanone was added in a second organic extraction to remove more of the heme. This process was repeated until the organic layer was colourless (ca. 4-5 times). The aqueous layer was then dialyzed against three times against 1 L volumes of 6 mM NaHCO₃ (i.e. 3 x 12 h dialyses, over a 48 hour period), and finally against 2 L of 10 mM Tris pH 8 overnight at 4 °C. Extraction of heme from APX was carried out exactly as for the HRP extraction, with the following modifications: APX (4mg/mL) was dissolved in ice cold water pH 1.7; following heme extraction, apo-APX was dialysed sequentially against 1 L of 1mM NaHCO₃, 1 L of 1 mM EDTA, 1 L of 1mM NaHCO₃ (12 hours each, over a 48 hour period), and finally against 2 L of 10 mM sodium phosphate buffer pH 8 overnight at 4 °C. Both apo-enzymes were concentrated and stored at 4°C. Apo-enzyme concentrations were determined using the absorption coefficients (A₂₈₀) of 20,000 M⁻¹cm⁻¹ for HRP and 21,430 M⁻¹cm⁻¹ for APX.
**Heme affinity assay**

Stock hemin solutions (dissolved in a minimal volume of 0.1 M NaOH) were filtered through a 0.2 µM filter and diluted to 100 µM with the appropriate titration buffer. The concentration of hemin was determined using an absorption coefficient of $\varepsilon_{385} = 58,400 \text{ M}^{-1}\text{cm}^{-1}$. The binding of hemin to the apo-peroxidases was carried out using difference absorption spectroscopy. Difference absorption spectra were recorded (250 - 700 nm) using a double beam spectrophotometer (Perkin-Elmer Lambda 40). Microlitre volumes of a hemin solution were added to a sample cuvette containing the apo-peroxidase and to a reference cuvette containing buffer only. Both HRP (8) and APX (9) have a dependency on metal ions for formation of the fully folded structure. Thus, the buffer used for the HRP titration was 50 mM Tris pH 8.0 containing 100 µM CaCl$_2$, and that for the APX titration was 10 mM potassium phosphate buffer pH 7 containing 150 mM KCl. Spectra were recorded after each hemin addition until no further spectral change was observed. Changes in the Soret peak ($A_{403}$ for HRP and $A_{406}$ for APX) were plotted as a function of hemin concentration; spectra at the end of the titrations were the same as those expected for the corresponding ferric enzymes. Data were fitted (by non-linear regression) to a one site binding model to yield equilibrium ($K_d$) constants.

**Total cellular heme quantification**

Hemin was extracted from the aqueous sample homogenate using four volumes of an extraction solvent. The extraction solvent was made by mixing four volumes of ethyl acetate and one volume of glacial acetic acid. The resulting phases were separated by spinning in a microcentrifuge for 10 seconds at maximum speed. Ten microliters of supernatant was injected
into a Waters (Millford, MA) Acquity ultra-performance liquid chromatography (UPLC) system that consisted of a binary solvent manager, sample manager, column heater, a photodiode array (PDA) detector and an Acquity UPLC BEH C18, 1.7 µM, 2.1 x 100 mm column. The hemin peak was measured at an absorption maximum of 398 nm and quantified relative to a standard solution subjected to the same extraction method. Quantitation of biliverdin and bilirubin was performed similarly.

To determine total protein concentration, the cell pellets were mixed with 200µL 100mM Tris pH 7.65 and sonicated 3 x 5 seconds at about 30 watts (low power, just enough to make an almost clear solution) using a Sonicare W-380 Ultrasonic Processor by Heat Systems - Ultrasonics, Inc., Farmingdale, NY. The resulting homogenate was assayed for protein using a Pierce BCA protein assay kit (Thermo Sicentific, Rockford, IL) and a SpectraMax 190 microplate reader paired with SoftMax Pro v5.0b7 software, both from Molecular Devices Corp (Sunnyvale, CA).

**In-gel DAB staining and immunoblotting**

Cells expressing HRP and APX reporters were collected 42 hours after transfection, washed twice with cold DPBS, lysed in buffer containing 1% Triton X-100, 20 mM Hepes pH 7.4 and 150 mM NaCl with Protease Inhibitor Cocktail Set III. For each lane, 50 µg of protein was loaded and separated on 10% native or SDS-PAGE gel at 4°C. For in-gel activity staining, the gel were equilibrated in 10mM HEPES, pH6.2 and 100µM CaCl₂ for 30 min at room temperature. Then peroxidase activity was detected by incubating the gel in 0.1 mg/mL 3,3′-
diaminobenzidine (DAB), 0.003% H$_2$O$_2$, 10mM HEPES, pH6.2 and 100 µM CaCl$_2$ for 1 hour to overnight at 4°C in the dark.

For immunoblotting, the gel was transferred to nitrocellulose membrane (BioRad). Membranes were blocked with 5% nonfat milk for one hour, and then incubated overnight at 4°C in primary antibody (mouse-α-GFP, 1:1,000, Covance; rabbit-α-HRP, 1:1,000, Sigma; rat-α-RFP/mCherry, 1:1,000, Chromotek) in 5% milk. After six washes in phosphate buffered saline with 0.05% Tween-20, blots were incubated with anti-mouse horseradish peroxidase conjugated secondary antibody in 5% milk for 1 hour. Blots were washed again six times in phosphate buffered saline with 0.05% Tween-20, and then developed using SuperWest Pico Chemiluminescent Substrate (Thermo Scientific).

**Peroxidase reporter activity assay**

HEK293 cells were transfected as described above. For heme depletion treatments, basal growth medium was replaced by HD, SA or HD + SA medium 30 minutes before transfection, and replaced again with the same media 24 hours after transfection, inoculated for additional 18 hours before harvesting. For heme repletion treatments, 24 hours after transfection and heme depletion, cells were treated with 100µg/mL cycloheximide for 30 min, the medium was then replaced by HD + SA with 2, 4, 8, 16µM heme or 0.125, 0.25, 0.5, 1mM PBG and 1mM Fe-NTA, and inoculated for additional 18 hours in the presence of 100µg/mL cycloheximide. For inhibitor treatment, transfected cells were deprived of heme for 24 hours, repleted with either endogenous heme (HD medium) or exogenous heme (HD + SA + 4µM heme) in the presence of 100µg/mL cycloheximide and 80µM dynasore or 50µM Mdivi-1 for additional 18 hours.
Cells were then harvested and washed twice with cold DPBS, lysed in 50µL lysis buffer (150mM NaCl, 20mM HEPES, 0.5% Triton X-100, with Protease Inhibitor Cocktail Set III). Then, 10µL cell lysate was mixed with 190µL freshly prepared peroxidase assay buffer (0.1mg/mL o-dianisidine, 0.02% H₂O₂ in 0.1M NaH₂PO₄/Na₂HPO₄ buffer, pH 6) in 96-well plate, and immediately determined absorbance at 440nm (A₄₄₀) and 600nm (A₆₀₀) using plate reader (SynergyHT, BioTek). For apoHRP reconstitution assay, 10µL cell lysate was mixed 10µL 50µM purified apoHRP solution, sit on ice for 10 minutes and then added 180µL peroxidase assay buffer to detect activity. EGFP and mCherry intensity of the lysates were determined by plate reader (SynergyHT, BioTek) using filter sets (EX 485/20nm, EM 528/20nm) and (EX 590/20nm, 620/15nm), respectively. Protein concentrations were measured by Bradford method. In cellula peroxidase activity was calculated by subtracting blank readings at baseline (A₆₀₀) and activity (A₄₄₀), than normalized to assay time, followed by a second normalization for reporter expression levels using fluorescence measurements (RFU) from the EGFP/mCherry tags or total protein (TP) as:

\[
\frac{(A_{440}\text{(sample)} - A_{600}\text{(sample)}) - (A_{440}\text{(blank)} - A_{600}\text{(blank)})}{(TP\text{sample or RFU}_{\text{sample}}) \times T_{\text{min}}}
\]

For worm peroxidase assay, 10,000 synchronized L1 larvae were grown in 10mL mCeHR-2 axenic liquid medium supplemented with 1.5, 4, 10, 20, 50, 100, 200 and 500µM heme for 72 hours, harvested at young adult stage, washed twice with M9 buffer, lysed in 100µL buffer containing 150mM NaCl, 20mM HEPES, 0.5% Triton X-100, with Protease Inhibitor Cocktail Set III and Lysing Matrix C beads (MP Biomedicals) in a FastPrep-24 Beadbeater (MP Biomedicals), and subjected to apoHRP reconstitution and reporter activity analysis as described above.
Endocytic assay

For transferrin uptake, inhibitor treated or Dynamin transfected HEK293 cells were incubated with 5 µg/mL DyLight-549-ChromPure-Human-Transferrin (Jackson Immunoresearch) for 10 minutes at 37˚C, washed in ice cold PBS, fixed in 4% PFA for 1 hour at room temperature, stained with DAPI and mounted on coverslips with Prolong Antifade (Invitrogen). Images were taken on a Zeiss LSM710 confocal microscope using argon lasers and a 63x oil immersion lens.

Heat-shock and RNAi knockdown assay

For heat-shock induction, synchronized L1 worms expressing Phsp-16.2::ER-HRP (IQ9005) were placed mCeHR-2 axenic liquid medium supplemented with 4, 20 and 100 heme at 20°C. Worms were heat-shocked for 30 min at 37 ºC, allowed to recover at 20°C for 0, 0.5, 1, 2, 4, 8, and 24 hours, then harvested at young adult stage (72 hours) by placing on ice, followed by washing twice with cold M9 buffer and peroxidase activity assay.

HT115 RNAi feeding bacteria were grown in LB broth for 5.5 hours and spotted on NGM plates with IPTG to induce dsRNA expression for 24 hours at room temperature. Prior to RNAi knockdown, transgenic worms maintaining in mCeHR-2 axenic liquid medium with 20 µM heme were bleached to synchronize the population, hatched overnight in M9 buffer. Synchronized L1 larvae were exposed to RNAi by feeding with HT115 bacteria expressing dsRNA against control vector, hrp and mrp-5 for 72 hours, harvested at young adult stage, lysed and subjected to peroxidase activity assay.
Bioinformatics and statistics

Structure information of HRP (1H5A(10)) and APX (1APX(11)) was acquired from RCSB Protein Data Bank, and the 3D structures were generated using PyMOL. Statistical significance was calculated by using one-way ANOVA with the Student–Newman–Keuls multiple comparison test in GraphPad INSTAT version 3.01 (GraphPad, San Diego). Data values were presented as mean ± SEM. A p value < 0.05 was considered as significant.
**Figure S1. Determination of heme affinity.** The binding of hemin to the apo-peroxidases was carried out using difference absorption spectroscopy. Microliter volumes of a hemin solution were added to a sample cuvette containing the apo-peroxidase and to a reference cuvette containing buffer only. Both (A) HRP and (B) APX have a dependency on metal ions for formation of the fully folded structure. Thus, the buffer used for the HRP titration was 50 mM Tris pH 8.0 containing 100 µM CaCl₂, and that for the APX titration was 10 mM potassium phosphate buffer pH 7 containing 150 mM KCl. Spectra were recorded after each hemin addition until no further spectral change was observed. Changes in the Soret peak (A₄₀₃ for HRP and A₄₀₆ for APX) were plotted as a function of hemin concentration; spectra at the end of the titrations were the same as those expected for the corresponding ferric enzymes. Data were fitted (by non-linear regression) to a one site binding model to yield equilibrium (Kₐ) constants. The affinity of HRP for heme was determined as Kₐ = 270 ± 40 nM; the corresponding value for APX was Kₐ = 360 ± 40 nM. Results are represented as mean ± SEM from three biological independent experiments.
A  

[Graph showing the change in ΔA403 vs. [Hemin] μM for HRP.]

B  

[Graph showing the change in ΔA406 vs. [Hemin] μM for APX.]
Figure S2. HRP reporters are not active in non-secretory pathway.  (A) Localization of targeted HRP reporters in HEK293 cells. C-terminus mCherry tagged HRP reporters were targeted to cytosol (Cyto), nucleus (NLS), mitochondrial matrix (Mito) and peroxisome (Pero), respectively. HEK293 cells were fixed 42 hours post-transfection, counterstained with DAPI, and imaged using a Zeiss LSM710 confocal microscope under a 63x oil immersion objective. Scale bar = 10 μm.  Transfected HEK293 cell lysates were then analyzed by (B) in-gel peroxidase activity staining of SDS-PAGE gel; (C) immunoblotting of SDS-PAGE gel with anti-HRP antibody (upper right); (D) immunoblotting of SDS-PAGE gel with anti-mCherry antibody. For each lane, 50 μg of protein was loaded and separated on 10% native or SDS-PAGE gel at 4°C.
Figure S3. Localization of targeted HRP and APX reporters in HEK293 cells.  (A) CD3δ-EGFP (CD3δ) (12), EGFP-TGN38 (TGN38) (13), Alexa-Fluor-488-conjugated wheat germ agglutinin (WGA), were employed as co-localization markers for ER, Golgi, plasma membrane (PM) targeted HRP reporters, respectively.  (B) Nuclear export sequence fused mCherry (NES), DAPI and MitoTracker Red were employed as co-localization markers for cytosol (Cyto), nucleus (NLS) and mitochondrial matrix (Mito) targeted APX reporters, respectively.  HEK293 cells were fixed 42 hours post-transfection, counterstained with DAPI, and imaged using a Zeiss LSM710 confocal microscope under a 63x oil immersion objective. Boxed regions in the reporter images have been enlarged for clarity and shown as merged images with co-localization markers. Scale bar = 10 μm.
Figure S4. Additional APX reporters for interrogating labile heme in the secretory pathway.  (A) APX reporters are tagged with EGFP at the C-terminus. The sorting signal used for ER targeting is Calreticulin secretion signal + KDEL. (B) Localization of targeted APX reporters in HEK293 cells. HEK293 cells were transfected with engineered APX constructs, fixed and stained with DAPI 42 hours after transfection, and imaged using a Zeiss LSM710 laser scanning confocal microscope with a 63x oil immersion objective. Scale bar = 10 μm. (C) Transfected HEK293 cells were heme deprived for 24 h, then grown at the indicated conditions in for additional 18 hours. Cells were lysed and assayed for peroxidase activity using o-dianisidine. In cellula peroxidase activity was calculated as described in Fig. 3. Reporter activity of different growth conditions was baseline corrected for activity observed in basal medium. Error bars represent SEM from three biological independent experiments.
Figure S5. Reporters do not “steal” heme following cell lysis. HEK293 Cells transfected with (A) ER-HRP and (B) Cyto-APX were grown at HD+SA condition to acquire apo-reporters. Cells were lysed in 50μL lysis buffer (150mM NaCl, 20mM HEPES, 0.5% Triton X-100, with Protease Inhibitor Cocktail Set III). Then, 10 µL cell lysate was mixed with equal volume of increasing hemin solution (0.2nM – 20nM), sat on ice for 30 min and assayed for peroxidase activity with o-dianisidine and H₂O₂ as the substrates. Calibrated by the standard curve in (A), the labile heme in the ER in HEK293 cells grown at basal condition was found to be 0.036 ± 0.006 pmol / 10⁶ cells. Assuming the volume of HEK293 cells is 2000 fl (volume of COS-7 and HeLa cells is 2000-2500 fl) (14), the total volume of one million cells is 2 µl. The ER occupies around 10% of total cellular space (15), so the total ER volume of one million cells is 0.2 µl. Therefore the labile heme concentration in the ER is calculated to be 0.182 ± 0.030 μM. Similarly, calibrated by the standard curve in (B), the cytosolic labile heme in HEK293 cells grown at basal condition was found to be 0.237 ± 0.042 pmol / 10⁶ cells. Since the cytosol occupies around 50% of total cellular space (15), the total cytosol volume of one million cells is 1 µl. The cytosolic labile heme concentration is calculated to be 0.237 ± 0.042 μM. To examine heme incorporation during cell lysis, HEK293 cells transfected with (C) (E) ER-HRP, or (D) (F) Cyto-APX were grown at indicated conditions, mixed with corresponding untransfected HEK293 cells at 1:1 ratio immediately before lysing, and measured peroxidase activity in the mixtures. Error bars represent SEM from three biological independent experiments. ns: non-significant, * p < 0.05, ** p < 0.01, *** p < 0.001.
A. ER-HRP

B. Cyto-APX

C. Basal

D. Basal

E. HD+SA

F. HD+SA

Hemin / fmoles

Reconstituted reporter activity

Peroxidase activity / RFU / min

ER-HRP

Cyto-APX

Basal

HD+SA

HEK293

+Heme/μM

0 5 20 0 0 0

0 0 0 0 5 20

0 0 0 0 5 20

0 5 20 0 0 0

+Heme/μM

0 5 20 0 0 0

0 0 0 0 5 20

0 5 20 0 0 0

0 5 20 0 0 0

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ns ns

***

ns ns

***

ns ns

***

ns ns
Figure S6. Evaluate total cellular heme in HEK293 cells. (A) HEK293 cells were grown at indicated conditions for 42 hours, homogenized in 100mM Tris pH 7.65 by sonication, and heme was extracted from the aqueous sample homogenate using in four volumes of extraction solvent (ethyl acetate : glacial acetic acid = 4:1). The resulting phases were separated by spinning and 10µL of supernatant was injected into a Waters (Millford, MA) Acquity ultra-performance liquid chromatography (UPLC) system. The hemin peak was measured at an absorption maximum of 398 nm and quantified relative to a standard solution subjected to the same extraction method. Total cellular heme is represented as pmol/mg protein. To determine heme uptake, HEK293 cells were grown at (B) basal or (C) HD+SA conditions for 24 hours, followed by exposing to 100µM Fe:NTA, 2µM or 16µM heme for additional 18 hours either at 4ºC (open bars) or 37ºC (black bars). Total cellular heme was determined as in (A). (D) To measure dose dependent reconstitution of apoHRP, 10 µl purified apoHRP (25 µM) in lysis buffer (150mM NaCl, 20mM HEPES, 0.5% Triton X-100, with Protease Inhibitor Cocktail Set III) was mixed with equal volume of increasing concentrations of hemin (0–2nM). The resulting activity of holoHRP was measured o-dianisidine and H2O2 as the substrates. Calibrated by the standard curve in (D), the labile heme in HEK293 cells grown at basal condition was found to be 0.606 ± 0.175 pmol / 10^6 cells. The volume of HEK293 cells is 2 µl / 10^6 cells as calculated in Figure S5, assuming cellular water fraction is 0.7 (16), the total aqueous volume of one million cells is 1.4 µl. Thus total cellular labile heme concentration is calculated to be 433 ± 125 nM. Error bars represent SEM from two biological independent experiments. Values with different letter labels are significantly different (P < 0.05).
Figure S7. Exogenous heme is incorporated into reporters. Transfected HEK293 cells were deprived of heme for 24 hours, followed by supplementation of (A)–(C) 16 μM metalloporphyrins or (D)–(F) increasing concentrations of MnPPIX in HD+SA media for additional 18 hours. Cells lysates were analyzed by (A) (D) immunoblotting with anti-EGFP antibody; (B) (E) in-gel DAB staining; and (C) (F) measuring peroxidase activity peroxidase activity with o-dianisidine and H$_2$O$_2$. (●: inactive APX monomers; ▲: active APX dimers).
Figure S8. Adding iron alone did not restore reporter activity. HEK293 cells were transfected with engineered HRP/APX constructs, deprived of heme for 24 hours, repleted with 0.1mM Fe:NTA for 18 hours, harvested and the lysates were assayed for peroxidase activity by adding o-dianisidine and H₂O₂ as the substrates. In cellula peroxidase activity was calculated as described in Fig. 3. Reporter activity of different growth conditions was baseline corrected for activity observed in basal medium. Error bars represent SEM from three biological independent experiments.
Figure S9. Exogenous heme is imported and incorporated in subcellular compartments. (A) Reporter transfected HEK293 cells were grown at HD+SA condition for 24 hours, treated with 0.1mg/mL CHX for 30 min and grown in HD+SA for additional 18 hours in the presence of 0.1mg/mL CHX. (B) Transfected HEK293 cells were grown at HD+SA condition for 24 hours, treated with 0.1mg/mL CHX for 30 min and grown in HD+SA+4µM heme for additional 18 hours in the presence of 0.1mg/mL CHX. Cells were then fixed with 4% PFA and stained for in situ peroxidase activity. Brightfield (DAB) and fluorescence (FP) images were taken on a Leica DMIRE2 microscope using 63x oil immersion lens. Scale bar = 10 µm.
Figure S10. Impaired clathrin-mediated endocytosis in dynamin inhibited cells. (A) HEK293 cells grown at basal condition were treated with DMSO, 80 μM Dynasore or 50 μM Mdivi-1 for 30 min, and then incubated with 5 μg/mL DyLight-549-Human-Transferrin for 10 min at 37°C, washed in ice cold PBS, fixed in 4% PFA for 1 hour at room temperature, stained with DAPI and mounted on coverslips with Prolong Antifade (Invitrogen). (B) Similarly, 24 hours post transfection, cells expressing dynamin WT and K44A protein were incubated with 5 μg/mL DyLight-549-Human-Transferrin for 10 min at 37°C, fixed in 4% PFA, stained with DAPI and mounted for imaging. Images were taken on a Zeiss LSM710 confocal microscope using argon lasers and a 63x oil immersion lens. The shape of two cells are outlined in (B), upper: untransfected cell, lower: cell expressing dynamin K44A mutant. Scale bar = 10 μm.
**Figure S11. Dynasore does not affect holo-peroxidase activity.** (A) HEK293 cells expressing reporters were harvested 42 hours post transfection, lysed in 50µL lysis buffer. Then, 9µL cell lysate was mixed with 1µL of inhibitor solution (200µM, 400µM and 800µM), sat on ice for 30 min and assayed for peroxidase activity. (B) Transfected HEK293 cells were heme deprived for 24 hours, treated with 0.1mg/mL CHX for 30min, repleted with either HD medium or HD+SA+4µM heme in the presence of 0.1mg/mL CHX for additional 18 hours, DMSO or Dynasore solution was then added to the cells and immediately harvested for peroxidase activity. For dose-responsive analysis, transfected HEK293 cells were heme deprived for 24 hours, treated with 0.1mg/mL CHX for 30min, repleted with either endogenous heme (HD medium, C) or exogenous heme (HD+SA+4µM heme, D) in the presence of 0.1mg/mL CHX and indicated concentrations of Dynasore for 18 hours prior to measuring peroxidase activity. *In cellula* peroxidase activity was normalized as in Fig. 3. Reporter activity of different treatments was baseline corrected for activity observed with DMSO condition. Error bars represent SEM from three biological independent experiments. ns: non-significant, * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure S12. Confocal images of transgenic worms. Transgenic worms expressing peroxidase reporters in (A) intestine: *Pvha-6::ER-HRP-mCherry*, (B) hypodermis: *Pdpy-7::ER-HRP-mCherry*, (C) muscle: *Pmyo-3::ER-HRP-mCherry*, and (D) neuron: *Punc-119::ER-HRP-mCherry* were collected from axenic mCeHR-2 medium supplemented with 20μM hemin, washed with M9, immobilized with 10mM levamisole and imaged using a Zeiss LSM710 laser scanning confocal microscope with a 63x oil immersion objective. Scale bar = 10 μm.
Figure S13. Measure heme homeostasis in worms.  (A) Total labile heme in WT worms determined with apoHRP directly correlates environmental heme levels. Synchronized L1 stage N2 worms were grown in mCeHR-2 medium supplemented with various concentrations of heme, harvested at young adult stage, lysed and subjected to labile heme analysis. Total labile heme was determined by reconstituting purified apoHRP activity and plotted against heme concentrations supplied in the mCeHR-2 medium. (B) Transgenic worm strains express ER-HRP reporter driven by tissue-specific promoters: intestine: *Pvha-6*; hypodermis: *Pdpy-7*; muscle: *Pmyo-3*; neuron: *Punc-119*. Synchronized L1 stage N2 and transgenic worms were grown in mCeHR-2 medium supplemented with various concentrations of heme, harvested at young adult stage, lysed and subjected to peroxidase activity analysis. Peroxidase activity was normalized to total cellular protein content. All the data are plotted against heme concentrations supplied in the mCeHR-2 medium. Error bars represent SEM from three biological independent experiments.
A

Relative holo-HRP activity

[Heme]/μM

B

HRP Activity/protein/minute

[Heme]/μM

N2
vha-6
dpy-7
myo-3
unc-119
Figure S14. The effect of transgene expression level can be minimized by internal calibration. Synchronized L1 stage reporter strains (Pmyo3::ER-HRP, muscle) were grown in mCeHR-2 medium supplemented with 20µM heme harvested at young adult stage. Reporter expression level was measured (A) with microplate reader using filter sets (EX 590/20nm, 620/15nm) and normalized by protein content; (B) by immunoblotting with anti-HRP (upper) and anti-tubulin (lower) antibody. (C) Reporter (Pmyo3::ER-HRP, muscle) activity is dependent on environmental heme availability and expression level. (D) Reporter (Pmyo3::ER-HRP) activity fold change (normalized to 1.5µM heme condition) is dependent on environmental heme availability but independent on expression level. Synchronized L1 stage reporter worms were grown in mCeHR-2 medium supplemented with various concentrations of heme, harvested at young adult stage, lysed and subjected to peroxidase activity analysis. Peroxidase activity was normalized to total cellular protein content (C) or to activity at 1.5µM heme (D). All the data are plotted against heme concentrations supplied in the mCeHR-2 medium. Error bars represent SEM from three biological independent experiments.
A  $P_{myo-3::ER-HRP-mCherry}$

B  $P_{myo-3::ER-HRP-mCherry}$

C  $P_{myo-3::ER-HRP-mCherry}$

D  $P_{myo-3::ER-HRP-mCherry}$
**Supplementary Table 1. Constructs of HRP and APX reporters.**

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Target</th>
<th>Targeting sequence</th>
<th>N-terminus added sequence</th>
<th>C-terminus added sequence</th>
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<td>PM-HRP</td>
<td>Plasma membrane</td>
<td>Human folate receptor (GPI anchored)</td>
<td>MAQRMTTQLLLLLLVWV AVVGEAQTRI</td>
<td>AAMSGAGPWAAWPFL SLALMLLLWLS</td>
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<tr>
<td>Cyto-HRP</td>
<td>Cytosol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NLS-HRP</td>
<td>Nucleus</td>
<td>2X NLS</td>
<td>MDPKKKRKVDPKKKRKV</td>
<td>-</td>
</tr>
<tr>
<td>Mito-HRP</td>
<td>Mitochondrial matrix</td>
<td>Subunit VIII of hCOX</td>
<td>MSVLTPLLRLGTGSAR RLPVRAKIHL</td>
<td>-</td>
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<tr>
<td>Pero-HRP</td>
<td>Peroxisome</td>
<td>SKL</td>
<td>-</td>
<td>SKL</td>
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<tr>
<td>Cyto-APX</td>
<td>Cytosol</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>MDPKKKRKVDPKKKRKV</td>
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</tr>
<tr>
<td>Mito-APX</td>
<td>Mitochondrial matrix</td>
<td>Subunit VIII of hCOX</td>
<td>MSVLTPLLRLGTGSAR RLPVRAKIHL</td>
<td>-</td>
</tr>
<tr>
<td>ER-APX</td>
<td>ER</td>
<td>Calreticulin SS + KDEL</td>
<td>MLLSVPLLGLLGLAVA</td>
<td>KDEL</td>
</tr>
<tr>
<td>NES-mCherry</td>
<td>Cytosol</td>
<td>NES</td>
<td>-</td>
<td>LQLPPLERLTD</td>
</tr>
</tbody>
</table>
Supplementary Table 2. Total intracellular heme quantification in HEK293 cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heme pmol/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>132.2 ± 8.1</td>
</tr>
<tr>
<td>HD</td>
<td>106.5 ± 0.9</td>
</tr>
<tr>
<td>Basal + SA</td>
<td>62.05 ± 3.75</td>
</tr>
<tr>
<td>HD + SA</td>
<td>38.1 ± 1.4</td>
</tr>
<tr>
<td>Basal + 100µM Fe:NTA (4ºC)</td>
<td>78.95 ± 2.35</td>
</tr>
<tr>
<td>Basal + 100µM Fe:NTA</td>
<td>102.85 ± 2.75</td>
</tr>
<tr>
<td>Basal + 2µM hemin (4ºC)</td>
<td>101.9 ± 1.6</td>
</tr>
<tr>
<td>Basal + 2µM hemin</td>
<td>112 ± 7.5</td>
</tr>
<tr>
<td>Basal + 16µM hemin (4ºC)</td>
<td>149 ± 1</td>
</tr>
<tr>
<td>Basal + 16µM hemin</td>
<td>166.3 ± 9.3</td>
</tr>
<tr>
<td>HD + SA + 100µM Fe:NTA (4ºC)</td>
<td>28.7 ± 1.4</td>
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<tr>
<td>HD + SA + 100µM Fe:NTA</td>
<td>36.7 ± 4.8</td>
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<tr>
<td>HD + SA + 2µM hemin (4ºC)</td>
<td>41.75 ± 1.65</td>
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<tr>
<td>HD + SA + 2µM hemin</td>
<td>94.5 ± 5</td>
</tr>
<tr>
<td>HD + SA + 16µM hemin (4ºC)</td>
<td>65.35 ± 5.25</td>
</tr>
<tr>
<td>HD + SA + 16µM hemin</td>
<td>116.2 ± 5.1</td>
</tr>
</tbody>
</table>

*HD: heme-depleted FBS, SA: succinylacetone, NTA: nitrilotriacetic acid.

**Total heme is presented as pmol/mg protein, mean and SEM are shown.