Table S1

Table S1. Related to Figure 4 - Morpholino injections resulting in anemia as measured by absence of o-dianisidine staining.

Experiment	Stage of Analysis	Control MO	Mrp5 MO	Mrp5 MO + Mrp5	Control MO + Mrp5
1	2 dpf	0/10 = 0%	29/37 = 78.4%	n/a	n/a
2	3 dpf	0/28 = 0%	18/18 = 100%	8/14 = 57.1%	0/27 = 0%
3	2 dpf	2/103 = 0.02%	54/62 = 87.1%	15/97 = 15.5%	11/78 = 14.1%
4	3 dpf	0/71 = 0%	28/37 = 75.7%	12/49 = 24.5%	n/a
	Total Embryos	2/212 = 0.01%	129/154 = 83.8%	35/160 = 21.9%	11/105 = 10.5%
	Average %	0.005%	82.8%	32.4%	7.1%

Table S2

Table S2. Related to Experimental Methods - List of *C. elegans* strains used in this study

Strain Name	Background	Transgene
IQ5051	*unc-119 (ed3) III*	P_{mrp-5}*::GFP::unc-54 3' UTR; unc-119 rescue*
IQ5151	*unc-119 (ed3) III*	P_{mrp-5}*::MRP-5-GFP::unc-54 3' UTR; unc-119 rescue*
IQ5351	*unc-119 (ed3) III*	P_{vha-6}*::MRP-5-GFP::unc-54 3' UTR; unc-119 rescue*
VC1599	*+/szT1[lon-2(e678)] I; mrp-5(ok2067)/szT1X.*	-
IQ5951	*mrp-5 (ok2067) X*	-
IQ6011	N2	P_{hrg-1}*::GFP::unc-54 3' UTR; rol-6 marker*
IQ8122	*unc-119 (ed3) III*	P_{hrg-2}*::HRG-2-YFP::hrg-2 3' UTR; unc-119 rescue*
IQ8031	*unc-119 (ed3) III*	P_{hrg-3}*::GFP::hrg-3 3' UTR; unc-119 rescue*
IQ8135	*unc-119 (ed3) III*	P_{hrg-3}*::HRG-3-mCherry::hrg-3 3' UTR; unc-119 rescue*
IQ8136	*unc-119 (ed3) III*	P_{hrg-3}*::HRG-3:ICS:GFP::hrg-3 3' UTR; unc-119 rescue*
VP303	*rde-1(ne213) V*	P_{nhx-2}*::rde-1; rol-6 marker*
WM118	*rde-1(ne300) V*	P_{myo-3}*::HA::RDE-1; rol-6 marker*
NR222	*rde-1(ne219) V*	P_{lin-26}*::nls-gfp; Plin-26::rde-1; rol-6 marker*

Extended Experimental Procedures

Hemin Chloride Preparation. For all experiments, heme was added in the form of hemin chloride (Frontier Scientific, Logan, UT). Hemin chloride was prepared 10 mM stock by dissolving in 0.3 M NH_4OH. The pH of the solution was adjusted to 8.0 by addition of HCl.

Worm Culture and Strains. Worms were maintained in axenic liquid mCeHR2 medium with continuous shaking or on nematode growth medium (NGM) plates seeded with OP50 or HT115(DE3) bacteria at 15°C or 20°C unless noted otherwise. Worms strains used in this study are listed in Table S2. VC1599, WM118, NR222, and VP303 strains were obtained from the Caenorhabditis Genetics Center (CGC, USA).

The deletion strain *mrp-5(ok2067)* was obtained by crossing VC1599 to wild type N2 worms on plates seeded with OP50 grown in media supplemented with 500 µM heme. F2 progeny were analyzed by single worm PCR to identify mutants and wild type broodmates. For single worm PCR, worms were lysed in lysis buffer (50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin, 1 mg/ml proteinase K) by incubation for 2 hours at -80°C, 1 hour at 65°C, and 30 min at 95°C. Worm lysates were added to PCR reactions with primers to detect wild type and mutant alleles. *mrp-5* strains were outcrossed three times to N2 worms, and maintained on plates seeded with OP50 bacteria grown in LB broth supplemented with 200 µM heme. Presence of the *mrp-5(ok2067)* allele was confirmed by sequencing; presence of a stable transcript containing the *ok2056* allele was confirmed with RT-PCR using primers flanking the deletion region.

The mrp-5 transcriptional and translational reporter constructs were generated using Multisite Gateway recombination (Invitrogen). These constructs, along with the unc-119 rescue

construct were introduced into *unc-119* worms using the PDS-1000 particle delivery system (Bio-Rad). At least two lines were analyzed per construct.

Worm sorting. Worms for each condition were analyzed for time of flight (length) and extinction (optical density) using a COPAS BioSort (Union Biometrica, Holliston, MA) with gating parameters for mixed worm populations as in Chen et al (Chen et al., 2011). The settings for measuring GFP intensity in IQ6011 (P_{hrg-1}::GFP) were gain = 2.5 and PMT voltage = 400 and in IQ8031 (P_{hrg-3}::GFP) were gain = 2.5 and PMT voltage = 600. The settings for measuring YFP intensity in IQ8122 (P_{hrg-2}::HRG-2-YFP) were gain = 2.5 and PMT voltage = 600.

Worm Fecundity and Growth Assays. To assay embryonic lethality, worms were grown to gravid stage on NGM plates seeded with OP50 bacteria. (Wild type N2 worms were grown on OP50 bacteria with no added heme, while *mrp-5(ok2067)* worms were grown on OP50 with >200 µM added heme.) Synchronized L1 larvae were placed on NGM plate containing IPTG, tetracycline, and carbenicillin that were seeded with HT115(DE3) control or RNAi bacteria with or without added heme, and allowed to reach young adult stage. Adult worms were moved to fresh plates, allowed to lay eggs for 24 hours, and then removed. Hatched L1 worms and eggs were counted 24 hours after removal of adults; eggs that did not hatch after 24 hours were considered dead.

To assay larval development, wild type N2 and *mrp-5(ok2067)* worms were grown to gravid stage on NGM plates seeded with OP50 bacteria grown in LB broth with the addition of >200 µM heme. Synchronized L1 larvae were seeded onto NGM plates seeded with OP50 bacteria grown with or without 200 µM added heme. Worms were incubated and DIC images were obtained when wild type N2 worms reached gravid stage (4 days).

Heme Analog Assays. Zinc mesoporphyrin, and gallium protoporphyrin IX were purchased from

Frontier Scientific, Inc (Logan, UT). Heme analog solutions were prepared as 10 mM stock by dissolving in 0.3 M NH_4OH and pH-adjusted to 8.0 by addition of HCl. To assess uptake of ZnMP, synchronized L1 worms were grown on NGM plates seeded with OP50 or HT115(DE3) bacteria until they reached L4 stage. Worms were washed off plates and rinsed three times in M9 buffer to remove bacteria. Worms were incubated in mCeHR2 medium with 60 μM ZnMP for 3 hours, rinsed to remove excess ZnMP, and imaged as described previously (Rajagopal et al., 2008). To assess toxicity of GaPPIX, synchronized L1 worms were grown on NGM plates seeded with OP50 or HT115(DE3) bacteria until they reached L4 stage. Worms were washed off plates and added to NGM plates containing various concentrations of GaPPIX and seeded with OP50 or HT115(DE3) bacteria. After 48 hours, worms were scored as dead if they were unresponsive to a physical stimulus.

Zebrafish Maintenance, Injection, and Staining. All zebrafish procedures were approved by the University of Maryland Animal Care and Use Committee. For morpholino knockdown of mrp5 (Genbank NM_001195613), MO^{mrp5} (5'-TCCCAAATCATGTCCTTTCATCTTC-3') was complementary to the ATG translation start site. Fertilized eggs were injected at the 1-cell stage with ~1.4nl/embryo of ~0.5 μM of either control MO or MO^{mrp5}. The morpholino-resistant rescue construct was generated as follows: The PCR product generated from the primer sequences, zfMRP5-EcoRI-MO2_ATG_fwd 5'-AGCTTCTGGAATTCGCCAGGATGAAGGGTCACGACTTAGGAAAGGACTGC-3', and zfMRP5-XbaI-TGA_rev, 5'-TTCAGACTTCTAGATCAGCCTCTTACAGAGATTTTGTT-3', was cloned into the pCS2+ vector using the EcoRI and XbaI restriction enzyme cut sites. 5' capped cRNA was synthesized using the SP6 mMessage mMachine kit (Ambion). Rescue injections were performed by co-injection with 175 pg of rescue construct. Live embryos at 2-3

dpf were stained using for hemoglobinized blood cells using o-dianisidine (Iuchi and Yamamoto, 1983). Pools of ~50 embryos were collected from wild type, control MO and mrp5 morpholino-injected LCR-GFP fish. Disaggregated cells were passed sequentially through 70 and 40 mm cell strainers, washed in Hank's Balanced Salt Solution (HBSS), and pelleted by centrifugation at low speed. The cells were resuspended in HBSS and analyzed by fluorescent-activated cell sorting (FACS) on a FACSaria III cell sorter (BD Biosciences).

Mouse Maintenance and Mammalian Cell Lines. All animal experiments were approved by the University of Maryland Institutional Animal Care and Use Committee. FVB/NJ mice were purchased from Jackson Laboratories (Bar Harbor, ME).

To generate mouse embryonic fibroblasts (MEFs), E12.5 embryos were isolated, and cultured as described (Spector D L, 1998). Immortalized $Mrp5^{+/+}$, $Mrp5^{+/-}$, and $Mrp5^{-/-}$ cell lines were generated by retroviral infection of MEFs with conditioned media from Ψ2U195 cells producing the SV40 large T antigen as in (Williams et al., 1988). MEFs were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin/glutamine for cell culture studies. SV40 large T antigen expression was confirmed in MEFs by Western blot. Mouse pups and cell lines were genotyped for Mrp5 using the following primers adapted from (de Wolf et al., 2007): 5' Mrp5 Genotype 5'-CTAGAGTCTAATCCGTATTGG-3', 3' Mrp5 Genotype 5'-CCCGCAAATACATTCAAACC-3', 5' Hygromycin Genotype 5'-GCTTTCAGCTTCGATGTAGG-3', 3' Hygromycin Genotype 5'-CGTCAGGACATTGTTGGAGC-3'.

Immunofluorescence. MEFs were transfected using the Lipofectamine 2000 transfection reagent (Invitrogen). For MRP5 staining, cells were fixed in acetone, blocked in 5% BSA in 1X PBS,

incubated with the primary antibody (anti-MRP5, 1:40 in blocking solution), secondary antibody (Alexa-conjugated anti-rat, 1:3000 in blocking solution), subjected to DAPI staining, and mounted using Prolong Gold Antifade (Invitrogen). Transformed yeast were grown to mid-log phase in 2% w/v raffinose SC (-Ura) liquid medium supplement with 0.4% galactose and 250 uM, fixed with 4% formaldehyde for 1 hour at room temperature. Immunofluorescence staining was performed as in (Burke et al., 2000). Images were taken using an LSM710 confocal microscope (Zeiss).

DNA Cloning. C. elegans promoters, ORFs, and 3' UTRs were amplified with sequence-specific Gateway attB primers. PCR products were recombined into donor plasmids, and then into expression plasmids, according to the manufacturer's instructions (Invitrogen). To generate yeast expression plasmids, a yeast codon optimized and flag-tagged ORF of CeMRP-5 was synthesized by Genscript, amplified with primers containing BamHI and XbaI sites, digested, and ligated into the pYES-DEST52 vector (Invitrogen) digested with the same enzymes. Human MRP5 (Genbank #U83661) was amplified with primers containing BglII and SpeI sites and a C-terminal HA tag, digested, and ligated into pYES-DEST52 digested with the compatible cohesive enzymes, BamHI and XbaI. The human MRP5 ORF cut with BglII and SpeI was also cloned into pcDNA3.1(+)zeo cut with BamHI and XbaI for mammalian expression.

Western Blotting. For detection of MRP5, cells were lysed in SDS lysis buffer (2% SDS, 1% Triton X-100, 1 mM EDTA, 62.5 mM Tris-Cl pH 7.5) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 4 mM benzamidine, 2 μg/ml leupeptin, and 1 μg/ml pepstatin), and sonicated twice. Cells were centrifuged at 14,000 x g for 15 min at 4°C, and total protein concentration in the supernatants was measured using the Pierce BCA assay kit (Thermo Scientific). Unboiled samples were mixed with Laemmli sample buffer and 200 mM

dithiothreitol (DTT), and 100 μg protein/lane were separated on 7% SDS-PAGE and transferred to a nitrocellulose membrane. The anti-MRP5 monoclonal antibody was used at a concentration of 1:200, goat anti-rat HRP-conjugated secondary was used at 1:30,000, and blots were developed in SuperWest Femto Chemiluminescent Substrate (Thermo Scientific).

Yeast Strains and Growth. S. cerevisiae strains used in this study contained the *hem1*Δ mutation in the W303 and YPH499 strains and have been described previously (Kaplan et al., 2006; Protchenko et al., 2008; Yuan et al., 2012). Cells were maintained at 30°C in yeast peptone dextrose (YPD) media supplemented with 250 μM δ-aminolevulinic acid (ALA) unless otherwise noted (Frontier Scientific). The *hem1Δ fre1Δfre2ΔPGK1-FRE1*strain was maintained on 2% w/v glucose SC (-Trp) plates supplemented with 250 μM ALA.

Yeast Growth Assays. The liquid and dilution spot assays were performed as described previously (Kaplan et al., 2006; Protchenko et al., 2008; Yuan et al., 2012). In short, plasmids encoding potential heme transporters were transformed into hem1Δ yeast using the lithium method (Ito et al., 1983) and selected on 2% w/v glucose SC (-Ura) plates supplemented with 250 μM ALA. Ten colonies were streaked onto 2% w/v raffinose SC (-Ura) plates supplemented with 250 μM ALA for 48 hrs. Cells were grown in 2% w/v raffinose SC (-Ura) liquid medium for at least 12 hours to deplete heme. For the liquid growth assay, cells were then diluted to OD600 of 0.05 in 96-well plate format, and grown in a humidity chamber at 30°C for 24 hours. OD600 was measured using a Synergy HT microplate reader (Biotek). For the spot growth assay, cells were then diluted to OD600 of 0.2, serially diluted, and spotted in 10 μl aliquots onto 2% w/v raffinose SC (-Ura) plates supplemented with varying amounts of heme or ALA and 0.4% w/v galactose to induce gene expression from the GAL1 promoter. Plates were incubated for 3 days before imaging.

β-Galactosidase Assay. Plasmids were co-transformed into the *hem1Δ* strain with the reporter plasmid pRS314m-CYC1-LacZ. Transformants were selected on 2% w/v glucose SC (-Ura -Trp) plates supplemented with 250 μM ALA. Cells were heme-depleted by growth in 2% w/v raffinose SC (-Ura -Trp) liquid medium with no added ALA for 12 hours. Cells were then resuspended to an OD600 of 0.1 in 2% w/v raffinose SC (-Ura -Trp) liquid medium supplemented with varying concentrations of heme or ALA and 0.4% w/v galactose for gene induction and cultured overnight. Cells were washed in washing buffer (2% BSA, 0.1% Tween-20 in 2X PBS), lysed, and assayed for β-galactosidase activity as described elsewhere (Adams A, 1997). Protein concentrations for each lysate were measured and β-galactosidase activity was normalized to total protein.

Ferric Reductase Assay. Plasmids were transformed into the *hem1Δ fre1Δfre2ΔPGK1-FRE1* strain as above and selected using 2% w/v glucose SC (-Ura -Trp) plates supplemented with 250 μM ALA. Cells were restreaked on 2% w/v raffinose SC (-Ura -Trp) plates supplemented with 250 μM ALA for 2 days. Cells were heme depleted by growth is in 2% w/v raffinose SC (-Ura -Trp) liquid media with no added ALA for at least 12 hours. Cells were resuspended in 2% w/v raffinose SC (-Ura -Trp) liquid medium supplemented with 0.4% w/v galactose, 0.1 mM Na_2S, and varying concentrations of heme or ALA and grown overnight. Cells were washed repeatedly in washing buffer, then in reaction buffer (5% glucose, 0.05 M sodium citrate buffer, pH 6.5) and resuspended in reaction buffer. The OD of the suspended cells was measured, equal volume assay buffer (2 mM bathophenanthroline disulfonate, 2 mM $FeCl_3$ in reaction buffer) was added (T=0 min), and cells were incubated at 30°C until a red color developed. OD535 and OD610 were measured and ferrireductase activity (nm/10^6 cells/min) was determined as follows:

HRP Assay. $Mrp5^{+/+}$ and $Mrp5^{-/-}$ MEFs were transfected with GolgiHRP expressed from the

pShuttle-IRES-hrGFP-1 vector using the Lipofectamine transfection reagent (Invitrogen). The following day, cells were incubated in heme-depleted media (DMEM with 10% heme depleted FBS and 1% PSG) and 0.5 mM succinylacetone (SA). The following day, cells were switch to HD media + SA with the addition of 1.5 or 2 μM heme. Cells were harvested a day later and lysed in 1% Triton X-100, 1 mM EDTA, 62.5 mM Tris-Cl pH 7.5, with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 4 mM benzamidine, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). Total protein concentration for each sample was measured using the BioRad Protein Assay Dye Reagent Concentrate solution. Peroxidase activity for each sample was measured as in (White et al., 2013). Each sample was normalized to the peroxidase activity in a corresponding empty vector control, as well as to total protein levels.

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