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Regulation of intracellular and interorgan heme trafficking revealed by subcellular reporters

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Abstract

Heme is an essential prosthetic group in proteins which 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. The current paradigm posits that cellular hemoproteins preferentially utilize de novo synthesized heme whereas extracellular heme is catabolized to release iron from the porphyrin. Here we show, using subcellular-targeted, genetically-encoded hemoprotein peroxidase reporters, that both extracellular and endogenous heme contributes to cellular labile heme, and that extracellular heme can be transported and utilized 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. We found that 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 *C. elegans* shows that environmental heme influences labile heme in a tissue-dependent manner; reporter activity in the intestine show a linear increase compared to 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 to serve as a heuristic paradigm for more sophisticated studies on the dynamics of heme trafficking in cellular and whole animal models.

Significance Statement

The intracellular trafficking of heme, a cytotoxic and hydrophobic cofactor in proteins such as hemoglobin, remains an underexplored area. The current view postulates that cellular hemoproteins, found in numerous subcellular organelles, acquire heme exclusively from de novo synthesis, while extracellular heme is catabolized to release iron. To critically examine this notion, we developed peroxidase-based enzymatic reporters for heme and deployed them in subcellular compartments in mammalian cells and in several tissues in the *C. elegans* animal model. Our results show that extracellular and endogenous heme is trafficked to virtually all intracellular compartments via distinct cellular routes, and that interorgan heme transport is essential for systemic regulation of heme homeostasis in *C. elegans*.

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Introduction

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, 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 organismal development (10-14). Given that heme is cytotoxic and hydrophobic (15, 16), the trafficking of heme for insertion and/or signaling is likely coordinated between heme transporters, chaperones, and carrier proteins. However the identity of these molecules is largely unknown.

The intracellular trafficking of heme remains an unexplored territory especially in light of the fact that hemoproteins reside in virtually every subcellular compartment (1). Regardless of whether heme is synthesized in the mitochondria or imported from the environment heme has to be translocated across membrane barriers (17, 18). It has been suggested that extracellular heme is entirely degraded through the heme oxygenase pathway to extract iron from the porphyrin (17, 19). Therefore, it is of significant interest to investigate whether exogenous heme is ever utilized in toto for cellular hemoproteins, 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.

While 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, 20, 21), 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 *C. elegans* animal model. Our results show that extracellular heme is utilized 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* validate the utility of these hemoprotein reporters and demonstrate that inter-organ heme transport is essential for organismal heme homeostasis.

Results

Subcellular targeting of peroxidase reporters

To determine whether heme is trafficked to various subcellular organelles, we needed to first develop appropriate reporters which would measure heme levels uniformly, irrespective of the intracellular membrane compartment. We employed 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 apo-protein and heme (22). Studies have employed purified apo-HRP to bind bioavailable heme in cell-free extracts as a way to measure regulatory heme (23-26). This *in vitro* HRP reconstitution method was found

to be more sensitive than the traditional pyridine hemochromogen method (27), with minimal interference from fluorescent porphyrins (28). However, a major drawback for this method was that the biological material must first be disrupted and then mixed with apo-HRP to measure the conversion of apo to holo thereby preventing analysis of subcellular heme distribution (23, 24).

HRP variants were created by using specific subcellular targeting sequences to restrict protein expression to the cytosol, mitochondrial matrix, peroxisome, endoplasmic reticulum (ER), Golgi complex, and the plasma membrane (PM, GPI anchored). However, only the ER, Golgi, and PM-targeted HRP was found to be active (data not shown), plausibly because HRP is a class III peroxidase that requires protein glycosylation and an oxidative, high calcium environment for proper folding in the secretory pathway (29) (Fig. 1A). To generate reporters for the remaining compartments, we used the cytosolic ascorbate peroxidase (APX), a class I heme-containing homodimer (30, 31) (Fig. 1B).

To ensure the correct targeting of these genetically-engineered probes, HRP and APX were tagged with mCherry and GFP, respectively (Fig. 1C). We first evaluated the heterologous expression of these two plant proteins in mammalian HEK293 cell lines and found them to be localized to the expected membrane compartment (Fig. 1D & E). In addition, targeting APX to the ER showed comparable expression levels as other APX probes, demonstrating that ER-HRP could be substituted by ER-APX in the secretory pathway (Supplementary Fig. 1A, B).

Subcellular labile heme dictates reporter activity

We tested the activity of our genetically-targeted probes as a function of heme concentrations by expression in HEK293 cell lines. Cells expressing engineered HRP exhibited 2,000 to 11,000-fold greater peroxidase activity. By contrast, APX reporters showed 50 to 60-fold greater activity (Fig. 2A); no APX activity was detected in a monomeric version of APX (APEX) or a mutant in which the active site has been mutated to mimic HRP (APXH; Fig. 2A) (32). This large signal-to-background ratio would provide sufficient range to detect changes in intracellular labile heme (LH).

Native HRP is a monomeric glycoprotein that only dimerizes when expressed as a non-glycosylated catalytically active protein in *Escherichia coli*. The equilibrium between monomeric and dimeric recombinant HRP is affected by the peroxidase substrates (33, 34). By contrast, APX is not glycosylated and forms a non-covalent homodimer in a concentration dependent manner (35, 36). Since the monomer-dimer equilibria of APX could interfere with our interpretation of changes in LH, we sought to characterize the active APX species in our cell-based assay conditions (36). We analyzed targeted APX reporters expressed in mammalian cells using an in-gel peroxidase activity assay and immunoblotting. APX reporters were only active on native PAGE, but not on SDS-PAGE (Fig. 2B, right panels). APX reporters migrated as monomers and dimers on native PAGE gels, and disassociated into monomers on SDS-PAGE (Fig. 2B, left panels); monomeric mutant APEX migrated exclusively as a monomer on both native and SDS-PAGE (Fig. 2B, lanes 4 and 9). Furthermore, immunoblotting revealed that the active APX on native PAGE corresponded to dimers, while the monomers had no detectable activity (Fig. 2B).

In order to investigate the heme dependence of APX dimerization, we examined APX reporters expressed in mammalian cells grown in different heme concentrations. We depleted cellular heme by first removing exogenous sources of heme from the media by replacing serum with heme-depleted serum (HD) and treating the cells with succinylacetone (SA), an inhibitor of heme synthesis which blocks the formation of porphobilinogen (PBG). In HD + SA conditions, APX migrated almost exclusively in its monomeric form (Fig. 2C, lane 4). Supplementation by increasing concentrations of exogenous heme resulted in gradual conversion of APX from a monomer to a dimer. At 16 μM heme, the majority of APX in the cytoplasm, nucleus and mitochondria was active and migrated as a dimer (Fig. 2C, 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

The current view posits that cellular hemoproteins acquire their prosthetic groups exclusively from *de novo* heme synthesis, while extracellular heme taken-up by the cell is catabolized to release iron. We, therefore, sought to determine the source of LH by first creating a heme deficiency condition using HD+SA, followed by heme supplementation. Total LH 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 LH, whereas cells treated with SA reduced LH by one-third (Fig. 3A). However, the combination of HD+SA decreased LH to 40% of basal values, and 2 μM heme completely reconstituted apo-HRP (Fig. 3A). The incomplete depletion of intracellular LH by the HD+SA combination treatment could be because cellular heme depletion may

require longer incubation periods (> 40h), or cellular LH can be depleted only to a certain level. Nonetheless, the LH concentration in HEK293 cells as measured by apoHRP in basal condition was estimated to be 433±125 nM, similar to a recently published study on *in vitro* reconstitution of HRP (24).

We next determined the effect of intracellular heme perturbation on subcellular-heme dynamics. Unlike the *in vitro* reconstitution of apoHRP, genetically-encoded HRP and APX reporter activities were undetectable in HD+SA (Fig. 3B). The heme depletion was highly effective across all the intracellular compartments, and persisted even in the presence of iron supplementation (Supplementary Fig. 2B). PM-HRP activity was the only reporter which was significantly reduced by HD alone, while the remaining reporters showed greatly reduced activity only when heme synthesis was inhibited by SA (Fig. 3B).

Supplementation with 2 to 4 μM heme restored ≥75 % of HRP activity across all six compartments (Fig. 3C), implying that extracellular heme can be imported and incorporated into these subcellular-targeted hemoproteins. Moreover, restoration of HRP activity by exogenous heme appeared to be compartment-specific with an overall hierarchy of Golgi > PM > ER > cytoplasm ≥ nucleus ≥ mitochondria (Fig. 3C). A similar preference was observed when PBG was supplemented in the grown medium to bypass the SA block. Between 250 to 500 μM PBG restored ≥50 % of the reporter activity in the following order: Golgi > ER > cytoplasm ≥ mitochondria ≥ nucleus ≥ PM (Fig. 3D). Together, these results indicate that both exogenous and endogenous heme contributes to cellular LH, and that exogenous heme can be utilized intact by hemoproteins.

Exogenous and endogenous heme are trafficked by distinct pathways

To differentiate how exogenous versus endogenous heme is transported and trafficked, we utilized two small molecule inhibitors: Dynasore, a small molecule GTPase inhibitor that targets dynamin (37); and MitoBloCK-6 (MB6), an inhibitor of mitochondrial protein Erv1 which is proposed to regulate redox-dependent protein translocation and heme export from the mitochondria (38, 39) (Fig. 4A). To restrict the source of intracellular heme, we grew the cells in either HD (source is endogenous heme) or HD + SA + 4 μM heme (source is exogenous heme). Total LH measured by *in vitro* reconstitution of apoHRP activity showed no difference between control and inhibitor treated cells under HD condition (Fig. 4B). However, Dynasore treated cells grown in the presence of 4 μM heme showed a significantly greater HRP activity raising the possibility that more LH is bioavailable when the formation or division of dynamin-dependent endocytic vesicles are inhibited (Fig. 4B).

We next examined the effects of these inhibitors on heme trafficking and compartmentalization using HRP/APX reporters. Even though the overall LH had not changed in the HD conditions, with the exception of mitochondrial APX, all the other reporters showed a significant reduction in activity in MB6-treated cells, consistent with the notion that Erv1 inhibition could interfere with extra-mitochondrial heme proteins and export (Fig. 4C). Reporter activities were fully restored in cells supplemented with 4 μM heme (Fig. 4D). Unlike plasma membrane HRP, all the other reporters showed significant reduction in activity when cells were treated with Dynasore in HD conditions (Fig. 4C). This could be because Dynasore, in addition to inhibiting dynamin-1 and

dynamin-2, also disrupts Drp1, the mitochondrial dynamin, and consequently interferes with mitochondrial fission and formation of the ER–mitochondria encounter structure (ERMES) (37, 40). Surprisingly, cells treated with 4 μM 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). Given that total LH was significantly higher in Dynasore treated cells (Fig. 4B), our results imply that hemoproteins residing in the secretory pathway preferentially acquire exogenous heme via a dynamin-dependent endocytic pathway. By contrast, cytoplasmic, nuclear and mitochondrial hemoproteins could acquire exogenous heme through a dynamin-independent process. These results also support the notion that the trafficking pathways for exogenous and endogenous heme are distinct with intrinsic preference for specific subcellular compartments (Fig. 4C, D).

Measuring interorgan heme homeostasis *in vivo*

To measure subcellular heme in various organs within an intact animal, we exploited *C. elegans* since it is a heme auxotroph. Heme levels can be experimentally manipulated by either nutritional supplementation or genetically regulating the heme trafficking pathways mediated by HRGs (13, 14, 20, 21, 41). We generated transgenic worms expressing HRP and APX reporters under the control of tissue-specific promoters. Since transgenic worms expressing the HRP reporters showed greater activity compared to APX reporters (not shown), we further characterized worms expressing ER-targeted HRP driven from the intestinal (*Pvha-6*), hypodermal (*Pdpy-7*), muscle (*Pmyo-3*) and pan-neuronal (*Punc-119*) promoters (Fig. 5A and Supplementary Fig. 3).

Wildtype (WT) worms were grown in mCeHR2 liquid axenic culture supplemented with varying concentrations of heme and total worm homogenates were incubated with apoHRP to measure LH (41). Although there was a direct correlation between apoHRP reconstitution and increasing heme (R^2=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 LH under these conditions (Supplementary Fig. 4A).

Transgenic worms expressing ER-HRP showed a heme-dependent increase in activity that was tissue dependent (intestine > hypodermis > neuron > muscle; Supplementary Fig. 4B). 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. The high-copy number strain showed > 5 fold greater activity at 20 μM heme compared to the low-copy strain (Supplementary Fig. 5A). However, when HRP activity at various heme concentrations was normalized to activity at 1.5 μM, the lowest concentration of heme which permits worm growth, no discernable differences were observed in both transgenic strains in their response to environmental heme (Supplementary Fig. 5B), suggesting that the effect of transgene copy numbers can be minimized by appropriate baseline correction.

Worms expressing intestinal HRP showed a linear increase in activity at concentrations beyond ≥ 50 μM heme, although not as rapidly as apoHRP reconstitution by LH (Fig. 5B). Activity of HRP reporters expressed from extra-intestinal 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 to worms grown at 1.5 μM (Fig. 5B), indicating that the neuronal threshold for heme is lower than non-neuronal tissues.

We next assessed the *in vivo* temporal kinetics for heme incorporation into HRP by expressing ER-HRP-mCherry from the heat-shock inducible promoter (*Phsp-16.2*). HRP was transiently expressed by exposing the worms to 37°C for 30 min. Expression of HRP-mCherry reporter activity could be easily quantified within 1 h post induction and maximal activity was observed at 4 h in worms grown at the optimal 20 μM heme (Fig. 5C). Notably, varying the concentration of heme by 5-fold did not show a proportional change in HRP activity probably because, at 4 μM and 100 μM heme, heme and apo-HRP might become limiting, respectively.

Regulation of interorgan heme homeostasis by intestinal heme export

We employed the transgenic worm reporter strains to directly measure tissue heme perturbations in genetically altered worms. In *C. elegans*, knockdown of *mrp-5* causes embryonic lethality that is suppressed by heme supplementation leading us to propose that MRP-5 exports heme from the intestine to extraintestinal tissues (13). Depletion of the *hrp* transgene suppresses HRP reporter 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 (42). 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 extra-intestinal tissues.

Discussion

Free heme is not readily soluble at neutral pH and forms cytotoxic radicals causing inflammatory tissue damage (15, 16, 43). These chemical properties of heme necessitate that cellular heme levels are tightly regulated whilst retaining transitory pools of regulatory heme. In accordance with the attributes of the well-documented labile iron pool (44), we termed this regulatory form of heme as labile heme (LH) – metabolically active form of heme associated with macromolecules or low molecular weight ligands and exchangeable with acceptor proteins. It is unclear whether LH is in dynamic equilibrium between unbound and bound states, or bound to putative heme-binding protein(s) such as heme chaperones for targeted delivery, or sequestered within subcellular organelles that serve as reservoirs. Intracellular LH 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 to 0.15 μM in normal human erythrocytes (45). Given that the affinity of heme to intra- and extra- cellular heme-binding proteins varies from 100 nM to <1 pM (46), and the Km of heme oxygenase-1 (HO-1), the major heme-degrading enzyme in mammals, is ~1 μM, cytosolic LH concentrations are likely to be less than 1 μM (47). Since heme can be transferred from one protein to another with a higher heme affinity (48, 49), *in vitro* reconstitution of purified apoHRP, which has a heme affinity

lower than housekeeping proteins that bind proto-heme or heme *b*, has been previously used to determine LH in IMR90 lung fibroblast cell extracts (23, 24). This method estimated LH to be 614 ± 214 nM, in close approximation to our estimate of 433 ± 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 LH by the *in vitro* reconstitution method, while *in cellula* activity for subcellular-targeted HRP and APX reporters are virtually undetectable (Fig. 3A and B). One simple explanation for this discrepancy could be that there are intrinsic differences in the process for *in vitro* versus subcellular processes hemylation i.e. conversion of apo to holo HRP by heme. Another plausible explanation could be that LH is stored in an intracellular compartment and inaccessible to the subcellular HRP/APX reporters during heme deprivation, but cell lysis disrupts this compartment thereby releasing the heme for apoHRP reconstitution.

Early heme absorption studies using dog intestines showed that heme is translocated intact across the membrane and collected in secondary endo-lysosomes in a subapical region colocalizing extensively with heme oxygenase-2 (HO-2) (50-54). 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 (55). We found that HEK293 cells supplied exclusively with exogenous heme accumulated 2.5-fold more LH when treated with Dynasore, resulting in attenuation of secretory pathway reporters without effect on non-secretory pathway reporters (Fig. 4B and D). These results suggest that a dynamin-

dependent endocytic pathway may exist for heme compartmentalization in mammalian cells. In support of this model, worms concentrated zinc mesoporphyrin IX, a fluorescent heme analog, in intracellular membrane-bound vesicles surrounded by the endo-lysosomal heme transporter CeHRG-1 (14, 56). *C. elegans* expressing the intestinal reporter showed a well-fitted correlation between ER-HRP activity and environmental heme indicating greater heme accumulation in the intestine than any of the other extraintestinal reporters (Fig. 5B). This would be expected as *C. elegans* does not synthesize heme and extra heme absorbed through the intestine might be either sequestered for detoxification or stored in specialized compartments for later use. While we don't know the precise nature or composition of this compartment, developing additional heme sensors that can report LH dynamically in these heme-storage compartments would be of great importance.

Extracellular heme was previously believed to be entirely degraded to liberate iron for endogenous heme synthesis in mammalian cells. Our results counter this 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 the six subcellular compartments examined in the absence of endogenous heme synthesis (Fig. 3C). These findings may at least partially explain the phenomenon for why intravenous heme, administered as a therapeutic treatment to acute porphyria patients, increases heme-dependent enzyme activity in the liver as hepatocytes may import and utilize the injected heme *in toto* (57). Additionally, even yeast may have the capability to distinguish endogenously produced or exogenously acquired heme as overexpression of

Pug1p, a heme transporter in *S. cerevisiae*, selectively suppressed cell growth when exogenous heme was the sole heme source (58).

We found that the Golgi and ER appear to be important locales 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 (59). Extracellular heme uptake in eukaryotes has been proposed to be either mediated by transporters (14, 60), or through endocytosis of hemoproteins (61-65). Inhibition of endocytosis by low temperatures showed that the heme analog palladium mesoporphyrin IX could still reach reservosomes, a lysosome-related organelle in *Trypanosoma cruzi* epimastigotes, through a endocytosis-independent mechanism within few seconds (66). Using Dynasore to inhibit dynamin we found that LH 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 not surprising 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. While 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 (MAMs) or the ER–Mitochondria Encounter Structure (ERMES) (1, 67, 68). These structures facilitate calcium transport and lipid trafficking between the ER and mitochondria. Importantly, ferrochelatase, heme-binding protein-1 (HBP1) and HO-2

were all detected at these interorganelle contact points (69, 70). 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 its counterparts in other compartments. Likewise, MB6-treated cells showed a greater heme reduction in the secretory pathway, presumably because Erv1 inhibition 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 (71, 72), how heme exits the mitochondria is still poorly understood. The precise location of FLVCR1b on the mitochondria or its contact points with other organelles have not been determined. Moreover, organisms such as yeast lack FLVCR homologs raising the possibility that inter-organelle transport may serve as alternate modes of mitochondrial heme transport.

C. elegans and helminthes are unable to synthesize heme *de novo* and need environmental heme to sustain growth and reproduction (41, 73). Consequently, coordination of heme homeostasis is essential for worm survival. Depletion of the heme importer *hrg-4* or exporter *mrp-5* upregulates a heme deficiency signal in the intestine even though they cause intestinal heme deficiency and excess, respectively (13, 14, 56). These contrasting results imply that intestinal heme transport does not function independently but is coordinated with extraintestinal heme requirements (13, 74). Our results show that HRP reporter activity in the intestine exhibited the sharpest change following increase in environmental heme (Fig. 5B). By contrast, neuronal cells maintained heme levels within a narrow range. Being the primary organ for heme uptake, storage and delivery, the worm intestine may also serve as an organ that

integrates autonomous and non-autonomous signals to provide and prioritize heme to other tissues such as neurons under low heme conditions, as well as to sequester excess heme to prevent cytotoxicity under high heme conditions. Inter-organ communications have been proposed in higher organisms, for instance, the "gut-brain axis" (75, 76). It is therefore reasonable to speculate an inter-organ hierarchy and prioritization, originally proposed for iron and copper, may also exist for heme trafficking and homeostasis at the organismal level (77, 78).

Materials and Methods

Full methods are described in detail in the SI Materials and Methods.

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Figure legends

Figure 1 Synthesis of peroxidase-based reporters for interrogating intracellular LH. (A) Structure and heme binding sites of HRP (from PDB IDs 1H5A(79)) and **(B)** APX (from PDB IDs 1APX(30)). The heme cofactor is shown in yellow. Residues involved in heme binding are indicated and displayed with side-chains. APX forms a non-covalent homodimer with each monomer binding one heme. **(C)** HRP and APX reporters were fused to mCherry and EGFP, respectively. The sorting signals used for intracellular targeting are described in the Methods section. **(D)** Localization of targeted HRP and **(E)** APX reporters in HEK293 cells. HEK293 cells were fixed 42 h post-transfection, counterstained with DAPI, and imaged using a Zeiss LSM710 confocal microscope under a 63x oil immersion objective. Scale bar = 10 μm.

Figure 2 Subcellular LH dictates reporter activity. (A) HEK293 cells were harvested 42 h after transfection with engineered HRP/APX constructs and assayed for peroxidase activity by adding *o*-dianisidine and H_2O_2 as the substrates. Peroxidase activity was normalized for total cellular protein content. hpAPEX: humanized monomeric version of APX; hpAPXH: humanized mutant in which the active site has been mutated to mimic HRP. Error bars represent SEM from three independent experiments. ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(B)** Transfected HEK293 cell lysates were analyzed by in-gel peroxidase activity staining of native PAGE gel (upper left); or immunoblotting of native PAGE gel with anti-EGFP antibody (upper right); in-gel activity staining of SDS-PAGE gel (lower left); immunoblotting of SDS-PAGE gel with anti-EGFP antibody (lower right). **(C)** Transfected HEK293 cells were deprived of heme for 24 h followed by heme supplementation for the indicated heme concentrations for 18 h. Cells

were lysates were analyzed by in-gel DAB staining and immunoblotting with anti-EGFP antibody (●: inactive APX monomers; ▲: active APX dimers).

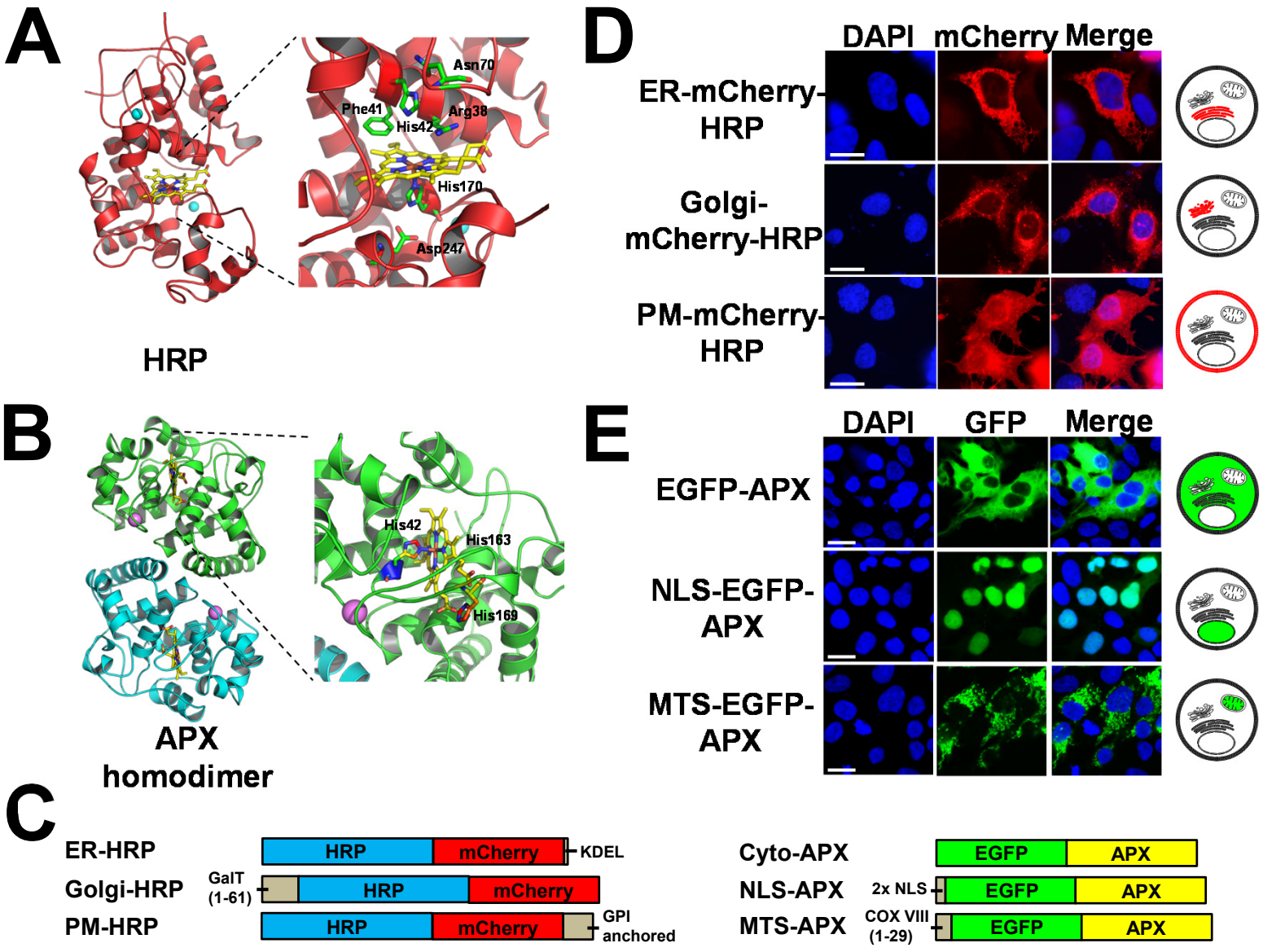
Figure 3 Intracellular LH is influenced by both endogenous and exogenous heme. (A) HEK293 cells were deprived of heme for 24 h followed by repletion with indicated concentrations of heme for 18 h. Cells were harvested and total LH in the lysates was analyzed by reconstituting purified apoHRP activity using o-dianisidine and H_2O_2 as the substrates. (B) Transfected HEK293 cells were grown in (B) the indicated conditions for 42 h or (C) after 24 h were exposed to varying concentrations of heme or (D) PBG plus 1mM Fe for 18 hours. Cells were lysed and assayed for peroxidase activity using o-dianisidine. *In cellula* peroxidase activity was calculated by subtracting blank readings at baseline (OD_{600}) and activity (OD_{440}), than normalized to assay time, followed by a second normalization for reporter expression levels using fluorescence measurements (RFU) from the GFP/mCherry tags or total protein (TP) $\frac{(OD_{440(sample)} - OD_{600(sample)}) - (OD_{440(blank)} - OD_{600(blank)})}{(RFU_{sample} \text{ or } TP_{sample}) \times T_{min}}$. Reporter activity of different growth conditions was baseline corrected for activity observed in basal medium. Error bars represent the standard error of the mean (SEM) from three independent experiments.

Figure 4 Endogenous and exogenous heme trafficking are differentially allocated at the subcellular level. (A) Illustration of pathways affected by Dynasore and mitoblock-6 (MB-6). **(B)** Untransfected HEK293 cells were deprived of heme for 24 h, repleted with either endogenous heme (HD medium) or exogenous heme (HD + SA + 4 μM heme) in the presence of 80 μM dynasore or 50 μM MB-6 for 18 h, harvested and total LH in the lysates was analyzed by

reconstituting apoHRP activity. Transfected HEK293 cells were grown in either endogenous **(C)** or exogenous heme **(D)** 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 the standard error of the mean (SEM) from three independent experiments. ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5 Tissue-specific peroxidase reporters in *C. elegans* show regulation of interorgan heme trafficking. (A) Tissue-specific expression of reporters in transgenic worms. Live young adult worms on OP50 seeded NGM plates were imaged using a Leica MZ16FA fluorescent stereomicroscope. Scale bar = 50 μm. **(B)** Synchronized L1 larvae were grown in mCeHR-2 axenic liquid medium supplemented with various concentrations of heme (x-axis), harvested at young adult stage, lysed and subjected to LH and reporter activity analysis. Total LH (black circles, right y-axis) was determined by reconstituting purified apoHRP activity. Reporter activity in different tissues (left y-axis) was baseline corrected for activity observed at 1.5 μM heme. **(C)** Synchronized L1 larvae carrying the transgene for the heat-shock inducible reporter (*Phsp-16.2::ER-HRP-mCherry*) were grown in mCeHR-2 axenic medium supplemented with different concentrations of heme at 20° C. Worms were heat-shocked for 30 min at 37 °C, allowed to recover at 20° C for varying lengths of time, harvested and the lysates were analyzed for peroxidase activity. **(D)** Synchronized transgenic L1 larvae were exposed to RNAi by feeding HT115 bacteria expressing dsRNA against control vector, *hrp* and *mrp-5*, harvested at young adult stage, lysed and subjected to peroxidase activity assay. Peroxidase activity was normalized

by RFU. Error bars represent the standard error of the mean (SEM) from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.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C
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Golgi-HRP
PM-HRP
GalT
(1-61)
HRP
mCherry
KDEL
GPI
anchored
Cyto-APX
NLS-APX
MTS-APX
2x NLS
COX VIII
(1-29)
EGFP
APX
D
DAPI
mCherry
Merge
ER-mCherry-
HRP
Golgi-
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DAPI
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APX

Figure 1

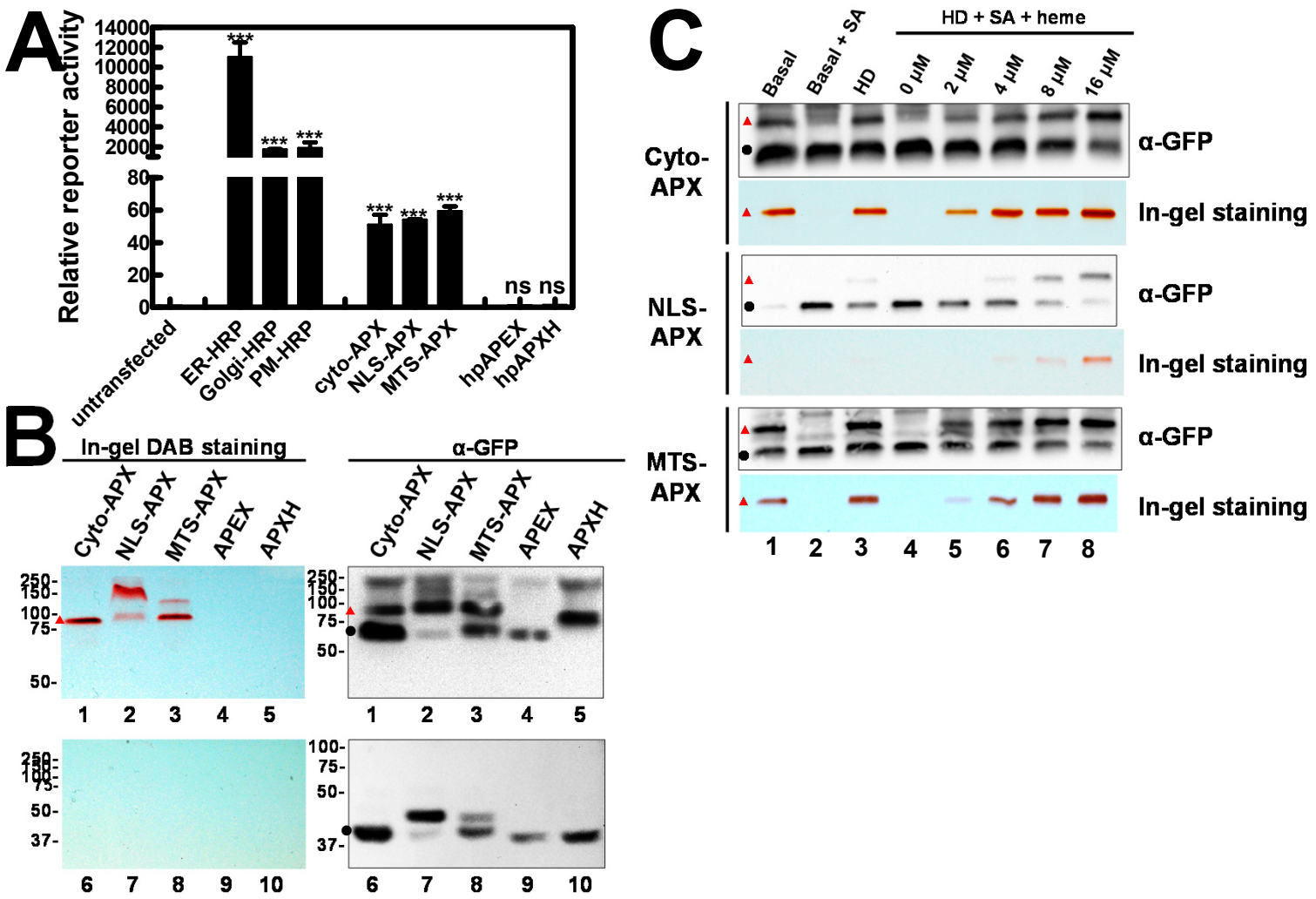
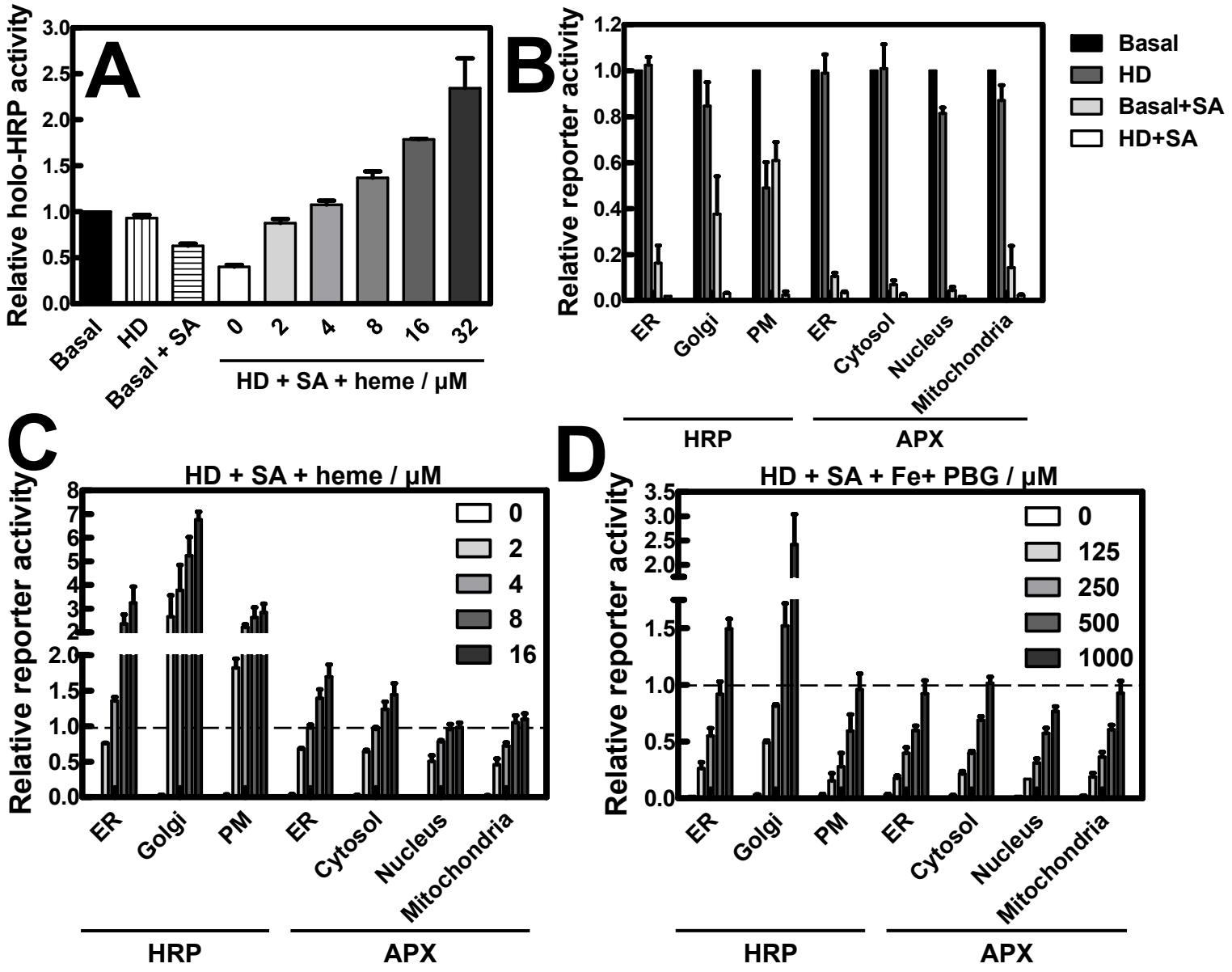

A
Relative reporter activity
14000
12000
10000
8000
6000
4000
2000
80
60
40
20
0
***
***
***
***
***
***
ns ns
untransfected
ER-HRP
Golgi-HRP
PM-HRP
cyto-APX
NLS-APX
MTS-APX
hpAPEX
hpAPXH
B
In-gel DAB staining
α-GFP
Cyto-APX
NLS-APX
MTS-APX
APEX
APXH
250-
150-
100-
75-
50-
37-
1 2 3 4 5
6 7 8 9 10
C
Basal
Basal + SA
HD
HD + SA + heme
0 μM
2 μM
4 μM
8 μM
16 μM
Cyto-APX
NLS-APX
MTS-APX
α-GFP
In-gel staining
1 2 3 4 5 6 7 8

Figure 2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Relative holo-HRP activity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Basal
HD
Basal + SA
0
2
4
8
16
32
HD + SA + heme / μM
B
Relative reporter activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ER
Golgi
PM
ER
Cytosol
Nucleus
Mitochondria
HRP
APX
Basal
HD
Basal+SA
HD+SA
C
Relative reporter activity
HD + SA + heme / μM
0
2
4
8
16
ER
Golgi
PM
ER
Cytosol
Nucleus
Mitochondria
HRP
APX
D
Relative reporter activity
HD + SA + Fe+ PBG / μM
0
125
250
500
1000
ER
Golgi
PM
ER
Cytosol
Nucleus
Mitochondria
HRP
APX

Figure 3

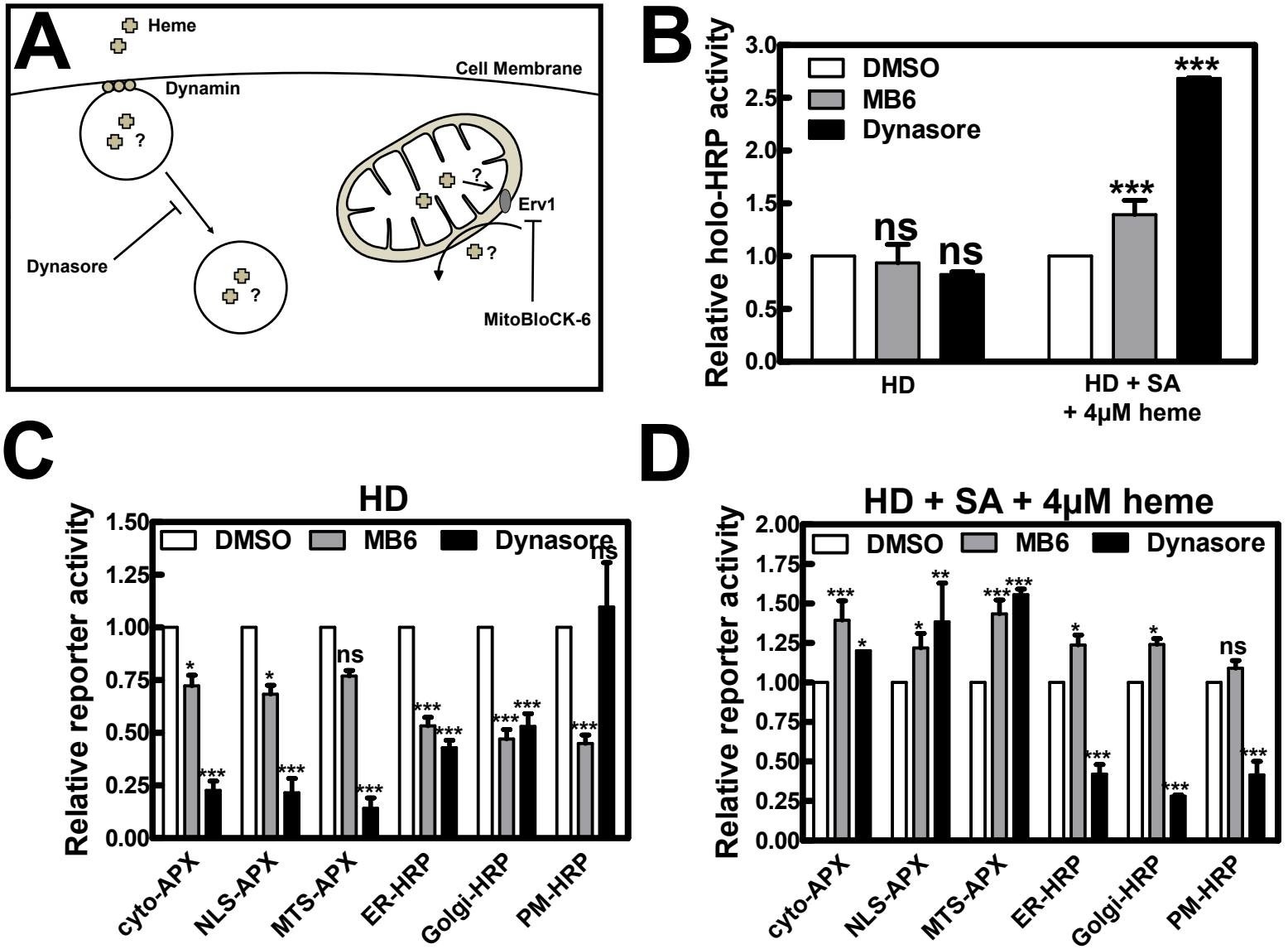
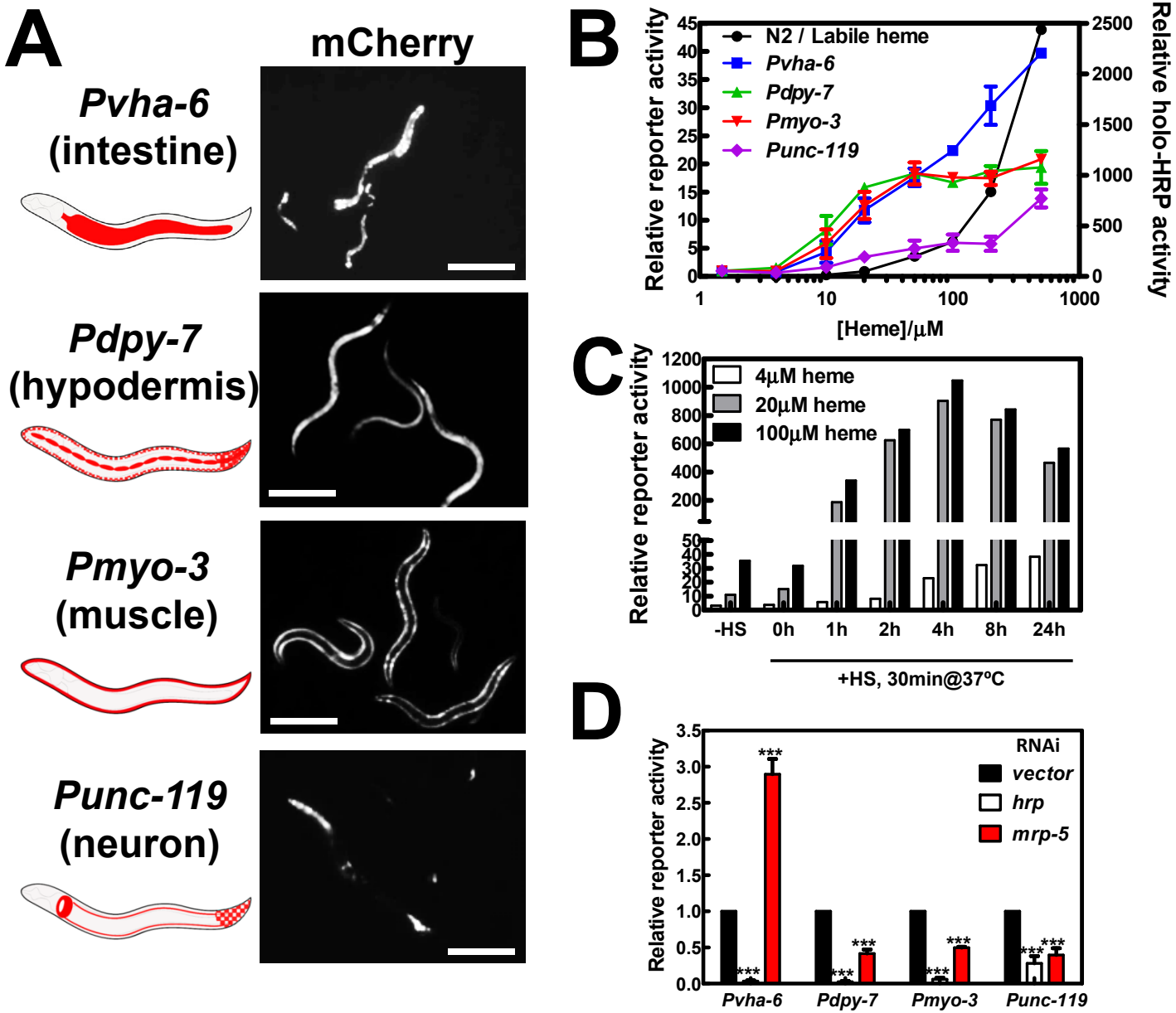

A
Heme
Cell Membrane
Dynamin
?
Dynasore
Erv1
MitoBloCK-6
B
Relative holo-HRP activity
DMSO
MB6
Dynasore
ns
ns
***
***
HD
HD + SA
+ 4μM heme
C
HD
Relative reporter activity
DMSO
MB6
Dynasore
cyto-APX
NLS-APX
MTS-APX
ER-HRP
Golgi-HRP
PM-HRP
D
HD + SA + 4μM heme
Relative reporter activity
DMSO
MB6
Dynasore
cyto-APX
NLS-APX
MTS-APX
ER-HRP
Golgi-HRP
PM-HRP

Figure 4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mCherry
Pvha-6
(intestine)
Pdpy-7
(hypodermis)
Pmyo-3
(muscle)
Punc-119
(neuron)
B
Relative reporter activity
Relative holo-HRP activity
N2 / Labile heme
Pvha-6
Pdpy-7
Pmyo-3
Punc-119
[Heme]/μM
C
Relative reporter activity
4μM heme
20μM heme
100μM heme
-HS
0h
1h
2h
4h
8h
24h
+HS, 30min@37°C
D
Relative reporter activity
RNAi
vector
hrp
mrp-5
Pvha-6
Pdpy-7
Pmyo-3
Punc-119

Figure 5