Supporting Information

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SI Materials and Methods

Strain and Plasmid Construction. HEM3 and HEM2/3/12 overexpression plasmids were provided by L. Liu (Chalmers Institute of Technology, Gothenburg, Sweden). In the plasmid-based overexpression assays, those plasmids were used unchanged. For the integrations, the constructs were recloned into the pIS385 disintegration vector (1). *HEM3* was amplified from the overexpression plasmid using primers HEM3-FWD and HEM3-REV and cloned into the EcoRI/AvrII site of pCS1585 (2), acquiring a TEF promoter and ADH1 terminator. The SacI/KpnI fragment of this plasmid was then cloned into pIS385 to produce pCS2342. *HEM2* and *HEM12* were amplified as a single amplicon from the overexpression plasmid using primers HEM212-FWD and HEM212-REV and cloned into the AvrII/XhoI site of HEM3-pCS1585. The integration plasmid pCS2342 was cut NotI/ClaI and blunted using T4 DNA polymerase (New England Biolabs) to remove an unwanted AvrII site. The AvrII/XhoI fragment from HEM2/3/12-pCS1585 was then cloned into the AvrII/XhoI site of pCS2342-Blunt to produce pCS2343. Plasmids pCS2342 and pCS2343 were digested with NruI and integrated into the lys2 locus of W303 (1). Rescue of the URA marker produced strains CSY851 and CSY852, respectively. CTT1 was amplified off the chromosome of W303 using primers CTT1-FWD and CTT1-REV and cloned into the SacII/NotI site of pCS782 (3). To move a caffeine demethylase from either a high-copy plasmid to a low-copy plasmid or vice versa, the EcoRI/AgeI fragment was cloned out of the original plasmid and into the desired plasmid (4).

Metabolite Analysis. Supernatant theophylline production was assayed on an Agilent 1200 series liquid chromatograph using a Poroshell 120 SB-C18 2.1 × 50 mm, 2.7-μm column (Agilent). The mobile phase was 0.50 mL/min of 20% methanol/80% water with 0.1% acetic acid. Theophylline eluted at 0.70 min and was detected at 274 nm. For each sample, 3 μL was injected onto the columns. The identity of the theophylline peak was confirmed with each assay by the use of an authentic standard (Sigma-Aldrich), and the concentration of theophylline in each sample was determined by comparison with a series of reference standards.

Supernatant salutaridine production was assayed on an Agilent 1200 series liquid chromatograph using a Zorbax SB-Aq 3.0 × 50 mm, 1.8-μm column (Agilent). The mobile phase was 0.60 mL/min of a mixture of water (buffer A) and methanol (buffer B), both with 0.1% acetic acid. The mobile phase started at 100% buffer A for 1 min, followed by a gradient to 75% buffer A/25% buffer B over 3 min, then held at 75% buffer A/25% buffer B for 3 min. After a total of 7 min, there was a further gradient to 100% buffer B over 1 min, then held at 100% buffer B for 4 min. Finally, the mobile phase was switched back to 100% buffer A and re-equilibrated for 6 min. Salutaridine was detected using an Agilent 6320 Ion Trap mass spectrometer, measuring the 265 m/z fragment of the 328 m/z ion.

DNA Microarray Experiments. Each strain for microarray analysis was grown overnight in appropriate dropout media. Each culture was diluted to OD 0.05 in 30 mL of fresh media, with four biological replicates per strain. When the cultures reached OD 0.3–0.4, they were quenched by decanting into a 50-mL centrifuge tube filled with ice. The cultures were centrifuged for 3 min at 4 °C and 5,000 × g, washed with 1 mL of water, transferred to a 1.5-mL centrifuge tube, and centrifuged for 2 min at 4 °C and 8,000 × g. The resulting cell pellet was frozen in liquid nitrogen and stored at −80 °C in preparation for analysis.

For each strain, three cell pellets representing three biological replicates were lysed using the RNeasy kit (Qiagen) following the manufacturer's instructions. cDNA synthesis followed by amplified (a)RNA synthesis and fragmentation were performed using the 3′ IVT Express kit (Affymetrix) following the manufacturer's instructions. aRNA synthesis and fragmentation were monitored using an Agilent 2100 Bioanalyzer and RNA 6000 Nano chips (Agilent Technologies). Fragmented aRNA was hybridized to Yeast Genome 2.0 DNA chips and scanned using a GeneChip 3000 7G Scanner (Affymetrix), according to the manufacturer's instructions.

Microarray data were analyzed using the BioConductor suite in R. Principal components analysis, treating the samples as the variables and gene expression data as observations (5), was used to identify genes with consistent patterns of expression between the different strains. Before PCA, the microarray data were normalized to correct for the steady state expression level (6). The ∼400 genes with the highest magnitude scores for PC1 were used as input to Reporter Features (7) to identify transcription factors whose targets were overrepresented.

Proteasomal Activity Measurements. Proteasomal activity was measured using the Proteasome-Glo kit (Promega), according to the manufacturer's directions. Cultures were grown to midlog phase (OD 0.2–0.4) and then diluted in fresh media to OD 0.1, 0.05, and 0.02 (corresponding to ∼100,000–20,000 cells per 100 μL, respectively). A total of 100 μL of the resulting cell suspension was mixed with 100 μL of the assay reagent, prepared according to the manufacturer's directions, and agitated on a Kuhner LT-X plate shaker at 480 rpm for 2 min. After a 10-min incubation at room temperature, the luminescence was measured on a Wallac 1420 Victor3 microplate reader (PerkinElmer).

Heme Measurements. For each sample, a 25-mL culture was grown to OD 0.4 and then centrifuged at 4 °C and 5,000 × g for 5 min. The pellet was washed with water, transferred to an amber centrifuge tube, and centrifuged again at 4 °C and 8,000 × g for 5 min. The pellet was then resuspended in 500 μL of 20 mM oxalic acid (Sigma-Aldrich) and stored at 4 °C in the dark for 16 h. After the acid extraction, 500 μL of 2 M oxalic acid was added to each tube. Half of the resulting suspension was transferred to a new centrifuge tube. The original centrifuge tube was heated to 95 °C for 30 min, removing the iron from nonfluorescent heme and producing a fluorescent porphyrin ring. Two replicate 200-μL aliquots of each sample (heated and unheated) were measured in a microplate reader (Safire; Tecan), exciting at 400 nm and measuring emission at 620 nm. A standard curve was constructed using variable concentrations of hemin (Sigma-Aldrich).

Quantitative Western Blots. Yeast strains harboring the appropriate enzyme expression constructs were grown overnight in 5 mL of uracil dropout media. Protein extraction was carried out using 0.1 M NaOH (8), followed by lysis in protein loading buffer (Invitrogen). Samples and ladder (P7711S;New England Biolabs) were resolved on 4–12% Bis-Tris SDS/PAGE gels in 1× Mops (Invitrogen). Protein was transferred to a nitrocellulose membrane using semidry transfer (Bio-Rad) in 2× NuPAGE transfer buffer (Invitrogen) plus 10% MeOH. After transfer, the membrane was cut in half at ∼55 kDa. Both membrane halves were blocked in 5% BSA for 1 h. The membrane with higher-molecular-mass proteins was blotted with an anti-V5 HRP antibody according to the manufacturer's instructions (Invitrogen). The membrane with lower-molecular-mass proteins was blotted with a mouse anti-actin antibody (8224; Abcam) and

a rabbit anti-mouse HRP (6728; Abcam) according to the manufacturer's instructions. Both HRP antibodies were detected by chemiluminescence, following the manufacturer's instructions, (Pierce) using a Chemi-Doc XRS imager (Bio-Rad). Blots were analyzed using the QuantityOne analysis software (Bio-Rad).

Catalase Activity Assays. Cells containing the catalase-overexpression construct, as well as various heme-overexpression constructs, were grown to saturation overnight. The cultures were diluted 20× into 50 mL of fresh dropout media and regrown to midlog (OD, ~0.4). Five-milliliter samples were taken to measure heme content as described previously. The remaining culture volume was centrifuged at 4 °C and 6,000 × *g* for 5 min, washed once with 1 mL of resuspension buffer (0.1 M potassium phosphate, 0.5 mM EDTA), and centrifuged again. The pellet was then resuspended in 1 mL of resuspension buffer plus protease inhibitor (HALT; Pierce) and transferred to a tube containing 500 mg of acid washed glass beads (Sigma). The samples were lysed by five cycles of 1-min vortexing, followed by 1 min on ice. After vortexing, the crude lysate was centrifuged at 4 °C and 16,000 × *g* for 5 min, and the supernatant was transferred to a new tube. The total protein concentration was measured using a Bradford reagent (Bio-Rad) according to the manufacturer's instructions, using 160 μL of sample dilutions and 40 μL in a microwell plate. Absorbance was assayed using a Tecan Safire microplate reader. Sample values were compared with a standard curve was constructed using BSA to determine the total protein concentration.

Next, the catalase activity of each sample was measured. Samples were diluted to ~10 μg/mL. A total of 40 μL of protein was mixed with 160 μL of 250 μM H_2O_2. Aliquots were taken at 30, 60, and 120 s and quenched in 200 μL of Peroxide Assay Reagent (Pierce), according to the manufacturer's instructions. Absorbance was measured on a Tecan Safire microplate reader. Residual peroxide was calculated by comparison with a standard curve of H_2O_2 dilutions. One unit of catalase activity was calculated as the amount of active protein necessary to degrade 1 mM H_2O_2 in 1 min.

qRT-PCR Measurements. Cells containing various combinations of heme- and P450-overexpression plasmids were grown to saturation overnight. They were then diluted in triplicate 30 mL cultures and regrown to midexponential phase (OD_{600}, ~0.5). A total of 10 mL of each culture was centrifuged for 5 min at 6,000 × *g* and 4 °C, washed with 1 mL of water, and repelleted for 5 min at 8,000 × *g* and 4 °C. The supernatant was removed and the cells were frozen in liquid nitrogen and stored at −80 °C.

The cell pellets were resuspended in 500 μL of buffer AE (50 mM NaOAc, 10 mM EDTA) with 1.5% (wt/vol) SDS. A total of 500 μL of acid phenol was added to each suspension, and the mixture was heated at 65 °C for 10 min with regular vortexing. The tubes were cooled on ice for 5 min and then centrifuged for 12 min at 10,000 × *g* and 4 °C. The supernatant was transferred to a new tube and mixed with an equal volume of chloroform. The tubes were again centrifuged, and the supernatant was transferred to a new tube. Nucleic acids were precipitated with 1/10th volume NaOAc and 2 volumes 100% ethanol. Tubes were stored at −20 °C for 30 min and then centrifuged for 30 min at 16,000 × *g* and 4 °C. The supernatant was removed, the pellets were washed with 500 μL of 70% ethanol, and the tubes centrifuged again for 20 min at 16,000 × *g* and 4 °C. The supernatant was removed, and the pellets were allowed to air dry. The pellets were then resuspended in 20 μL of water. A total of 2 μL of DNaseI buffer and 1 μL of DNaseI (New England Biolabs) was added to each tube. The tubes were incubated at 37 °C for 10 min. Next, EDTA was added to a final concentration of 5 mM, and the tubes were incubated at 75 °C for 10 min. Finally, the ethanol precipitation procedure was repeated to remove the EDTA.

The RNA was quantified using a Nano-Drop spectrophotometer. Total RNA was reverse-transcribed using SuperScript III (Invitrogen) and gene-specific primers for *HEM13* and *ACT1*, according to the manufacturer's instructions. Approximately 1.5 μg of total RNA was loaded into each reverse-transcription reaction. Following reverse transcription, qPCR was performed according to the manufacturer's instructions using the iQ SYBR Green Supermix (Bio-Rad) and 3 μL of cDNA in a 20-μL reaction. The qPCR reactions were monitored on a Bio-Rad iCycler. For each biological replicate, three technical replicates were performed for each of the gene-specific primers. A dilution series was conducted for one sample, using both primer pairs, to measure the cycle efficiency. For each biological replicate, the technical replicates were averaged, and the measured *HEM13* level was normalized to the *ACT1* level. The normalized expression was then averaged for the three biological replicates.

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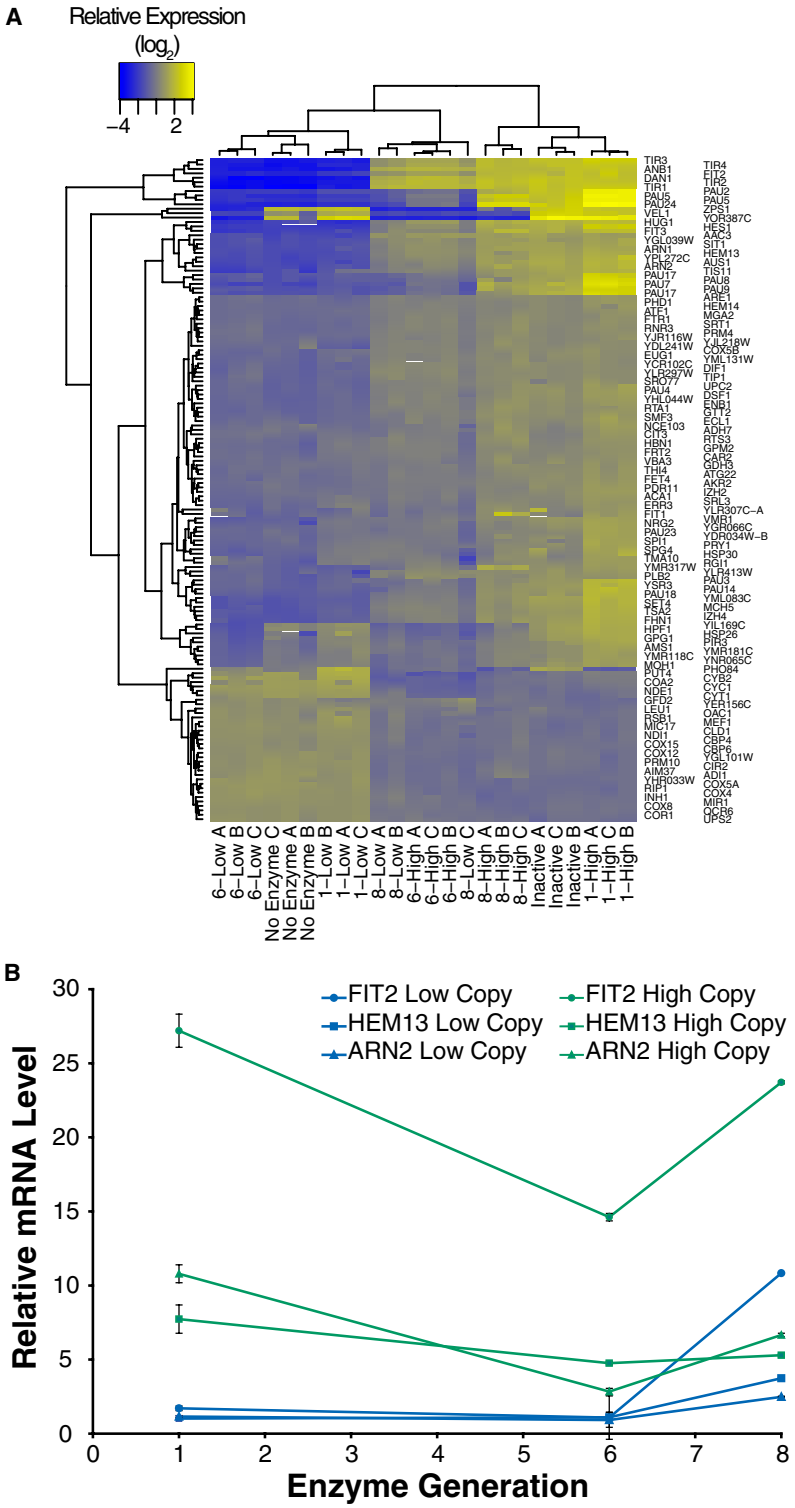

Fig. S1. (*A*) Heat map showing mRNA expression profiles of the genes most highly associated with PC1. Strains are shown along the horizontal axis, where a label of "1-High A" represents the first sample of yCDM1 expressed from a high-copy plasmid. Clustering strains by expression profile recapitulates the ordering of the PC1 loadings. (*B*) Expression profiles of representative iron- and heme-regulated genes. FIT2 produces a cell wall mannoprotein that facilitates iron-siderophore binding. Its expression is regulated by iron. HEM13 is involved in the biosynthesis of heme. Its transcription is repressed by heme. ARN2 is an iron-siderophore transporter whose expression is iron-dependent.

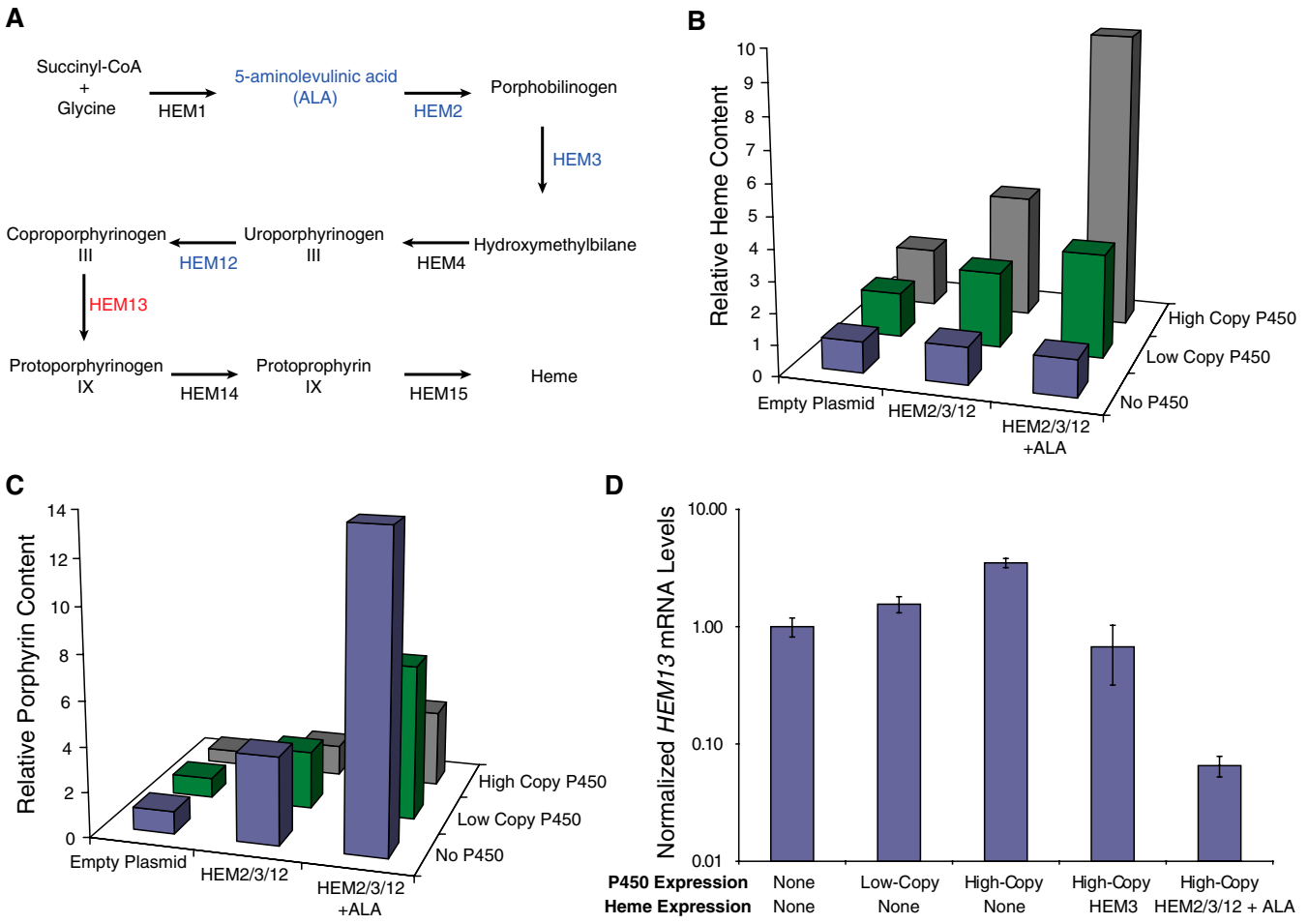

Fig. S2. Heme synthesis and use must both be elevated to produce large changes in heme content. (*A*) Heme biosynthetic pathway in yeast. Genes overexpressed in this study are labeled in blue. The first committed substrate, ALA, is also labeled in blue. HEM13, labeled in red, is transcriptionally regulated by the intracellular heme level. (*B*) Cells containing various combinations of heme synthesis constructs and heme-binding enzyme constructs were tested for total heme content. Increasing heme synthesis and heme use both led to higher cellular heme content. SDs, calculated from three biological replicates, are given in Table S3. (*C*) In contrast to the heme levels, porphyrin levels increase as more flux is routed through heme biosynthesis and decreases as heme is bound by the P450. SDs, calculated from three biological replicates, are given in Table S3. (*D*) *HEM13* mRNA levels, as measured by qRT-PCR, increase as the P450 expression increases, responding to a decrease in the amount of free heme. The levels then decrease when the upstream portion of the heme biosynthetic pathway is overexpressed, demonstrating that the free-heme levels have recovered and the cell is now restricting heme biosynthesis at HEM13 and accumulating porphyrins. The error bars show ±1 SD, calculated from three biological replicates.

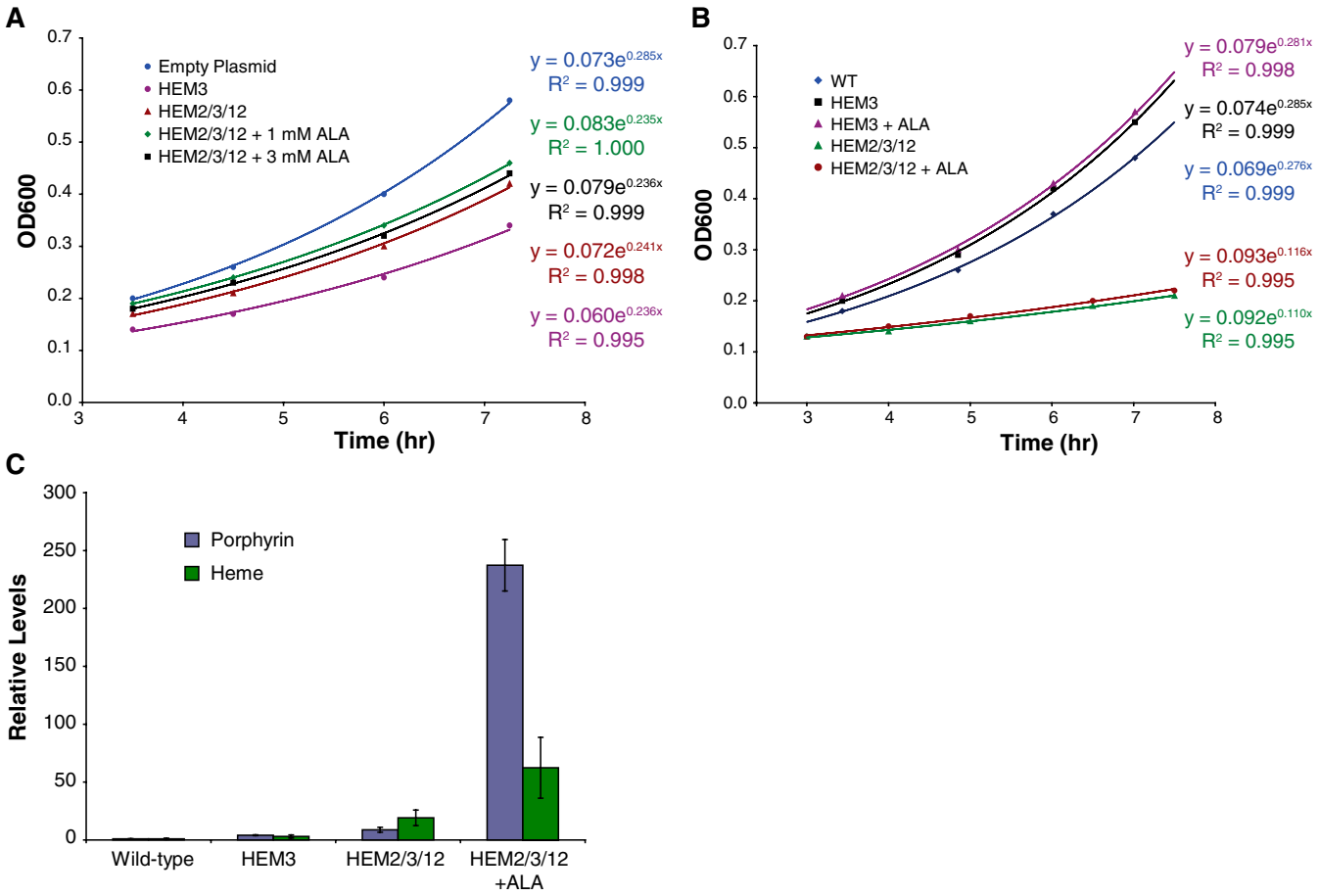

Fig. S3. Growth curves for cells overexpressing the heme biosynthetic pathway. (*A*) Cells expressing yCDM8 from a high-copy plasmid, as well as heme-overexpression constructs from a second high-copy plasmid, were grown in appropriate dropout media. Overexpression of *HEM3* led to a decrease in growth rate, although additional overexpression or *HEM2* and *HEM12*, even in combination with ALA, did not further slow growth. (*B*) The final demethylase yCDM8 was expressed from a high-copy plasmid in a wild-type strain (blue), a *HEM3* overexpression strain (black and purple), or a *HEM2/3/12* overexpression strain (green and red). Integrated expression of *HEM3* did not affect growth, although simultaneous overexpression of *HEM2/3/12* produced a severe growth defect. Addition of ALA did not change the growth rate. (*C*) Iron and porphyrin levels were measured for cells containing yCDM8 expressed from a high-copy plasmid and integrated heme-overexpression constructs. *HEM2/3/12* produced enormous concentrations of porphyrin and heme, perhaps explaining the slow growth rate.

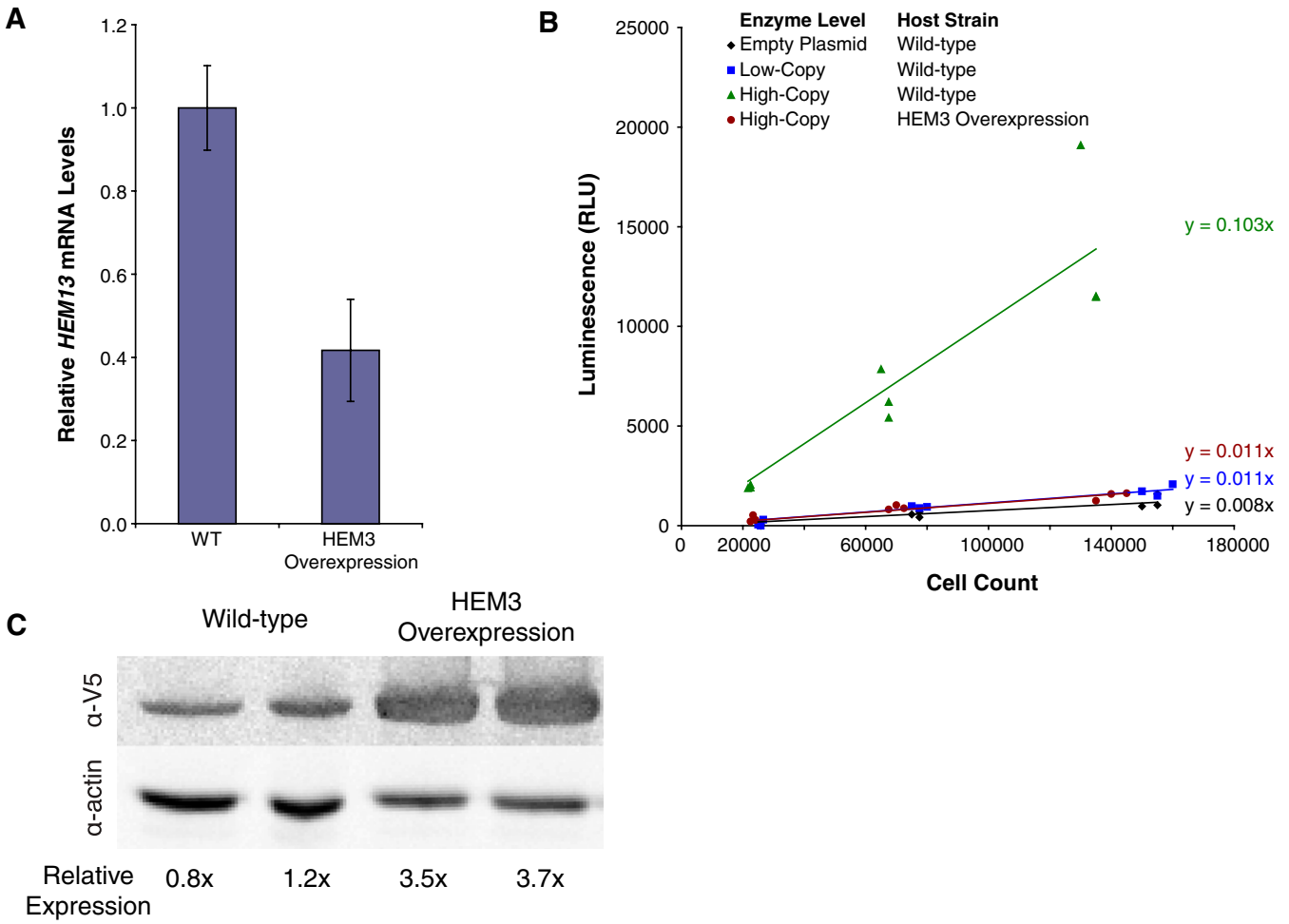

Fig. S4. Integrated overexpression of *HEM3* decreases *HEM13* mRNA levels, decreases proteasomal activity, and increases P450 expression. (*A*) yCDM8 was expressed from a high-copy plasmid either in a wild-type strain or a strain overexpressing *HEM3*. *HEM3* expression led to a decrease in *HEM13* mRNA levels, reflecting an increase in the amount of free heme. (*B*) As in the dual-plasmid system, overexpression of yCDM8 increases the proteasomal activity. However, combining enzyme and heme overexpression returns the proteasomal activity to background levels. Three biological replicates were assayed at each of three dilutions. Each data point represents a single measurement, and the lines are a linear fit to the data. (*C*) *HEM3* overexpression leads to an increase in P450 expression. yCDM8 was tagged with a V5 epitope and expressed from a high-copy plasmid either in a wild-type strain or a *HEM3*-overexpression strain. *HEM3* overexpression led to an increase in total P450 accumulation. Two biological replicates are shown for each condition. Note that this assay cannot distinguish between holo- and apoenzyme forms and that the increase in total P450 expression is larger than the increase in theophylline accumulation.

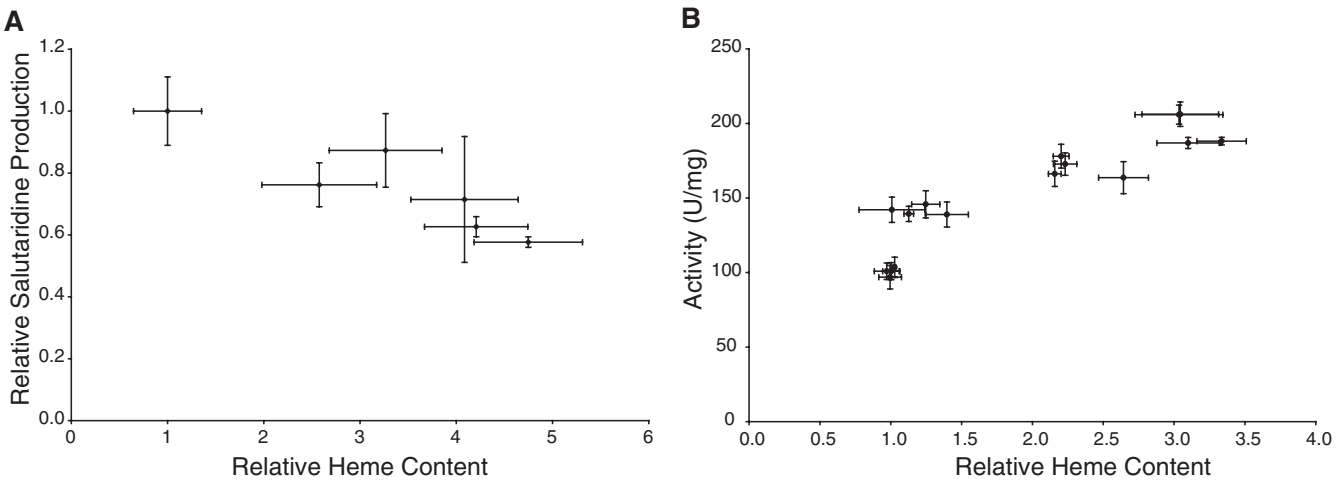

Fig. S5. Heme overexpression increases the activity of soluble, but not membrane-associated, hemoproteins. (*A*) Cells expressing membrane-associated CYP2D6 from a high-copy plasmid were cotransformed with high-copy heme-overexpression plasmids. Cultures were grown in the presence of norlaudanosoline and varying amounts of iron and ALA. After 48 h, the salutaridine concentration was measured in the supernatant, and the cell pellets were assayed for heme content. Each point represents the average of three biological replicates with error bars showing ±1 SD. (*B*) Cells expressing CTT1 from a high-copy plasmid were cotransformed with heme-overexpression plasmids. Cultures were grown to midexponential phase (OD, ~0.4) and lysed with glass beads. Catalase activity was determined by monitoring the degradation of H_2O_2 in vitro. Heme content was measured as described previously. Each point represents a single biological replicate, and the error bars show ±1 SD calculated from technical replicates.

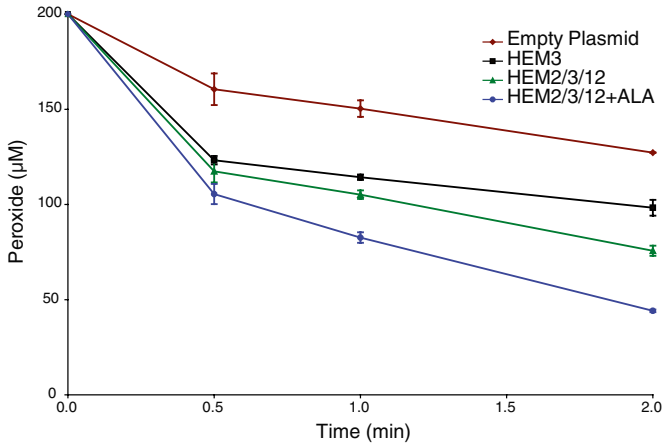

Fig. S6. Catalase time courses. The yeast cytosolic catalase CTT1 was overexpressed in cells containing various heme-overexpression constructs. Cultures were grown midexponential phase and lysed with glass beads. After measuring the total protein concentration, similar amounts of total protein (375, 349, 380, and 417 ng, respectively) were loaded into reactions containing 200 μM H_2O_2. Samples were taken after 30, 60, and 120 s and assayed for residual peroxide. A representative time course is shown. The activity of a lysate is calculated based on the H_2O_2 degraded by the 2-min time point. Error bars show ±1 SD, calculated from three technical replicates.

Table S1. Plasmids and strains used in this study

Strain	Genotype	Source
W303	MATα *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	
CSY409	W303 *his3::TEF-Ps6OMT, leu2::TEF-PsCNMT, ura3::TEF-Ps4'OMT, trp1::TEF-ATR1*	3
CSY492	W303 *lys2::P_{TEF}-GFP-L2Bulge8-ADH1T*	4
CSY851	W303 *lys2::P_{TEF}-HEM3-ADH1T*	This work
CSY852	W303 *lys2::P_{TEF}-HEM3-ADH1T-HEM2-P_{TEF}-P_{PGK}-HEM12-ADH1T*	This work
pCS782	2μ URA P_{TEF}-yCYP2D6	3
pCS2155	2μ URA P_{TEF}-yCDM1	4
pCS2160	2μ URA P_{TEF}-yCDM3	4
pCS2165	2μ URA P_{TEF}-yCDM5	4
pCS2166	2μ URA P_{TEF}-yCDM6	4
pCS2167	Centromeric URA P_{TEF}-yCDM6	4
pCS2168	Centromeric URA P_{TEF}-yCDM7	4
pCS2169	Centromeric URA P_{TEF}-yCDM8	4
pCS2170	2μ URA P_{TEF}-yCDM1 (A264H)	4
pCS2334	2μ URA P_{TEF}-CTT1	This work
pCS2335	2μ HIS P_{TEF}-HEM3	Courtesy of L. Liu, Chalmers Institute of Technology, Gothenburg, Sweden
pCS2336	2μ HIS P_{TEF}-HEM3, P_{TEF}-HEM2, P_{PGK}-HEM12	Courtesy of L. Liu
pCS2337	Centromeric URA P_{TEF}-yCDM1	This work
pCS2338	Centromeric URA P_{TEF}-yCDM3	This work
pCS2339	Centromeric URA P_{TEF}-yCDM5	This work
pCS2340	2μ URA P_{TEF}-yCDM7	This work
pCS2341	2μ URA P_{TEF}-yCDM8	This work
pCS2342	pIS385 + P_{TEF}-HEM3	This work
pCS2343	pIS385 + P_{TEF}-HEM3, P_{TEF}-HEM2, P_{PGK}-HEM12	This work
CSY821	CSY492+pCS2155	4
CSY853	CSY492+pCS2337	This work
CSY822	CSY492+pCS2160	4
CSY854	CSY492+pCS2338	This work
CSY823	CSY492+pCS2165	4
CSY855	CSY492+pCS2339	This work
CSY824	CSY492+pCS2166	4
CSY825	CSY492+pCS2167	4
CSY856	CSY492+pCS2340	This work
CSY826	CSY492+pCS2168	4
CSY857	CSY492+pCS2341	This work
CSY827	CSY492+pCS2169	4
CSY828	CSY492+pCS2170	4
CSY830	CSY492+pCS4 (empty centromeric plasmid)	4
CSY831	CSY492+pCS31 (empty 2μ plasmid)	4
CSY847	CSY3 + pCS9 (empty 2μ HIS plasmid) + pCS2334	This work
CSY848	CSY3 + pCS2335 + pCS2334	This work
CSY849	CSY3 + pCS2336 + pCS2334	This work
CSY858	CSY3 + pCS9 (empty 2μ HIS plasmid) + pCS31 (empty 2μ URA plasmid)	This work
CSY859	CSY3 + pCS2335 + pCS31 (empty 2μ URA plasmid)	This work
CSY860	CSY3 + pCS2336 + pCS31 (empty 2μ URA plasmid)	This work
CSY861	CSY3 + pCS9 (empty 2μ HIS plasmid) + pCS2169	This work
CSY862	CSY3 + pCS2335 + pCS2169	This work
CSY863	CSY3 + pCS2336 + pCS2169	This work
CSY864	CSY3 + pCS9 (empty 2μ HIS plasmid) + pCS2341	This work
CSY865	CSY3 + pCS2335 + pCS2341	This work
CSY866	CSY3 + pCS2336 + pCS2341	This work
CSY867	CSY851 + pCS2169	This work
CSY868	CSY851 + pCS2341	This work
CSY869	CSY852 + pCS2341	This work
CSY870	CSY3 + pCS2169	This work
CSY871	CSY3 + pCS2341	This work
CSY872	CSY409 + pCS782 + pCS9	This work
CSY873	CSY409 + pCS782 + pCS2335	This work
CSY874	CSY409 + pCS782 + pCS2336	This work

Table S2. Primers used in this study

Primer name	Primer sequence
ACT1-FWD	5′-CGGTGAAGGTGAAGGTGATGCTACT-3′
ACT1-REV	5′-GCTCTGGTCTTGTAGTTACCGTCATCTTTG-3′
HEM13-FWD	5′-GTCTCCGTTGTTTATGGTCAATTGAGCC-3′
HEM13-REV	5′-CAGTCTTTGGATCTTCTGGTAGACGCAG-3′
CTT1-FWD	5′-TAATCCGCGGAATTAATAAATGAACGTGTTCGGTAAAAAAGAAG-3′
CTT1-REV	5′-TAATGCGGCCGCTTAATTGGCACTTGCAATGGACCAAG-3′
HEM3-FWD	5′-TATAGAATTCGATATCAAGCTTGGAGATCTAAAAGAAAACAATGGGCCCTGAAACTCTAC-3′
HEM3-REV	5′-TATACCTAGGAGTCATTTGATTCTGTCTAAATTAATTTC-3′
HEM212-FWD	5′-TATACGCCAAACGACCTAGGAATTGG-3′
HEM212-REV	5′-TATACTCGAGTTACTTCGAACCAATTCTGTGGCAC-3′

Table S3. Heme-overexpression data

Enzyme expression	Heme overexpression	Relative total heme level	Relative total porphyrin level
No enzyme	Empty plasmid	1.00 ± 0.14	1.00 ± 0.14
No enzyme	HEM2/3/12	1.20 ± 0.20	3.91 ± 0.52
No enzyme	HEM2/3/12 + ALA	1.19 ± 0.33	13.81 ± 1.09
Low-copy P450	Empty plasmid	1.44 ± 0.14	0.89 ± 0.04
Low-copy P450	HEM2/3/12	2.44 ± 0.14	2.58 ± 0.26
Low-copy P450	HEM2/3/12 + ALA	3.35 ± 0.13	6.87 ± 0.15
High-copy P450	Empty plasmid	1.90 ± 0.11	0.62 ± 0.04
High-copy P450	HEM2/3/12	3.97 ± 0.17	1.37 ± 0.17
High-copy P450	HEM2/3/12 + ALA	9.54 ± 0.94	3.41 ± 0.71