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 NH_4OH, pH8.0. PBG was prepared as 50mM stock solution 0.3N NH_4OH, pH8.0 and stored at -80ºC. 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 -20ºC. Cycloheximide was prepared as 10mg/mL stock in water store at -20ºC. Dynasore was prepared as 80mM stock in DMSO and store at -20ºC. Mdivi-1 was prepared as 50mM stock in DMSO and store at -20ºC.

Succinylacetone (SA) solution was prepared as 200mM stock and stored at -20ºC. 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 (gtaagtttaaacatatatatactaactaaccctgattatttaaattttcag) 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

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 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 con-focal 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 $NaHCO_3$ (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 $NaHCO_3$, 1 L of 1 mM EDTA, 1 L of 1mM $NaHCO_3$ (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 (A_{280}) of 20,000 $M^{-1}cm^{-1}$ for HRP and 21,430 $M^{-1}cm^{-1}$ 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 ε_{385} = 58,400 $M^{-1}cm^{-1}$. 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 $CaCl_2$ 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_2O_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*-dianasidine, 0.02% H_2O_2 in 0.1M NaH_2PO_4/Na_2HPO_4 buffer, pH 6) in 96-well plate, and immediately determined absorbance at 440nm (A_{440}) and 600nm (A_{440}) 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 (A_{600}) and activity (A_{440}), 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:

$$\frac{(A_{440(\text{sample})} - A_{600(\text{sample})}) - (A_{440(\text{blank})} - A_{600(\text{blank})})}{(\text{TP}_{\text{sample}} \text{ or } \text{RFU}_{\text{sample}}) \times \text{T}_{\text{min}}}$$

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 Immuno Research) 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

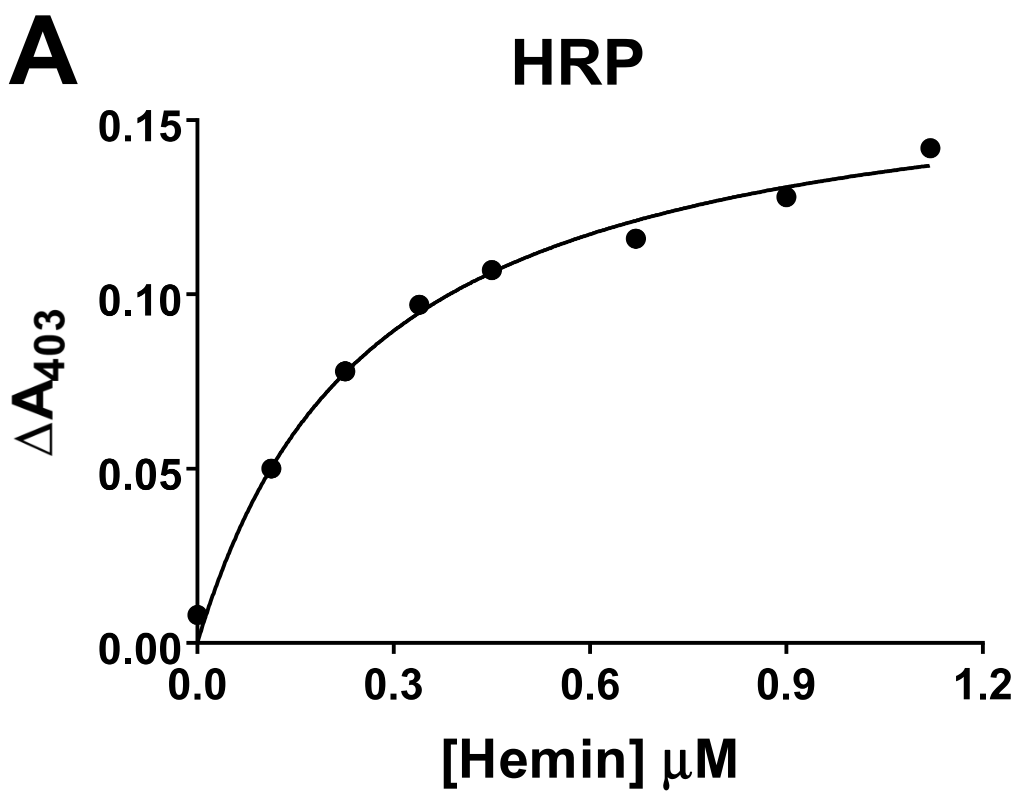
For heat-shock induction, synchronized L1 worms expressing *Phsp-16.2::ER-HRP* (IQ9005) were placed mCeHR-2 axenic liquid medium supplemented with 4, 20 and 100 heme at 20°C. Worms were heat-shocked for 30 min at 37 ºC, allowed to recover at 20ºC for 0, 0.5, 1, 2, 4, 8, and 24 hours, then harvested at 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 $CaCl_2$, 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. The affinity of HRP for heme was determined as $K_d = 270 \pm 40$ nM; the corresponding value for APX was $K_d = 360 \pm 40$ nM. Results are represented as mean ± SEM from three biological independent experiments.


A
HRP
0.15
0.10
0.05
0.00
ΔA403
0.0
0.3
0.6
0.9
1.2
[Hemin] μM

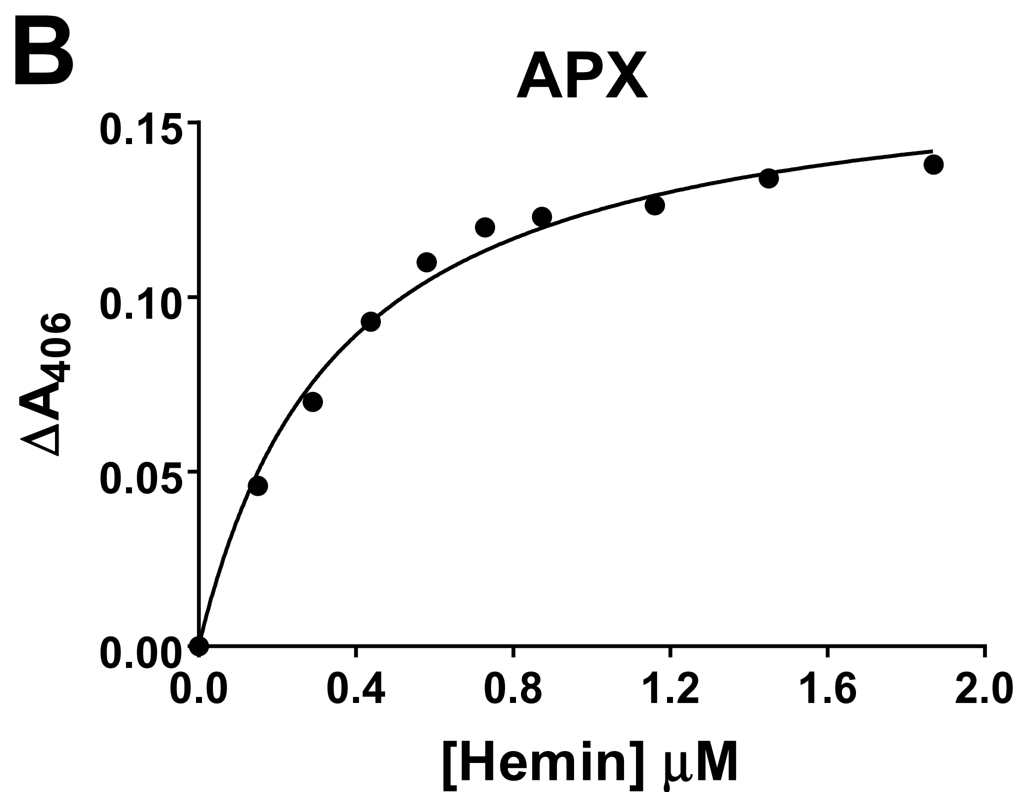

B
APX
0.15
0.10
0.05
0.00
ΔA406
0.0
0.4
0.8
1.2
1.6
2.0
[Hemin] μM

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.

A

mCherry DIC DAPI Merge

Cyto-HRP

NLS-HRP

Mito-HRP

Pero-HRP

B

HRP-mCherry

ER Golgi PM Cyto NLS Mito Pero

kD

250-
150-
100-
75-
50-
37-
25-

1 2 3 4 5 6 7

In-gel DAB staining

C

HRP-mCherry

ER Golgi PM Cyto NLS Mito Pero

kD

250-
150-
100-
75-
50-
37-

1 2 3 4 5 6 7

α-HRP

D

HRP-mCherry

ER Golgi PM Cyto NLS Mito Pero

kD

250-
150-
100-
75-
50-
37-

1 2 3 4 5 6 7

α-mCherry

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.

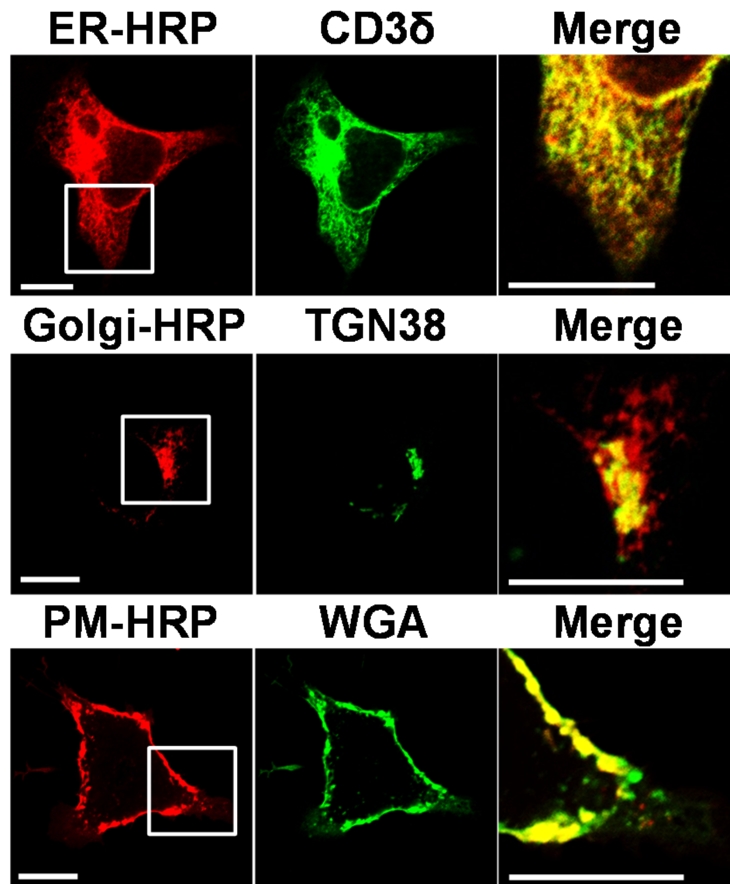
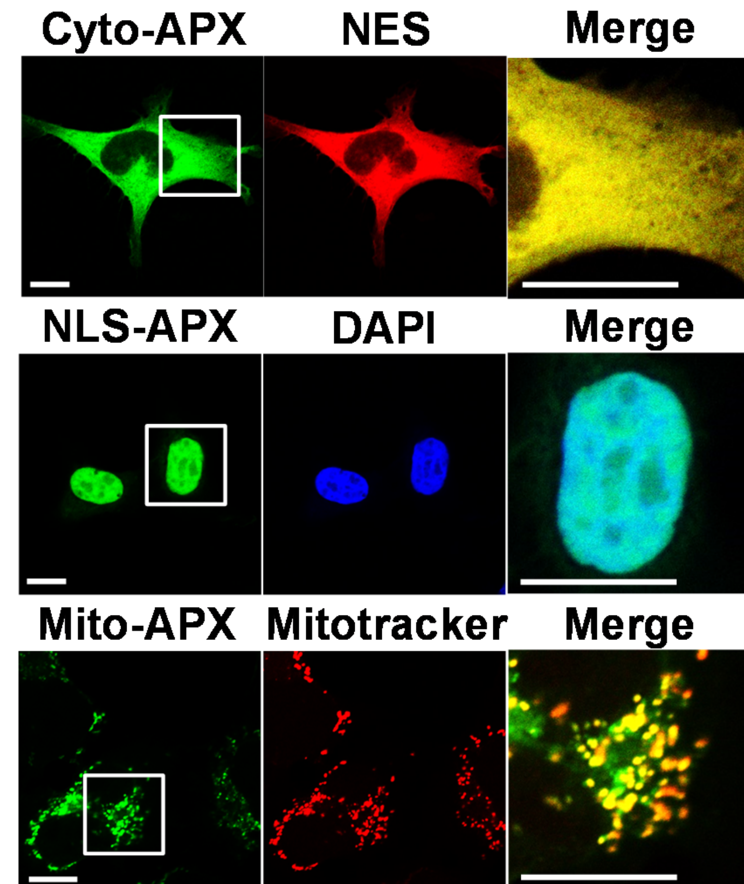
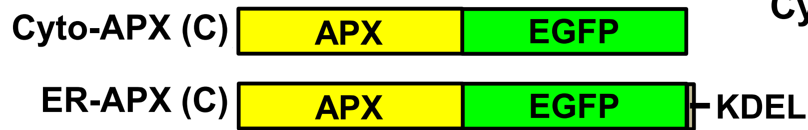
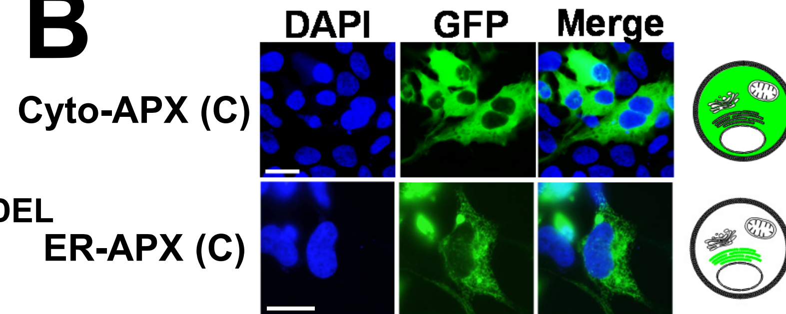

A
ER-HRP
CD3δ
Merge
Golgi-HRP
TGN38
Merge
PM-HRP
WGA
Merge

B
Cyto-APX
NES
Merge
NLS-APX
DAPI
Merge
Mito-APX
Mitotracker
Merge

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.

A



B



C

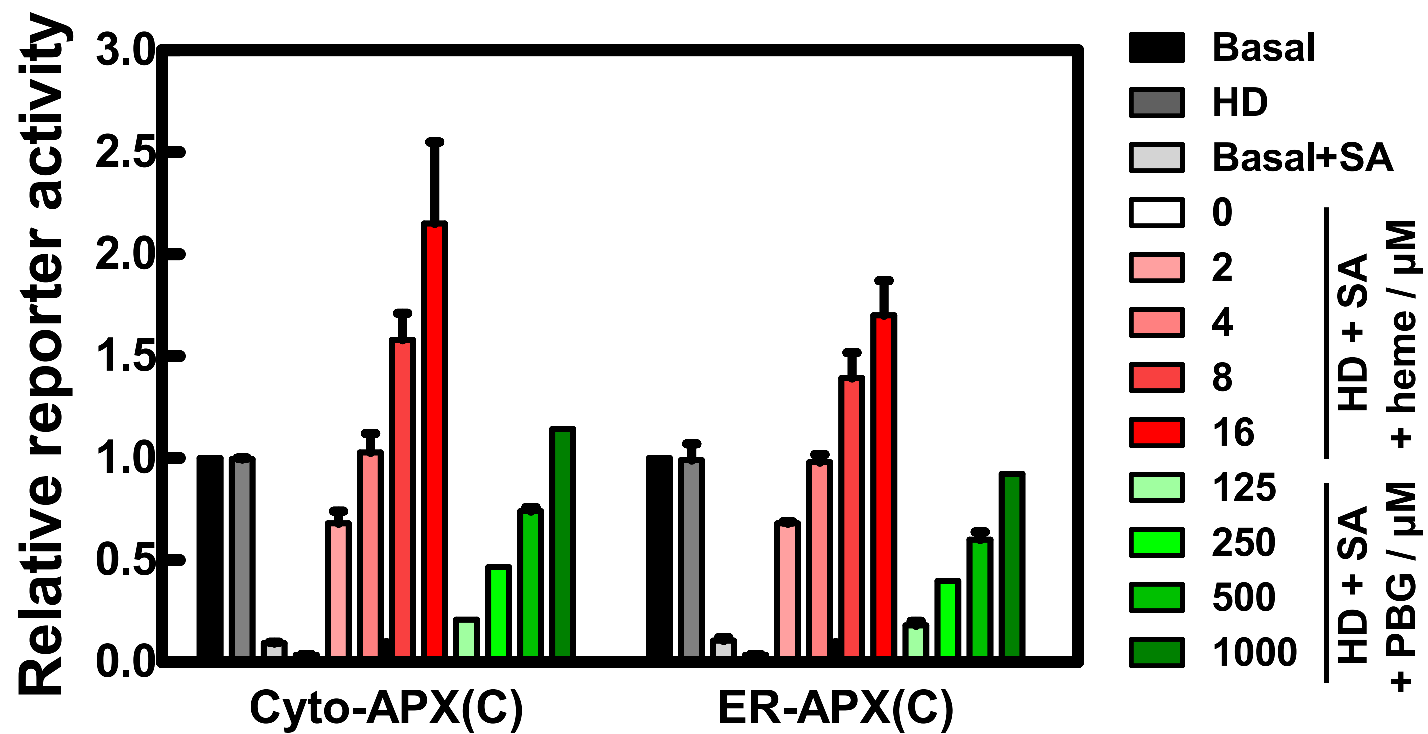
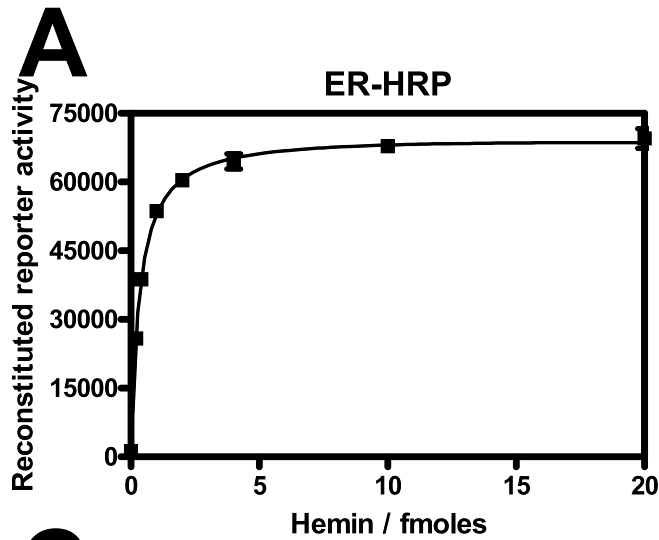
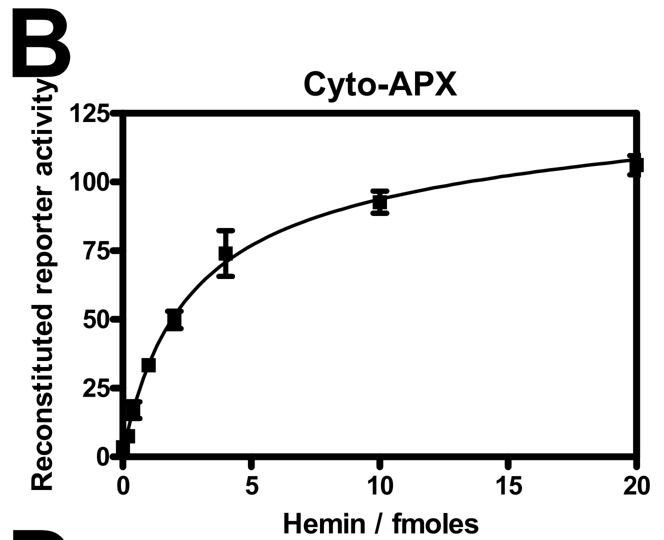


Figure S5. Reporters do not "steal" heme following cell lysis. HEK293 Cells transfected with **(A)** ER-HRP and **(B)** Cyto-APX were grown at HD+SA condition to acquire apo-reporters. Cells were lysed in 50μL lysis buffer (150mM NaCl, 20mM HEPES, 0.5% Triton X-100, with Protease Inhibitor Cocktail Set 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 H_2O_2 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 / 10^6 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 / 10^6 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
Reconstituted reporter activity
75000
60000
45000
30000
15000
0
0
5
10
15
20
Hemin / fmoles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Cyto-APX
Reconstituted reporter activity
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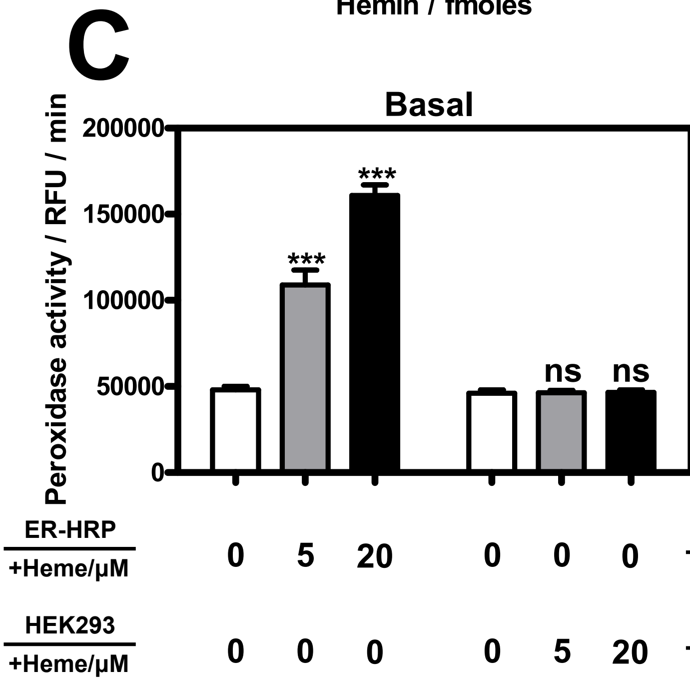
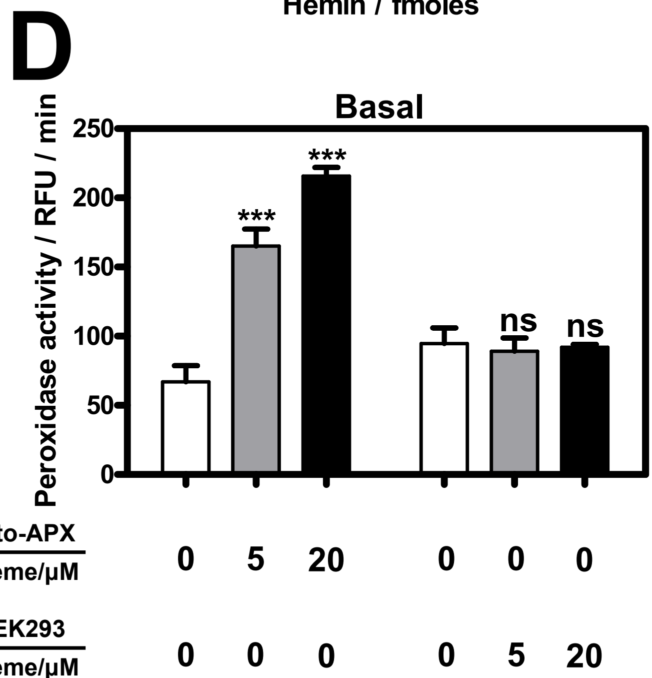
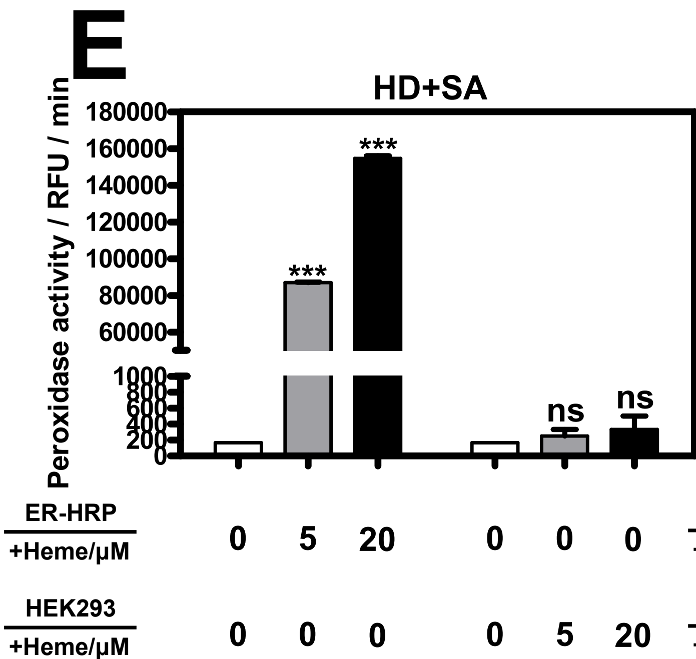
100
75
50
25
0
0
5
10
15
20
Hemin / fmoles


C
Basal
Peroxidase activity / RFU / min
200000
150000
100000
50000
0
***
***
ns
ns
ER-HRP
+Heme/μM
0 5 20 0 0 0
HEK293
+Heme/μM
0 0 0 0 5 20


D
Basal
Peroxidase activity / RFU / min
250
200
150
100
50
0
***
***
ns
ns
Cyto-APX
+Heme/μM
0 5 20 0 0 0
HEK293
+Heme/μM
0 0 0 0 5 20


E
HD+SA
Peroxidase activity / RFU / min
180000
160000
140000
120000
100000
80000
60000
1000
800
600
400
200
0
***
***
ns
ns
ER-HRP
+Heme/μM
0 5 20 0 0 0
HEK293
+Heme/μM
0 0 0 0 5 20

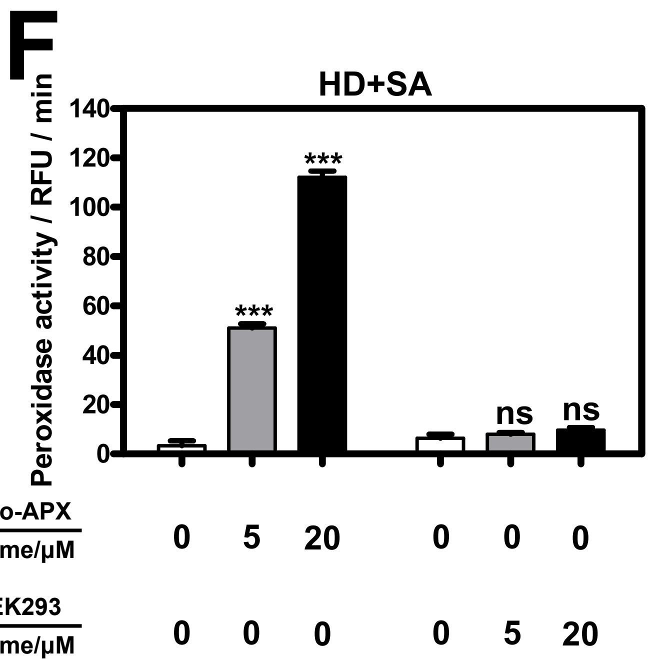
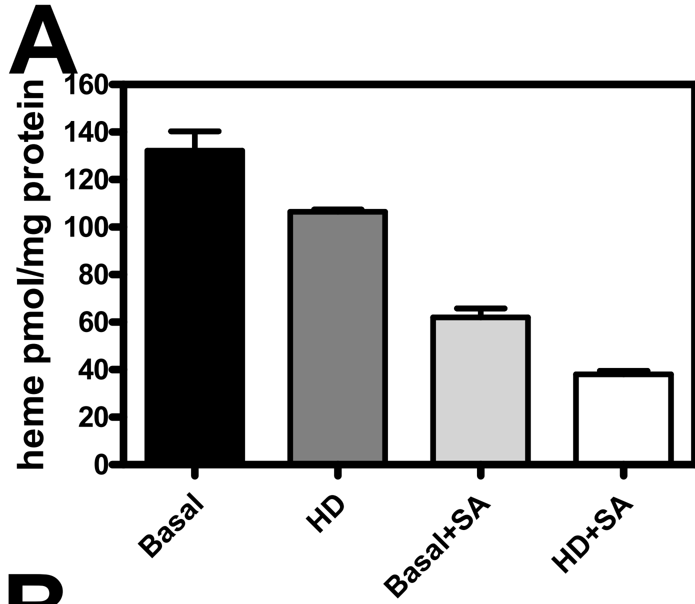
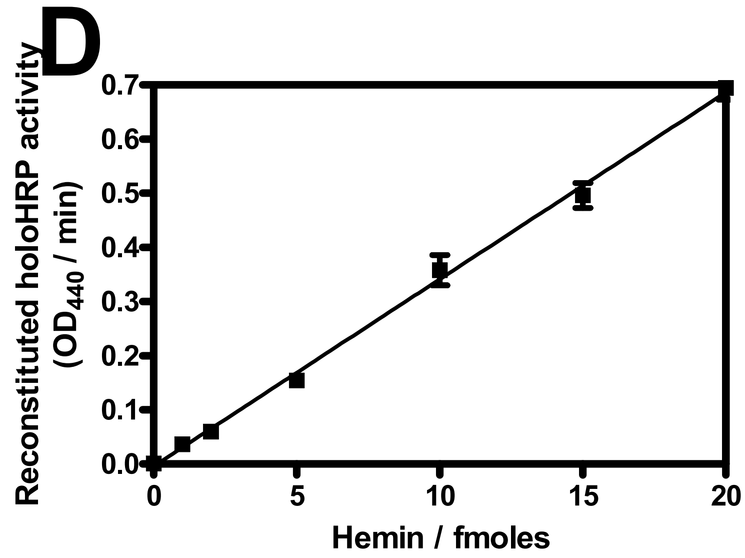
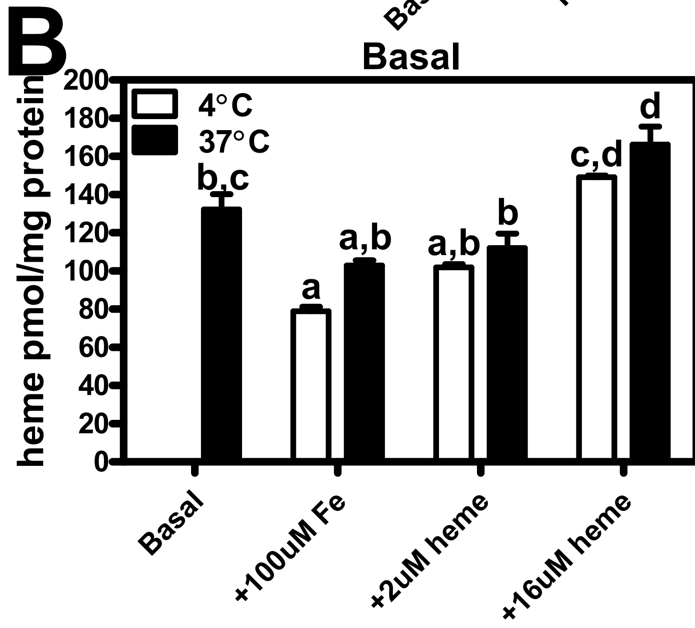

F
HD+SA
Peroxidase activity / RFU / min
140
120
100
80
60
40
20
0
***
***
ns
ns
Cyto-APX
+Heme/μM
0 5 20 0 0 0
HEK293
+Heme/μM
0 0 0 0 5 20

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 H_2O_2 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 ($P < 0.05$).


A
heme pmol/mg protein
0
20
40
60
80
100
120
140
160
Basal
HD
Basal+SA
HD+SA


D
Reconstituted holoHRP activity
(OD_{440} / min)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
5
10
15
20
Hemin / fmoles


B
Basal
heme pmol/mg protein
4°C
37°C
b,c
a
a,b
a,b
b
c,d
d
Basal
+100uM Fe
+2uM heme
+16uM heme

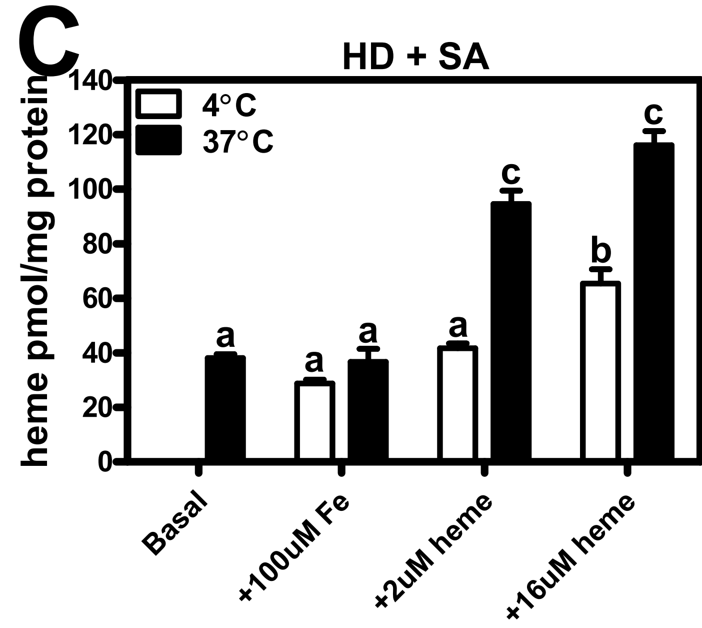

C
HD + SA
heme pmol/mg protein
4°C
37°C
a
a
a
a
c
b
c
Basal
+100uM Fe
+2uM heme
+16uM heme

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_2O_2. (●: inactive APX monomers; ▲: active APX dimers).

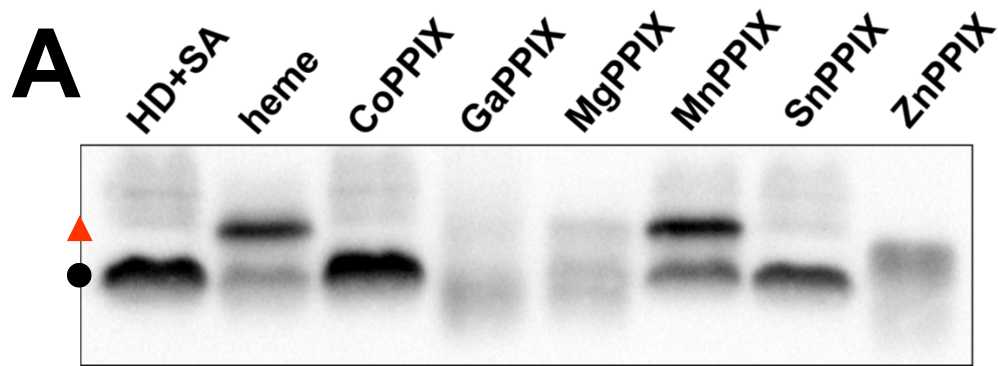
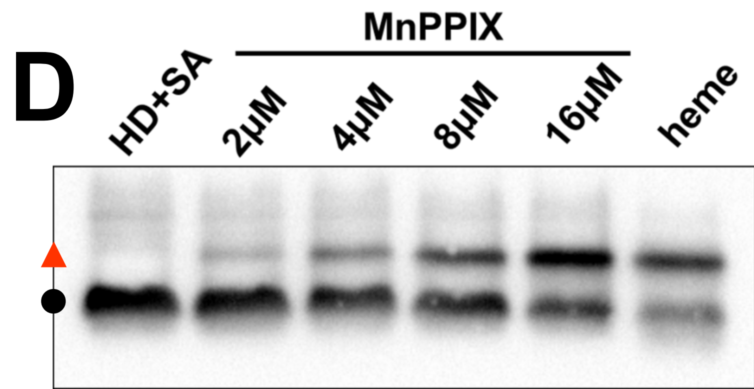
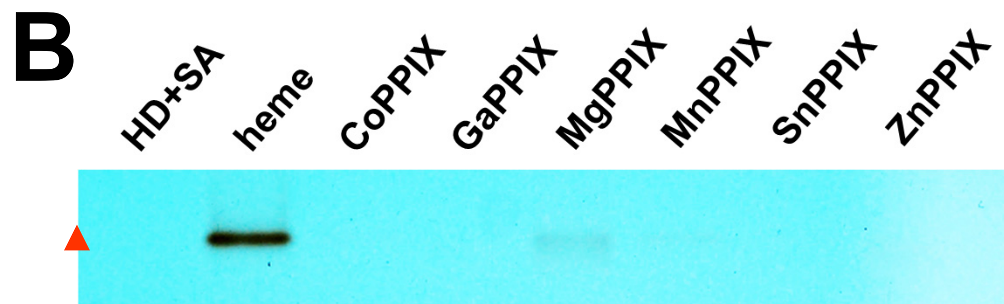
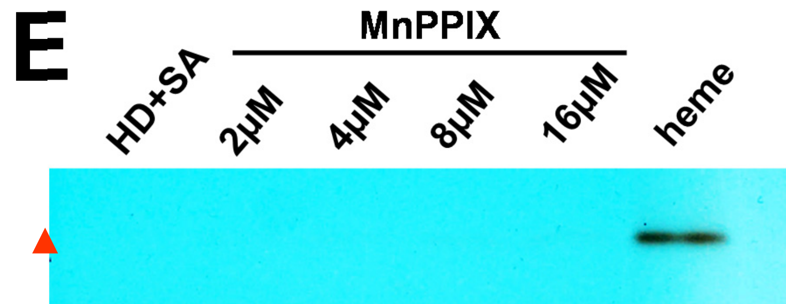
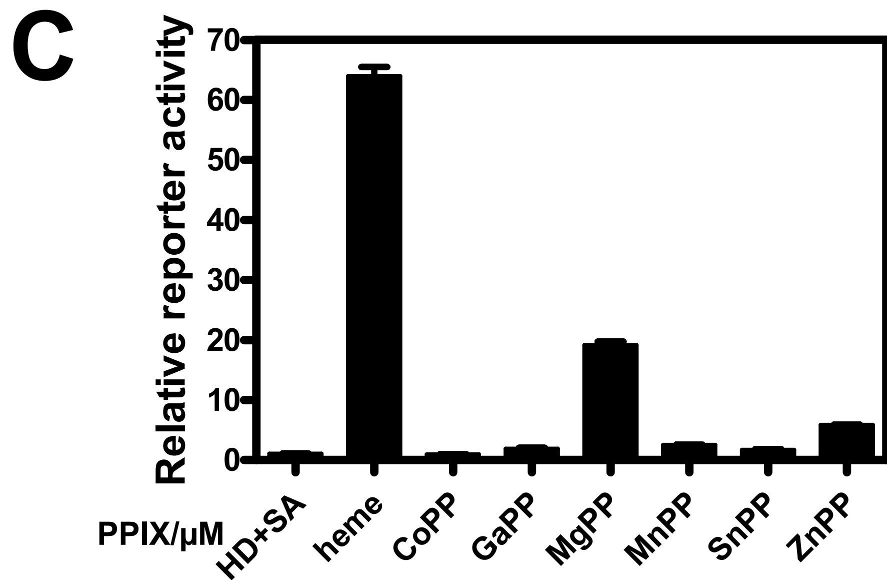
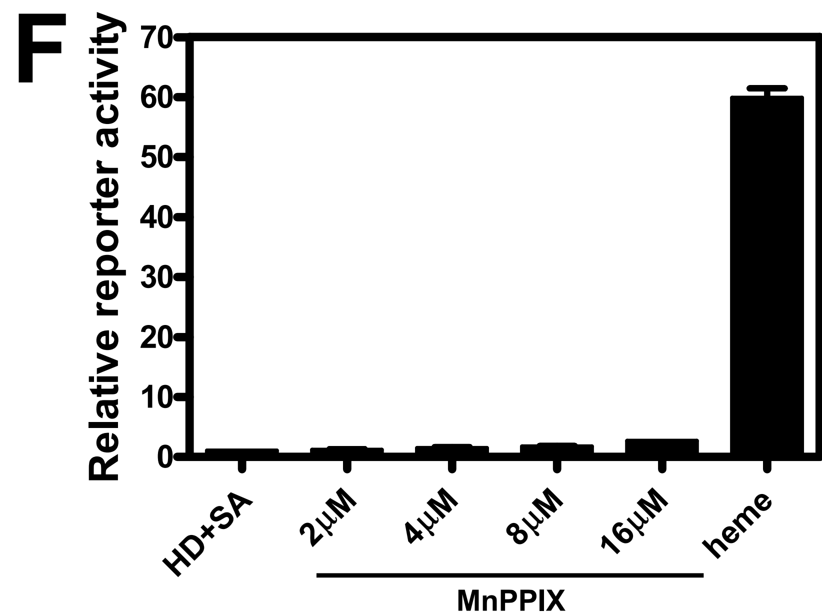

A
HD+SA
heme
CoPPIX
GaPPIX
MgPPIX
MnPPIX
SnPPIX
ZnPPIX

D
MnPPIX
HD+SA
2μM
4μM
8μM
16μM
heme

B
HD+SA
heme
CoPPIX
GaPPIX
MgPPIX
MnPPIX
SnPPIX
ZnPPIX

E
MnPPIX
HD+SA
2μM
4μM
8μM
16μM
heme

C
Relative reporter activity
70
60
50
40
30
20
10
0
PPIX/μM
HD+SA
heme
CoPP
GaPP
MgPP
MnPP
SnPP
ZnPP

F
Relative reporter activity
70
60
50
40
30
20
10
0
HD+SA
2μM
4μM
8μM
16μM
heme
MnPPIX

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 H_2O_2 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.

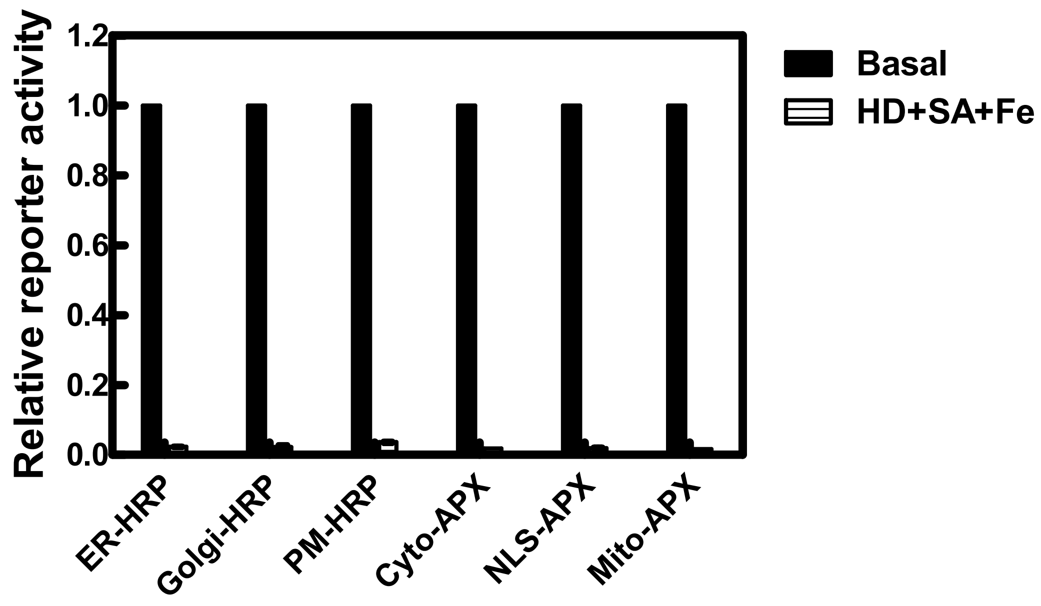
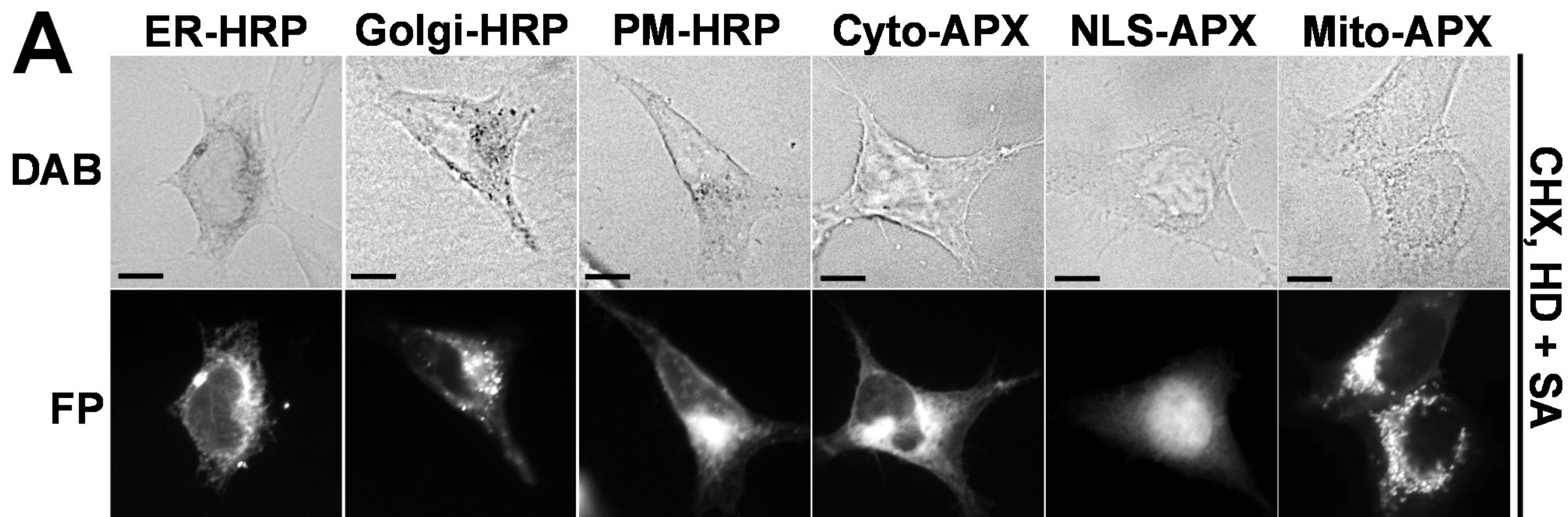

Relative reporter activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ER-HRP
Golgi-HRP
PM-HRP
Cyto-APX
NLS-APX
Mito-APX
Basal
HD+SA+Fe

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.


A
ER-HRP
Golgi-HRP
PM-HRP
Cyto-APX
NLS-APX
Mito-APX
DAB
FP
CHX, HD + SA

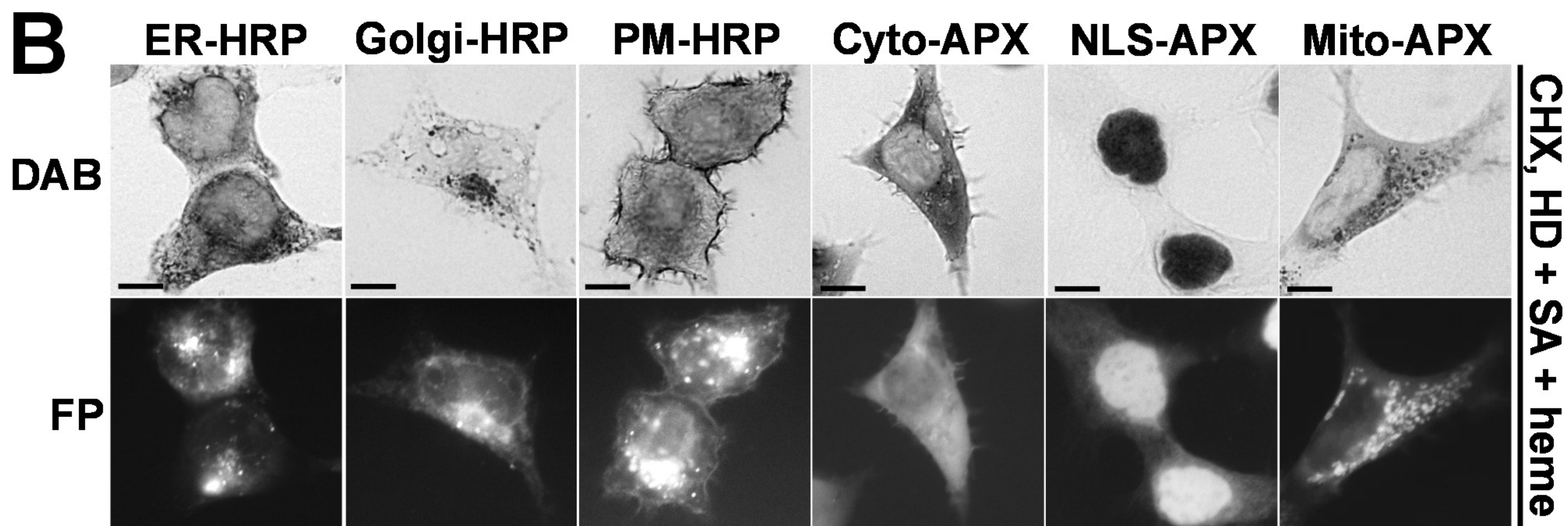
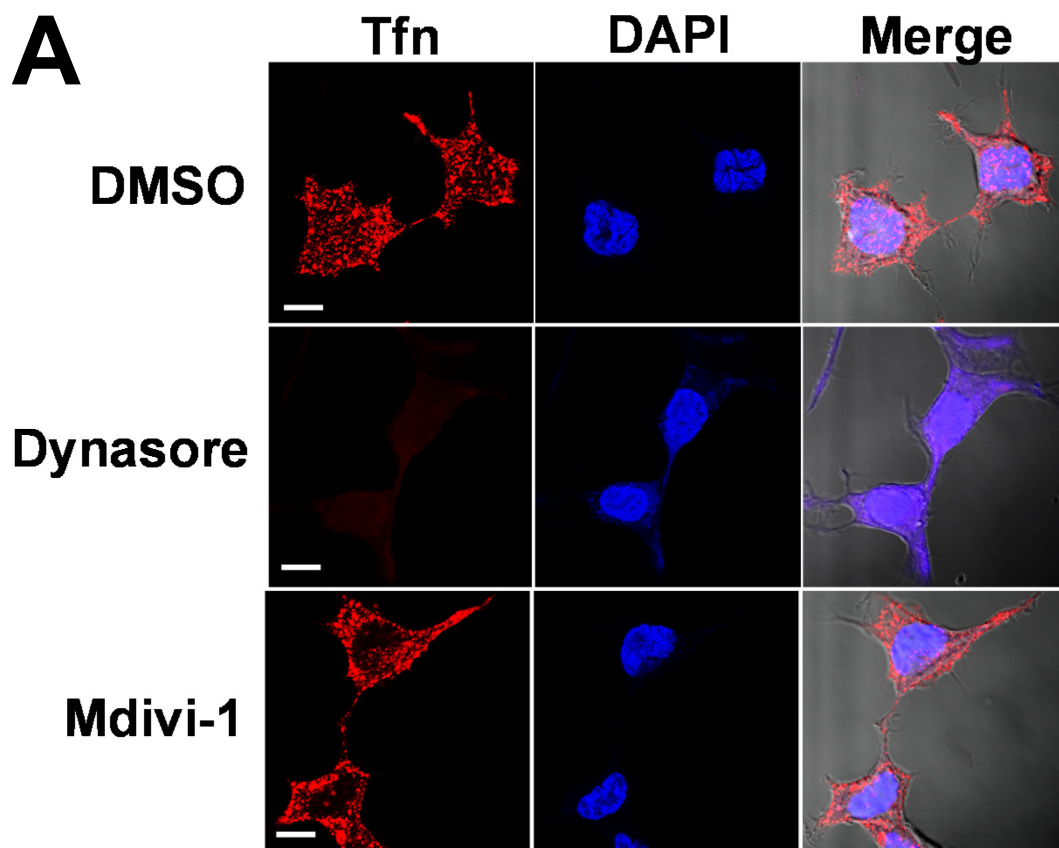

B
ER-HRP
Golgi-HRP
PM-HRP
Cyto-APX
NLS-APX
Mito-APX
DAB
FP
CHX, HD + SA + heme

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.


A
Tfn
DAPI
Merge
DMSO
Dynasore
Mdivi-1

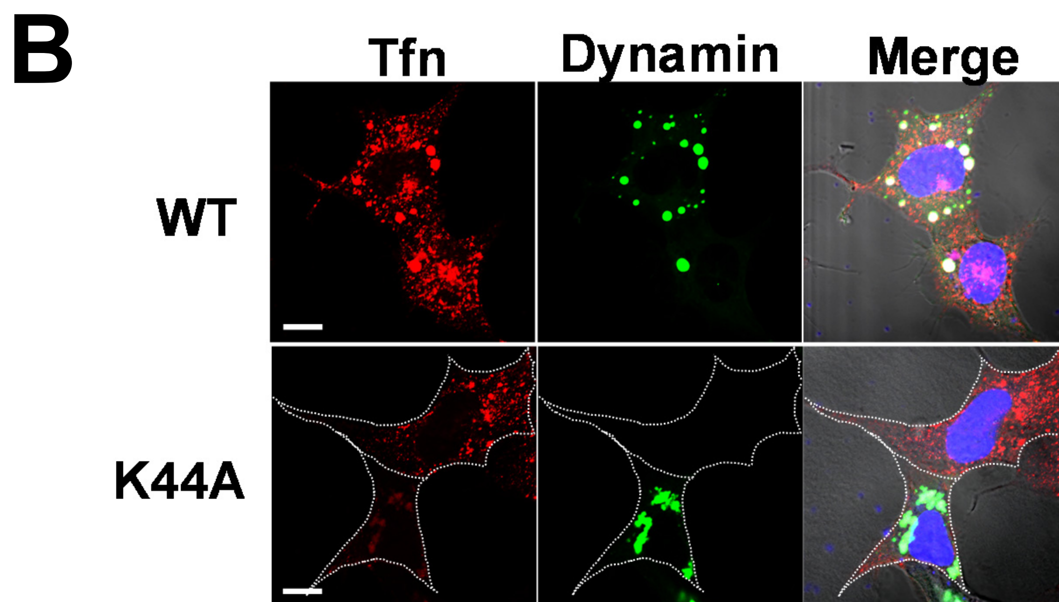
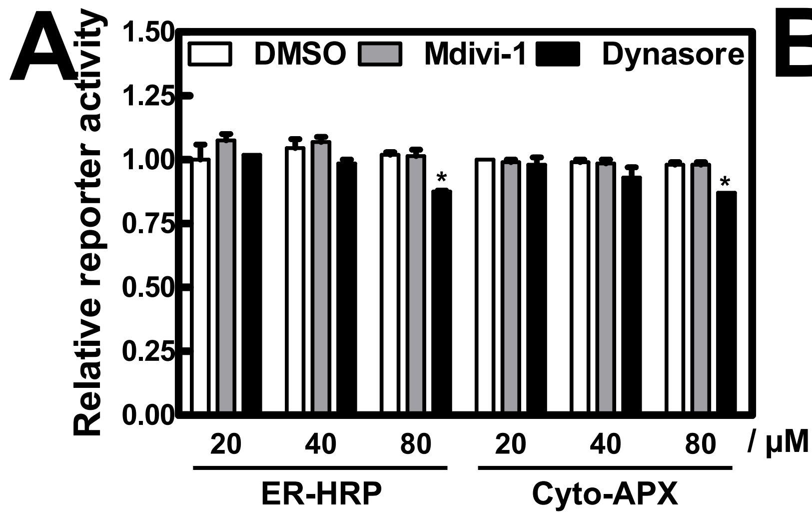
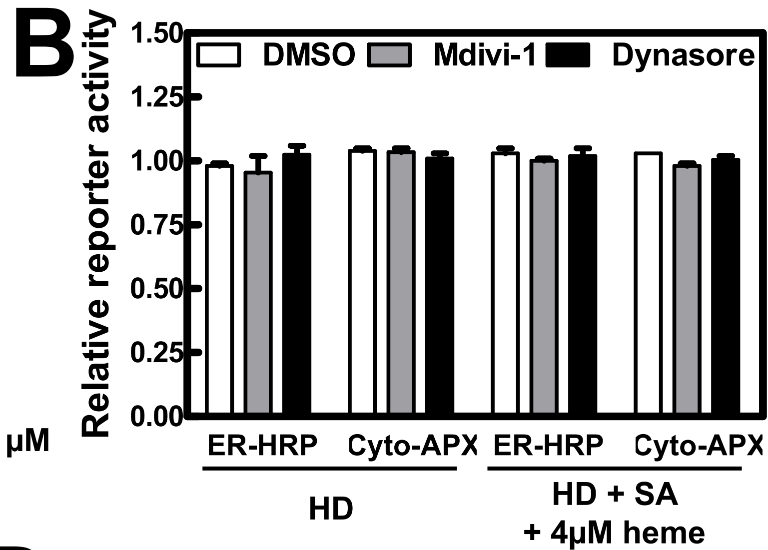
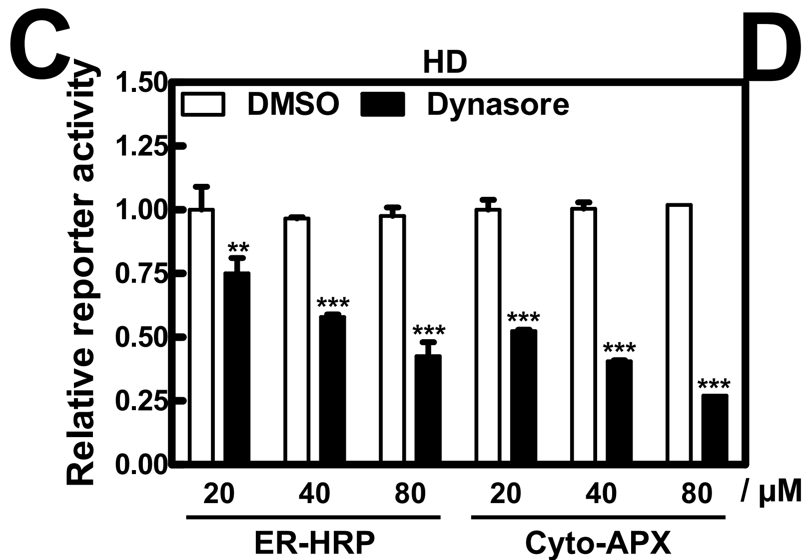

B
Tfn
Dynamin
Merge
WT
K44A

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+4 µM 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+4 µM 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, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


A
DMSO
Mdivi-1
Dynasore
Relative reporter activity
1.50
1.25
1.00
0.75
0.50
0.25
0.00
20
40
80
20
40
80
/ μM
ER-HRP
Cyto-APX


B
DMSO
Mdivi-1
Dynasore
Relative reporter activity
ER-HRP
Cyto-APX
ER-HRP
Cyto-APX
HD
HD + SA
+ 4μM heme


C
HD
DMSO
Dynasore
Relative reporter activity
20
40
80
20
40
80
/ μM
ER-HRP
Cyto-APX

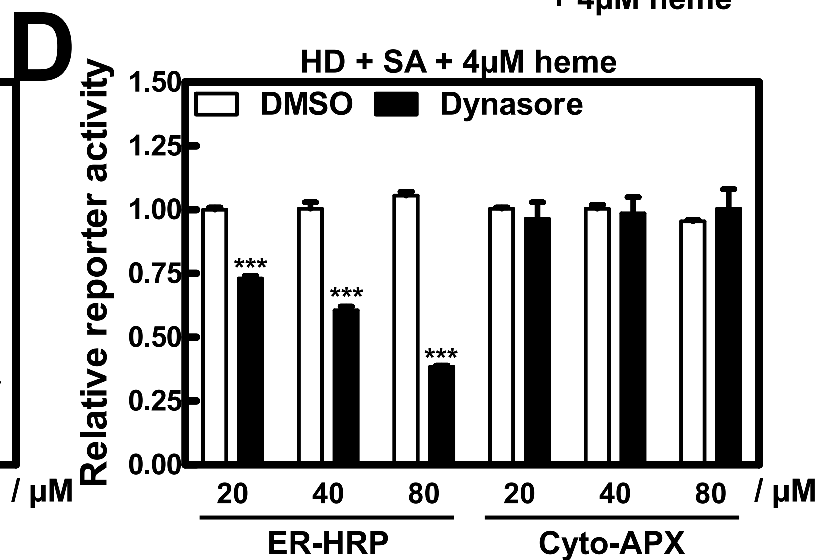
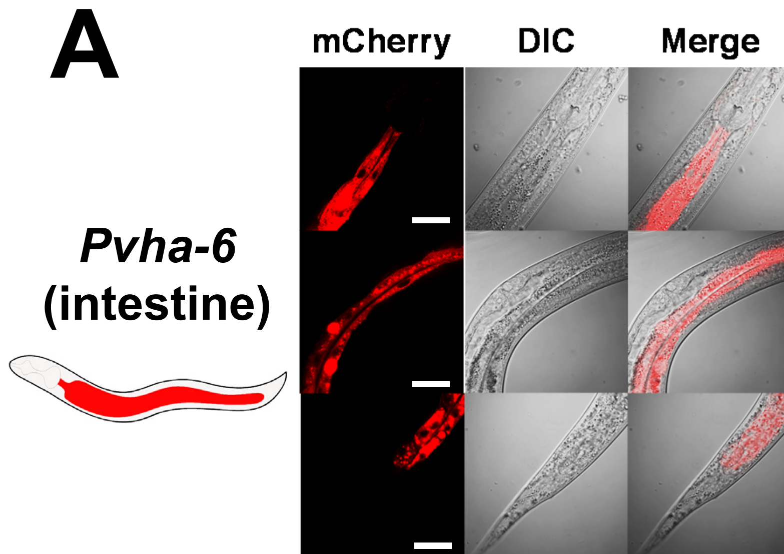
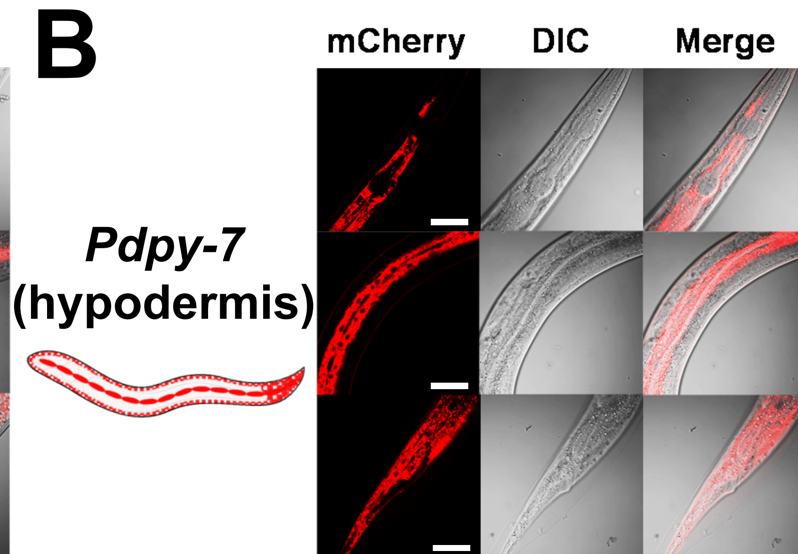
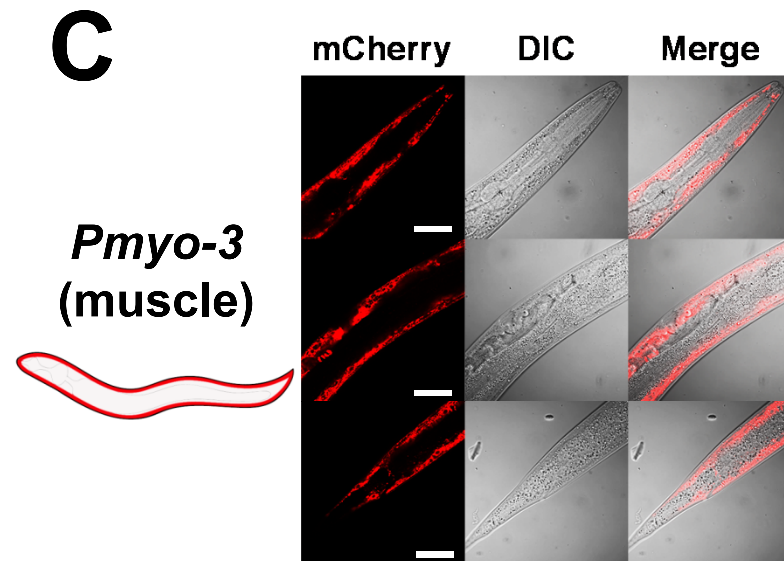

D
HD + SA + 4μM heme
DMSO
Dynasore
Relative reporter activity
20
40
80
20
40
80
/ μM
ER-HRP
Cyto-APX

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.


A
mCherry
DIC
Merge
Pvha-6
(intestine)


B
mCherry
DIC
Merge
Pdpy-7
(hypodermis)


C
mCherry
DIC
Merge
Pmyo-3
(muscle)

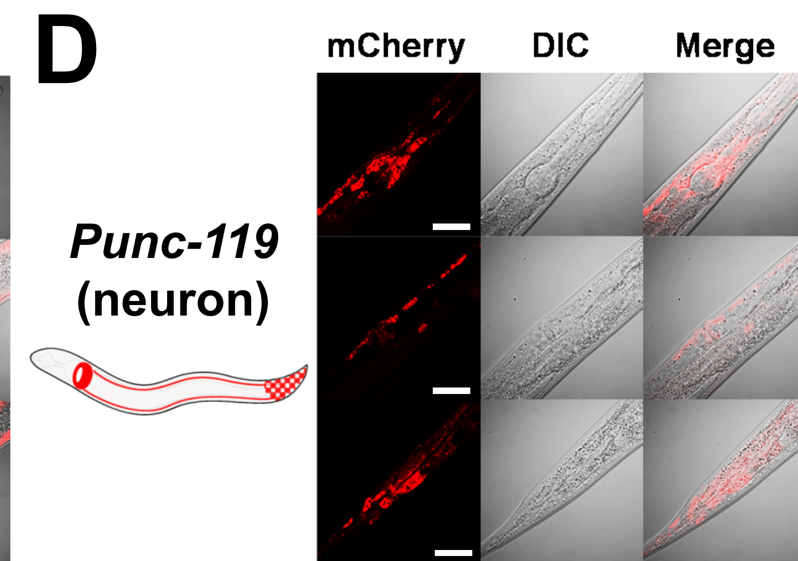
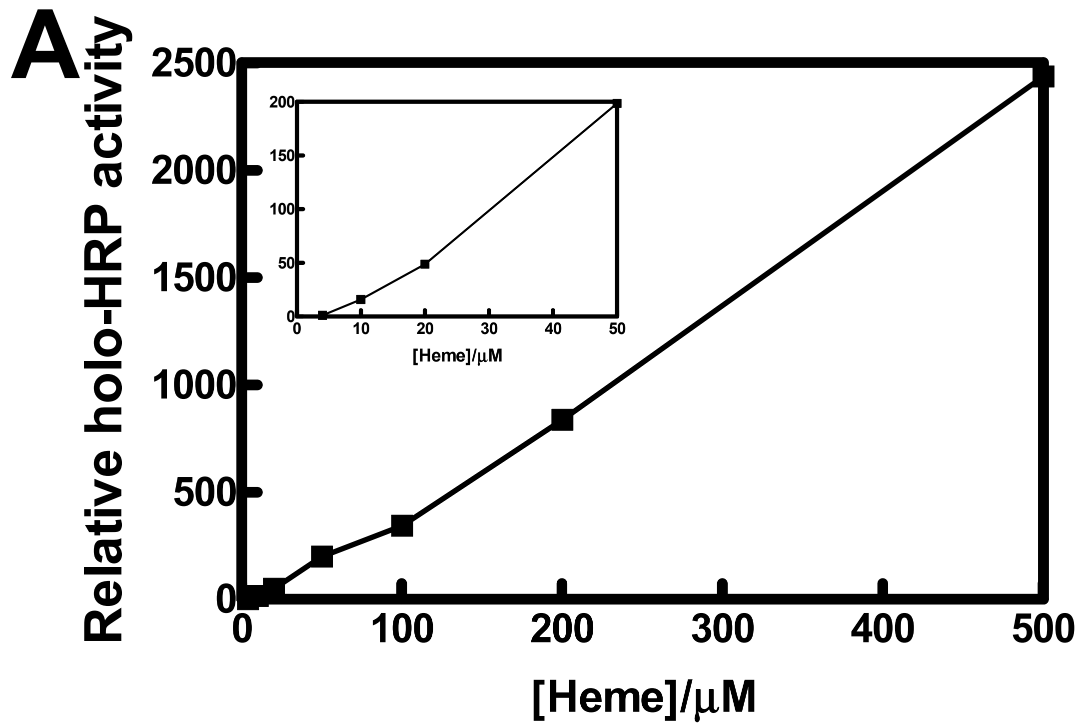
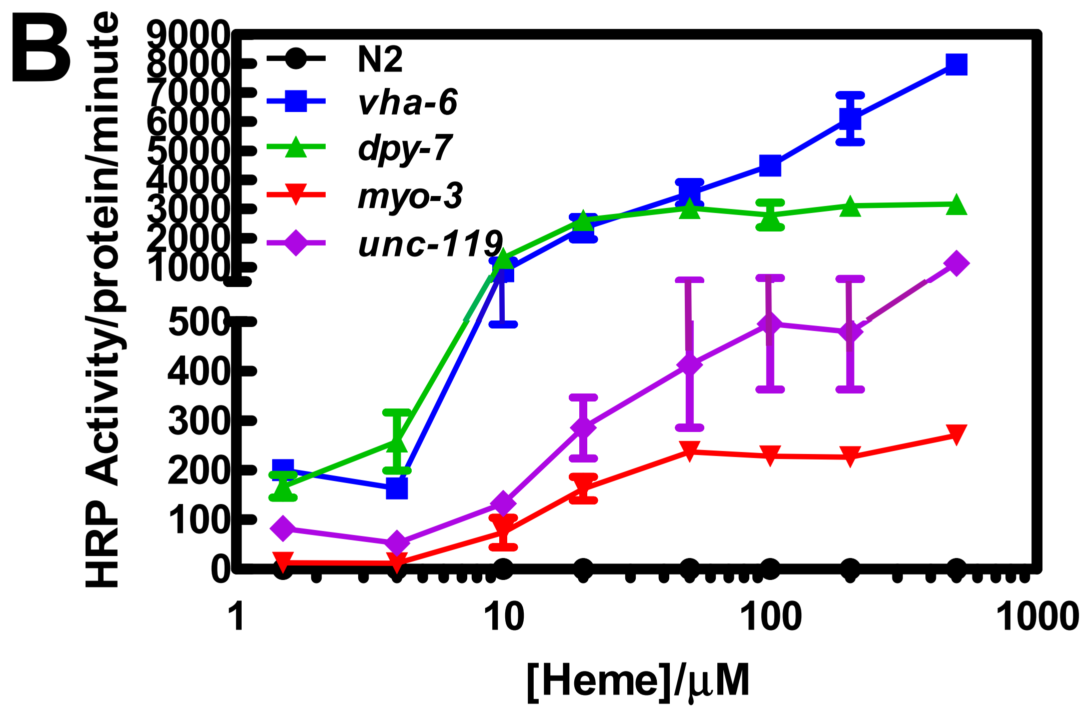

D
mCherry
DIC
Merge
Punc-119
(neuron)

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.

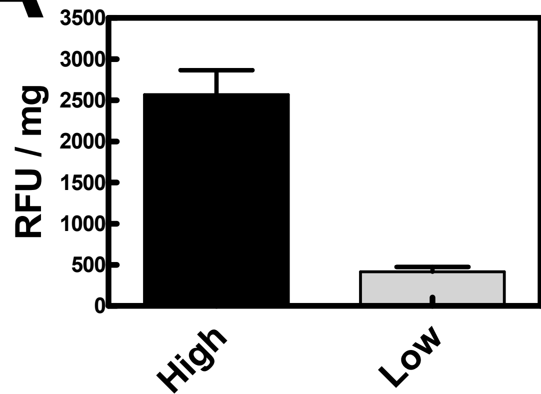


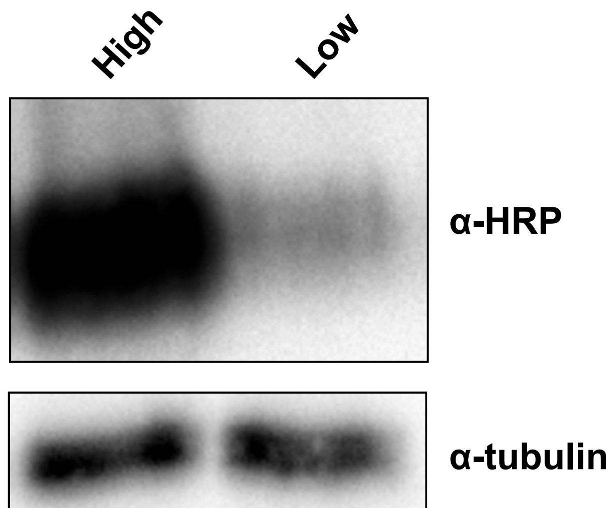
A
Relative holo-HRP activity
2500
2000
1500
1000
500
0
0
100
200
300
400
500
[Heme]/μM
200
150
100
50
0
0
10
20
30
40
50
[Heme]/μM

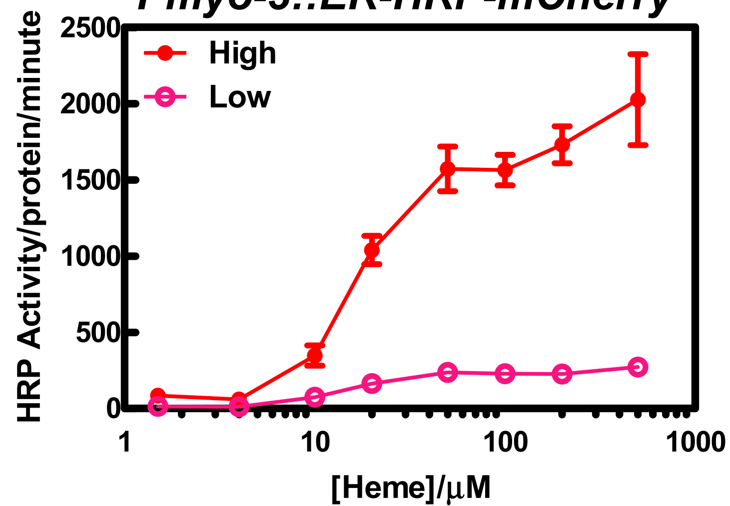


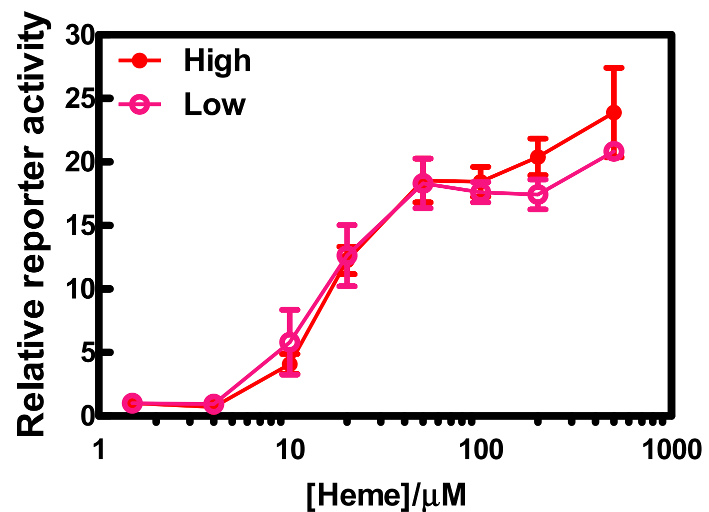
B
HRP Activity/protein/minute
9000
8000
7000
6000
5000
4000
3000
2000
1000
500
400
300
200
100
0
1
10
100
1000
[Heme]/μM
N2
vha-6
dpy-7
myo-3
unc-119

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
Pmyo-3::ER-HRP-mCherry
RFU / mg
3500
3000
2500
2000
1500
1000
500
0
High
Low


B
Pmyo-3::ER-HRP-mCherry
High
Low
α-HRP
α-tubulin


C
Pmyo-3::ER-HRP-mCherry
HRP Activity/protein/minute
2500
2000
1500
1000
500
0
High
Low
1
10
100
1000
[Heme]/μM


D
Relative reporter activity
30
25
20
15
10
5
0
High
Low
1
10
100
1000
[Heme]/μM

Supplementary Table 1. Constructs of HRP and APX reporters.

Reporter	Target	Targeting sequence	N-terminus added sequence	C-terminus added sequence
ER-HRP	ER	Calreticulin SS + KDEL	MLLSVPLLLGLLGLAVA	KDEL
Golgi-HRP	Golgi	N-GalT (1,4-galactosyltransferase)	MRLREPLLSGSAAMPGASLQRACRLLVAVCALHLGVTLVYYLAGRDLSRLPQLVGVSTPLQSTVPRARDPPV	-
PM-HRP	Plasma membrane	Human folate receptor (GPI anchored)	MAQRMTTQLLLLLVWVAVVGEAQTRI	AAAMSGAGPWAAWPFLLSLALMLLWLLS
Cyto-HRP	Cytosol	-	-	-
NLS-HRP	Nucleus	2X NLS	MDPKKKRKVDPKKKRKV	-
Mito-HRP	Mitochondrial matrix	Subunit VIII of hCOX	MSVLTPLLLRGLTGSARRLPVPRAKIHSL	-
Pero-HRP	Peroxisome	SKL	-	SKL
Cyto-APX	Cytosol	-	-	-
NLS-APX	Nucleus	2X NLS	MDPKKKRKVDPKKKRKV	-
Mito-APX	Mitochondrial matrix	Subunit VIII of hCOX	MSVLTPLLLRGLTGSARRLPVPRAKIHSL	-
ER-APX	ER	Calreticulin SS + KDEL	MLLSVPLLLGLLGLAVA	KDEL
NES-mCherry	Cytosol	NES	-	LQLPPLERLTLD

Supplementary Table 2. Total intracellular heme quantification in HEK293 cells.

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

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

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

1. Atamna H, Brahmbhatt M, Atamna W, Shanower GA, & Dhahbi JM (2015) ApoHRP-based assay to measure intracellular regulatory heme. *Metallomics* 7(2):309-321.
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3. Korolnek T, Zhang J, Beardsley S, Scheffer GL, & Hamza I (2014) Control of metazoan heme homeostasis by a conserved multidrug resistance protein. *Cell Metab* 19(6):1008-1019.
4. Samuel TK, Sinclair JW, Pinter KL, & Hamza I (2014) Culturing *Caenorhabditis elegans* in axenic liquid media and creation of transgenic worms by microparticle bombardment. *J Vis Exp* (90):e51796.
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6. Georgatsou E & Alexandraki D (1994) Two distinctly regulated genes are required for ferric reduction, the first step of iron uptake in *Saccharomyces cerevisiae*. *Mol Cell Biol* 14(5):3065-3073.
7. Teale FWJ (1959) Cleavage of the haem-protein link by acid methylethylketone. *Biochimica et Biophysica Acta* 35:543.
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