Glyceraldehyde-3-phosphate dehydrogenase is a chaperone that allocates labile heme in cells

Cellular heme is thought to be distributed between a pool of sequestered heme that is tightly bound within heme proteins and a labile heme pool required for signaling and transfer into proteins. A heme chaperone that can hold and allocate labile heme within cells has long been proposed but never been identified. Here, we show that the glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fulfills this role by acting as an essential repository and allocator of bioavailable heme to downstream protein targets. We identified a conserved histidine in GAPDH that is needed for its robust heme binding both in vitro and in mammalian cells. Substitution of this histidine, and the consequent decreases in GAPDH heme binding, antagonized heme delivery to both cytosolic and nuclear heme protein targets, including inducible nitric-oxide synthase (iNOS) in murine macrophages and the nuclear transcription factor Hap1 in yeast, even though this GAPDH variant caused cellular levels of labile heme to rise dramatically. We conclude that by virtue of its heme-binding property, GAPDH binds and chaperones labile heme to create a heme pool that is bioavailable to downstream proteins. Our finding solves a fundamental question in cell biology and provides a new foundation for exploring heme homeostasis in health and disease.

Intracellular heme trafficking enables heme proteins to mature and function outside the mitochondria and participate in diverse activities such as gas exchange, catalysis, electron transfer, transcription, and the initiation and propagation of signaling cascades (1–3). Details of the intracellular heme transport and delivery pathways and their regulation have remained elusive. In particular, what protein(s) may help traffic heme after it is made and released from the mitochondria has long remained a mystery, with a number of protein candidates proposed over the years (4–7). Our previous work (8, 9) implicated a most unlikely protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as having some role in the process. Knockdown of GAPDH in mammalian cells led to a diminished heme content and activity of the soluble hemeprotein-inducible nitric-oxide synthase (iNOS) (9), whereas a yeast knockout strain lacking the GAPDH homolog TDH3 showed increased intracellular "labile" heme levels as determined by a fluorescent heme sensor, yet had low Hap1 transcription factor activity, which requires Hap1 heme binding (8). This resulted in GAPDH joining the list of possible heme transport proteins (10, 11), but it did not lead to an understanding that GAPDH was in fact responsible for heme allocation in cells. Essentially, the previous work fell short because it did not test whether GAPDH heme binding is of central importance to its involvement. In fact, to date there has been no demonstration that GAPDH can even bind mitochondrially-generated heme in intact cells or whether such intracellular heme binding by GAPDH, if it exists, is actually required for downstream heme deliveries.

To address these gaps, we first identified a GAPDH histidine residue that is involved in its in vitro heme binding. This allowed us to create a GAPDH variant that demonstrated a decreased heme-binding affinity that we then used alongside wildtype (WT) GAPDH in mammalian cell assays to measure their intracellular heme-binding capacity and their impact on downstream heme delivery to distinct cytosolic and nuclear protein targets. Our findings provide the first demonstration

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that GAPDH binds heme that is naturally generated by mitochondria in cells and that its heme-binding function is the essential aspect that enables downstream heme deliveries, rather than any other property of GAPDH itself. Thus, our work reveals that GAPDH acts as a middleman to shuttle labile heme, in effect making it bioavailable to target proteins that reside outside the mitochondria in mammalian cells and yeast.

Results

Identifying a heme-binding residue in GAPDH

Our previous study with rabbit GAPDH (rGAPDH) showed that purified rGAPDH ligates heme through an unidentified histidine (His) residue (6). To identify this residue, we considered three highly-conserved His in human GAPDH (hGAPDH) (Figs. 1 and 2A). Of these, His-53 (His-51 in rGAPDH) is located on a flexible loop at the inter-protomer interface in the GAPDH tetramer. Our computer modeling showed that His-53 could form either a mono-coordinated heme complex or a bis-coordinated His heme complex with a His-53 located in the neighboring protomer (Fig. 2, A and B) or, alternatively, with the nearby nonconserved His-57. Accordingly, we generated H53A and H57A hGAPDH mutants and the corresponding H51A and H55A rGAPDH mutants, and we found that whereas none of the His mutations affected the in vitro glycolytic activity of GAPDH (Fig. 2C), the H51A substitution in rGAPDH did eliminate the characteristic Soret spectral shift to 415 nm that indicates His ligation to heme (Fig. 2D) (6). The failure of the H51A rGAPDH to produce this spectral shift indicates that...
His-51 is indeed the heme-ligating histidine. Changes to the heme spectrum in the presence of H51A rGAPDH indicate that there is residual binding either in the heme-binding pocket or at a secondary, nonspecific site. However, whatever the nature of this heme binding, it is no longer His-ligated.

Altering a specific histidine residue in GAPDH disables its heme binding

To further explore the effect of the histidine mutations on GAPDH heme binding, we measured in vitro heme binding to purified hGAPDH proteins. Only the H53A mutation significantly diminished hGAPDH heme–binding affinity, as judged by a 4.5-fold increase in heme $K_d$ values (Fig. 3A) and a 12-fold faster heme $k_{off}$ value (Fig. 3, B and C). Using these measured $k_{off}$ values and a $k_{on}$ value that was determined previously for heme binding to rGAPDH (17,800 M$^{-1}$ s$^{-1}$ (12)), we estimated $K_d$ values of heme binding to the WT, H57A, and H53A hGAPDH proteins to be 0.15, 0.06, and 1.9 $\mu$M, respectively. Thus, our binding studies showed that Ala substitution of His-53 but not His-57 caused an approximate 10-fold loss in heme-binding affinity toward hGAPDH. This matches the spectroscopic results we obtained with the analogous rGAPDH proteins and implies that His-53 is involved in heme binding to hGAPDH.

Cellular hGAPDH binds heme and its binding is destabilized by the H53A mutation

To examine whether GAPDH binds heme in cells, and whether the His mutations would affect its heme binding under this circumstance, we expressed hGAPDH proteins with a hemagglutinin (HA) tag in mammalian cells that were given the labeled heme biosynthetic precursor $[14C]$-aminolevulinic acid ($14C$-ALA), which can be naturally converted into $14C$-labeled heme (13). Pulldowns of the HA-tagged hGAPDH proteins showed that WT HA–hGAPDH displayed robust $14C$-heme binding (Fig. 4, A and B), which was severely diminished in the H53A mutant but only slightly diminished in H57A mutant (Fig. 4B). The $14C$ counts in the HA pulldowns were confirmed to be $14C$-heme through HPLC analysis (Fig. 4A). These data reveal that hGAPDH in mammalian cells binds endogenously-generated heme and that its heme binding is diminished by the H53A substitution.

GAPDH heme-binding mutant H53A antagonizes heme insertion into iNOS

To determine whether diminished GAPDH heme binding can impact heme delivery in mammalian cells, we assessed the effect of the H53A GAPDH mutant on iNOS heme insertion
GAPDH is a chaperone that controls bioavailable heme

![Graph A: Fraction bound vs. µM heme](image)

**A**  
Fraction bound  
- WT hGAPDH $K_c = 2.9 \pm 0.6 \text{µM}$  
- H57A hGAPDH $K_c = 3.8 \pm 0.7 \text{µM}$  
- H53A hGAPDH $K_c = 13.4 \pm 2.3 \text{µM}$

![Graph B: Normalized change in Abs @ 410nm](image)

**B**  
- WT hGAPDH $k_{off} = 2.8 \times 10^{-3} \pm 0.1 \text{s}^{-1}$  
- H57A hGAPDH $k_{off} = 1.0 \times 10^{-3} \pm 0.01 \text{s}^{-1}$

![Graph C: Normalized change in Abs @ 410nm](image)

**C**  
- H53A hGAPDH $k_{off} = 3.3 \times 10^{-2} \pm 0.8 \text{s}^{-1}$

Figure 3. Mutation of histidine 53 antagonizes heme binding in vitro.  
- A, heme binding to human GAPDH WT, H57A, and H53A measured using nanoscale thermophoresis on a label-free Nanotemper device. Values represent mean ± S.E. (H53A and H57A, n = 4, and WT, n = 7). Dashed lines represent 95% confidence interval.  
- B, $k_{off}$ measurement of WT and H57A GAPDH using heme binding to apo-myoglobin as measured at 410 nm. Difference in $k_{off}$ values is not statistically significant.  
- C, $k_{off}$ measurement of H53A GAPDH using heme binding to apomyoglobin as measured at 410 nm using stopped-flow.

and activity. After transfecting the various HA–hGAPDH constructs into heme-depleted mouse macrophage (RAW264.7) cells, we induced their apo-iNOS expression for 16 h, added in exogenous $^{55}$Fe-heme for the final 2 h, lysed the cells, and performed 2’5’-ADP resin pulldowns on the cell supernatants to measure the extent of iNOS heme binding (Fig. 5A). Expressing the WT HA–hGAPDH in cells led to robust $^{55}$Fe-heme binding by apo-iNOS. However, expressing the H53A and to a lesser extent H57A HA–hGAPDH proteins diminished the extent of $^{55}$Fe-heme binding by the apo-iNOS (Fig. 5A). This indicates that the heme-binding–defective hGAPDH mutant antagonized $^{55}$Fe-heme insertion into the accumulated apo-iNOS. To further explore this observation, we tested the effect of the HA–hGAPDH proteins on the NO synthesis activity of iNOS, which requires that iNOS obtain a bound heme (Fig. 5, B and C). This was done under two conditions: 1) in heme-depleted cells treated with exogenous heme as above (Fig. 5B), and 2) in cells grown in normal media with endogenous heme levels (Fig. 5C). Under both conditions, expressing the WT HA–hGAPDH as well as the H57A mutant supported development of full NO synthesis activity of iNOS. However, the expression of the H53A mutant suppressed iNOS activity significantly (Fig. 5, B and C). Because the HA–hGAPDH mutants must operate alongside the cell’s endogenous pool of WT GAPDH, the inhibitory effect of the H53A mutant is especially striking. The results imply that intracellular heme binding by GAPDH is an essential feature that enabled downstream heme delivery to iNOS.

**Heme binding allows GAPDH to buffer the intracellular labile heme concentration and to deliver heme to nuclear transcription factor Hap1 in yeast**

Next, we took advantage of a GAPDH yeast knockout strain to further explore the importance of GAPDH heme binding in heme homeostasis and allocation. *Saccharomyces cerevisiae* expresses three GAPDH isoforms, and through a gene knock-out approach, the expression of one isoform (TDH3) was previously found to be required for controlling the intracellular labile heme level and for enabling heme delivery to the heme-regulated nuclear transcription factor Hap1 (8). We utilized the *tdh3* yeast knockout strain (*tdh3Δ*) that also expressed a ratiometric fluorescent heme sensor (8) that can measure the level of intracellular labile heme. The heme sensor readings confirmed that labile heme levels are dramatically elevated in *tdh3Δ* compared with the parent yeast strain (Fig. 6A), as reported previously (8). Transforming the *tdh3Δ* cells to express either their native TDH3 or WT hGAPDH both resulted in the labile heme readings being restored to their normal lower levels. In contrast, expressing the TDH3 H51A mutant (the H53A equivalent in *S. cerevisiae*) or the hGAPDH H53A mutant did not rescue the *tdh3Δ* phenotype regarding its higher than normal labile heme level (Fig. 6A). This reveals that the heme-binding ability of GAPDH is what allows it to control the level of intracellular labile heme in yeast, with apparent conservation of the GAPDH heme-binding site and function between yeast and human. Regarding downstream heme delivery to the transcription factor Hap1, the *tdh3Δ* cells displayed the expected diminished Hap1 activity (Fig. 6B). This defect was rescued by expression of either the TDH3 WT or hGAPDH WT proteins but not by expression of either the TDH3 H51A or the hGAPDH H53A heme-binding mutants (Fig. 6B). Thus, GAPDH heme binding was also required for heme delivery to the nucleus-localized yeast protein, HAP1. The similar functional importance of GAPDH heme binding in our yeast and mammalian cell experiments suggests that these aspects are broadly conserved.

**Discussion**

Intracellular heme trafficking is a fundamental process in biology, but the transport and delivery pathways and their reg-
ulation are only recently being clarified. Besides GAPDH, several other proteins have been proposed to possibly serve in heme transport, including GSH $S$-transferase (4), heme-binding proteins (5, 6), and fatty acid–binding proteins (7), but in all cases their in-cell heme-binding abilities were not demonstrated nor found linked to heme delivery to downstream heme protein targets. We reasoned that if GAPDH is involved in intracellular heme trafficking, then it should be demonstrated to bind heme in cells, and its binding capacity should be directly linked to successful heme delivery to downstream target proteins. Our findings reveal that GAPDH fulfills both tenets: it binds endogenous and exogenous heme in cells, and its heme-binding capacity is directly linked to successful heme delivery to cytosolic and nuclear heme protein targets. This helps to clarify how we think about heme sequestration and allocation within the cell. Rather than acting as a passive heme sink to protect the cell from toxicity (14), GAPDH appears to be a critical middleman in a dynamic network that makes heme bioavailable for directed mobilization to downstream targets.

**Fig. 7** depicts a model that highlights a role for GAPDH in heme delivery and is consistent with the results to date. Heme that is generated naturally in cells (i.e. from $\delta$-ALA) is transported out of the mitochondria by membrane transporters such as FLVCR1b (15) to reach the cytosol, where it, either directly or through an unknown intermediary, binds to GAPDH to create a pool of safely-sequestered but bioavailable heme. If exogenous heme arrives at the cell surface, it can be internalized by cell membrane transporters like HRG-4 (16, 17) and also join the GAPDH heme pool. Our study suggests that the GAPDH heme pool is in equilibrium with the more general pool of labile heme that is detected by the fluorescent heme sensor and that GAPDH sequesters a significant portion of the cell-labile heme pool. Once heme is bound to GAPDH, it becomes bioavailable for insertion into cytosolic proteins like iNOS and for delivery to nuclear proteins like Hap1. Whether heme delivery into the nucleus involves membrane transporters like BVR or involves transfer of the GAPDH–heme complex itself, perhaps similar to GAPDH being imported into the nucleus during apoptosis (18, 19), is a fundamental question that can now be investigated.
Notably, our work reveals that cellular heme is distributed into at least three groups, instead of two, due to a functional splitting of the labile heme pool. Specifically, our findings distinguish the GAPDH heme pool, whose heme is available for transfer into the two target proteins that we studied, from the cell’s more general labile heme pool, whose heme is unavailable for transfer into the two targets in the absence of GAPDH.

Given that the fluorescent heme sensor is itself derived from a heme-binding protein (it contains the heme-binding domain of cytochrome b562) (8), our results imply that a subset of cellular hemeproteins rely on GAPDH for heme delivery, whereas another subset may obtain heme independent of GAPDH. It will now be important to confirm and investigate these two modes of heme delivery.

As noted above, the size of the GAPDH–heme pool appears to be physically significant, and GAPDH binds heme with an affinity that allows it to diminish the cell’s general pool of labile heme (as detected by the sensor) while at the same time allowing heme delivery to downstream targets. Indeed, deletion of TDH3 in yeast resulted in an increase in labile heme from 10 to 21 nM. This indicates that GAPDH binds ~50% of the cytosolic labile heme in yeast. Expression of either the H53A hGAPDH or H51A TDH3 proteins, which are both heme-binding defective, in the tdh3Δ yeast was unable to decrease the labile heme level to its normal value. This behavior is consistent with GAPDH functioning as an intermediary, with its heme-binding affinity poised to be in equilibrium with an upstream heme source, while being lower than the heme affinities of the downstream protein delivery targets. In this regard, our estimate of the hGAPDH heme-binding affinity (Kd of 1.5 × 10^{-7} M) derived from the k_{on} and k_{off} measures is similar to the heme Kd value estimated for rGAPDH (12), and it is poorer than the heme Kd values reported for hemeproteins like hemoglobin, myoglobin, eIF2α, and others (20). The GAPDH heme Kd is also consistent with the recent estimate of the labile heme concentration in cells that was determined using the fluorescent heme sensor that we used here (8). Apparently, a 10-fold loss in the hGAPDH heme-binding affinity that is caused by the H53A mutation disrupted the heme binding continuum within the cell, by preventing the buildup of a sufficient GAPDH–heme complex needed...
GAPDH is a chaperone that controls bioavailable heme

Supporting downstream heme deliveries. Given that mammalian GAPDH exists as a tetramer, it will be interesting to understand how the H53A mutation impacts the heme-binding affinity within a mixed tetramer that contains both WT and mutant GAPDH subunits, which are likely to form when the mutant is expressed in cells. The dynamics of the GAPDH heme flux (i.e., heme loading onto and transfer from GAPDH) is also an important parameter that needs to be studied. In preliminary experiments, we found that mammalian cells needed to be made heme-deficient in order for the expressed HA-hGAPDH to bind detectable quantities of newly-synthesized $^{14}$C-heme. This implies that the existing GAPDH needed to be (or become) depleted of bound heme before it could bind significant amounts of the newly-generated $^{14}$C-heme. Investigating the dynamics of GAPDH heme loading and depletion will help in understanding how this critical pool is utilized and regulated.

Exposure to NO causes the cell-labile heme level to temporarily increase (8). An intriguing open question is whether the GAPDH-bound heme is released upon such NO exposure and whether such change is related to NO’s ability to enable versus inhibit heme insertion into apo-protein targets such as the $\beta$ subunit of soluble guanylate cyclase (21) or into NOS enzymes, hemoglobin, cytochrome P450, and catalase (22, 23). We also do not know how GAPDH engages in heme transfer to the downstream targets. In this regard, there are likely to be mechanistic connections between GAPDH and the cellular chaperone hsp90, which participates in the process by binding to the heme-free forms of several proteins to help drive their heme insertion (21, 24). The discovery that GAPDH is a provider of bioavailable heme helps to build a foundation for ultimately understanding how intracellular heme trafficking and homeostasis is related to life, health, and disease.

Experimental procedures

Reagents

Chemicals were from Sigma unless otherwise noted. $^{14}$C-ALA was purchased from ChemDepo and $^{55}$Fe-heme from RI Consultants. HA-tagged hGAPDH and variants were cloned into pcDNA3.1 expression plasmids.

Cell lines

GlyA CHO were a gift from Dr. P. J. Stover, Cornell University, and were confirmed in-house to be glycine-auxotrophic for growth. RAW264.7 cells were obtained from ATCC.

GAPDH alignment

Sequences for GAPDH from a number of species were downloaded from the NCBI database and aligned using MacVector.

GAPDH–heme model building and molecular dynamics simulation

The structure of the heme group used to build the GAPDH–heme model was taken from the bis-ligated protoporphyrin IX containing iron of the NMR-solved cytochrome $b_6$ (PDB code 1jex). All cysteines (including the catalytic ones) were represented in their reduced form, whereas the protonation state of histidines was determined with the H/H11001/H11001 server (25). Using PyMOL version 1.7.6.3 (26), the heme group was manually accommodated in the inter-monomer cleft between the two flexible loops containing His-53 in the hGAPDH (PDB code 1znq). Using the Molefacture plug-in version 1.3 of VMD 1.9.2 (27), the histidines were rotated making both imidazole rings point toward the middle of the cleft, at bonding distance from the iron atom of the heme group. The imidazole group of His-53 was predicted by H/H11001/H11001 to be protonated in $\epsilon$ and $\delta$. to support downstream heme deliveries. Given that mammalian GAPDH exists as a tetramer, it will be interesting to understand how the H53A mutation impacts the heme-binding affinity within a mixed tetramer that contains both WT and mutant GAPDH subunits, which are likely to form when the mutant is expressed in cells. The dynamics of the GAPDH heme flux (i.e., heme loading onto and transfer from GAPDH) is also an important parameter that needs to be studied. In preliminary experiments, we found that mammalian cells needed to be made heme-deficient in order for the expressed HA-hGAPDH to bind detectable quantities of newly-synthesized $^{14}$C-heme. This implies that the existing GAPDH needed to be (or become) depleted of bound heme before it could bind significant amounts of the newly-generated $^{14}$C-heme. Investigating the dynamics of GAPDH heme loading and depletion will help in understanding how this critical pool is utilized and regulated.

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Figure 7. Model of GAPDH as a cellular depot and allocator of bioavailable heme. Heme can be synthesized endogenously by the cell or imported through the plasma membrane. Endogenous heme biosynthesis occurs from glycine through the intermediates δ-ALA and coproporphyrinogen III (CPgenIII) catalyzed by enzymes found in the cytoplasm and mitochondria and finally exported into the cytoplasm by the transporter feline leukemia virus subgroup C receptor 1b (FLVCR1b). Exogenous heme can be imported into the cell through transporters such as histidine-rich glycoprotein 4 (HRG-4). GAPDH can bind both endogenous and exogenous heme sources thereby sequestering heme into a bioavailable pool. Heme binding to the heme sensor HS1 causes a decrease in EGFP (green) fluorescence but has no effect on mKate (red) fluorescence. The labile heme pool detected by the heme sensor is distinct from the GAPDH-bound bioavailable heme pool. The GAPDH-bound heme can be delivered to soluble proteins such as iNOS or directly carried into the nucleus by GAPDH.

Consequently, the e proton was removed to make possible the binding of the NE2 atom of histidine with the iron center of the heme group. The model was then minimized in vacuo, neutralized (with 16 Cl\textsuperscript{−}), solvated (with explicit waters and 0.15 M NaCl), and minimized in solution with positional restraints on the solute using our well-established multistep protocol (28, 29). To produce the final model, the minimized structure was thermalized to 298 °C at NVT and then simulated during 150 ns by means of Molecular Dynamics simulations at NPT (p = 1 atm). GAPDH was represented by the state-of-the-art ff14SB force field (30), surrounded by a truncated octahedral box of ~22,000 TIP3P water molecules (31). Dang parameters for ions (32), Walker’s parameters for NAD\textsuperscript{+} (33), and the heme hexacoordinate parameters developed by Estrin and co-workers (34, 35). Ions were initially placed randomly, at a minimum distance of 5 Å from the solute and 3.5 Å from one another. All systems were simulated using the Berendsen algorithm (36) to control the temperature and the pressure, with a coupling constant of 5 ps. Center of mass motion was removed every 10 ps. SHAKE (37) was used to keep all bonds involving hydrogen at their equilibrium values, which allowed us to use a 2-fs step for the integration of Newton equations of motion. Long-range electrostatic interactions were accounted for by using the Particle Mesh Ewald method (38) with standard defaults and a real-space cutoff of 9 Å. All simulations were carried out using the PMEMD CUDA code module (39) of AMBER 16 (40) and analyzed with CPPTRAJ (41).

Protein purification

WT human GAPDH in a pGEX 4T-2 vector and mutants, made using site-directed mutagenesis, were purified from BL21 (DE3) Escherichia coli using standard techniques. Briefly, E. coli cells carrying the GAPDH construct were grown to an optical density of 0.8–1.0 and induced with 1 mM isopropyl β-D-galactopyranoside for 48 h at room temperature. After cell lysis, purification of WT and mutant GAPDH was carried out using GST affinity chromatography as per the manufacturer’s instructions (Sigma). After subsequent cleavage of the GST tag using thrombin, the GST and thrombin were removed using a GST column followed by a benzamidine FF column (GE Healthcare). Before use in heme-binding assays, the proteins were run over a Superdex 200 column (GE Healthcare) and assessed for purity by SDS-PAGE.

WT rabbit GAPDH and H51A mutant, made using site-directed mutagenesis, were purified as described previously (12) using nickel-nitritotriacetic acid affinity chromatography (Amersham Biosciences) followed by thrombin cleavage of the His\textsubscript{6} tag. Before use in heme-binding assays, the proteins were assessed for purity by SDS-PAGE.

UV-visible spectroscopy

UV-visible spectra of hemin, rGAPDH (WT and H51A), and rGAPDH-hemin complexes were measured on a Shimadzu UV-2600 using freshly prepared rGAPDH and hemin stocks. Heme binding via His ligation was determined by the shift in Soret peak from 390 nm (free heme) to 412 nm (WT rGAPDH–heme) (12).

Heme binding using nanoscale thermophoresis

Exact hemin concentration was determined by diluting the stock solution in pure DMSO and measuring absorbance at 406 nm on a Hitachi U-2000 spectrophotometer (extinction coefficient 170 mM\textsuperscript{−1} cm\textsuperscript{−1}) (42). Heme was diluted from the stock solution to 1.6 mM in PBS. Sixteen serial dilutions of heme were then made from 50 μM to 1.53 mM in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl\textsubscript{2}, 150 mM NaCl, and 1 mM DTT (TMND) with 0.65% DMSO. Purified hGAPDH WT and mutants were diluted to 1 μM in TMND with 0.1% Pluronic F-127 (Nanotemper). 10 μl of GAPDH protein was added to 10 μl of each heme dilution and after mixing transferred to capillary tubes (Nanotemper). Capillary tubes were loaded onto and analyzed using a Monolith NT. LabelFree Nanotemper device. Each capillary
series was analyzed at three different LED:MST settings, and binding curves were obtained using thermophoresis (movement of particles due to temperature gradients, which changes based on binding events) using the intrinsic tryptophan fluorescence of GAPDH. Data from individual Nano-temper runs were combined using Prism and fit with a one-site binding curve.

**Measurement of GAPDH dehydrogenase activity**

WT and GAPDH variants were assayed for their enzymatic activity by measuring conversion of NAD\(^+\) to NADH in 96-well clear plates as described in the literature (43). Briefly, GAPDH was diluted to a concentration of 6.9 nM in 10 mM tetrasodium pyrophosphate, pH 8.5, 20 mM sodium phosphate, 2 mM NAD\(^+\), and 0.3 mM DTT. The enzymatic reaction was initiated by addition of 2 mM D-l-glyceraldehyde 3-phosphate to the wells. After mixing, the absorbance at 340 nm was measured for 5 min. The initial linear portion of the curve was used to determine the rate of NADH production using the extinction coefficient for NADH of 6.22 mM\(^{-1}\) cm\(^{-1}\).

**Heme k\(_{on}\) measurements using apo-myoglobin**

The kinetics of ferric heme transfer from hGAPDH (10 \(\mu\)M GAPDH, 2 \(\mu\)M heme) to apo-myoglobin (50 \(\mu\)M) were measured as described (12) in either a Shimadzu UV-2600 or a Hi-Tech Scientific stop-flow using Kinetic Studio software for data acquisition and analysis. Normalized change in absorbance at 410 nm was plotted using Prism and fit using a one-phase exponential association.

**Cell culture**

RAW264.7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS and penicillin/streptomycin (100 units/ml and 100 \(\mu\)g/ml, respectively) at 37 °C with 5% CO\(_2\) in a humidified incubator. The day before transfection, RAW264.7 cells were seeded out at a density of 3 \(\times\) 10\(^6\) cells per 10-cm dish into DMEM with 10% FBS, 3 \(\mu\)M No-nitro-L-arginine methyl ester hydrochloride (L-NAME), and no antibiotic. Cells were transfected with 5 \(\mu\)g of HA-tagged GAPDH DNA and 15 \(\mu\)L of Lipofectamine 2000 (ThermoFisher Scientific) per sample. Sixteen hours after transfection, the media were changed to DMEM with 10% FBS, 3 \(\mu\)M L-NAME, and penicillin/streptomycin. Transfection efficiency was assessed by imaging an mVenus control plate. Efficiency was generally 85–95%. Cells were activated to induce iNOS expression 24 h after transfection with 10 units/ml interferon-\(\gamma\) (IFN-\(\gamma\), PeproTech) and 25 \(\mu\)g of lipopolysaccharide. After 16 h of activation, the cells were harvested and lysed in lysis buffer (40 mM EPPS, pH 7.6, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, and protease inhibitors). Crude lysates were cleared of insoluble debris by centrifugation at 13,000 rpm. Lysate protein concentration was assessed using a DC protein assay from Bio-Rad and subsequently used in HA-pulldown experiments.

**Measuring radiolabeled \(^{14}\)C and \(^{55}\)Fe counts**

Lysates containing equal amounts of protein were incubated with HA monoclonal antibody (GlyA or RAW264.7) overnight at 4 °C with rotation. For HA-pulldowns, protein G-agarose beads (Millipore) were washed twice with PBS and then equilibrated with RIPA buffer twice at 2000 \(\times\) g for 2 min. The overnight rotating cell lysate and antibody complexes were then incubated with equilibrated beads at 4 °C with rotation for 3 h. For iNOS, pulldowns using ADP-affinity resin (RAW264.7) beads were washed as above and incubated with lysates at 4 °C with rotation for 3 h. After incubation, the beads were washed three times with RIPA buffer. Washed beads (HA pulldowns and ADP pulldowns) were added to scintillation liquid and counted on a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter for \(^{14}\)C or \(^{55}\)Fe counts. Alternatively, heme was extracted from the beads using acidified acetone, and the extracts were run on an Agilent HPLC. Fractions were then assessed for radioactive counts as above.

**Measuring iNOS NO synthesis activity**

The oxyHb assay for NO was carried out in a 96-well clear plate in a 200-\(\mu\)L volume. The final reaction conditions included 5 \(\mu\)M tetrahydrobiopterin (Schircks), 5 \(\mu\)M FMN, 5 \(\mu\)M FAD, 0.3 mM DTT, 0.1 mg/ml BSA, protease inhibitor, 60 units/ml superoxide dismutase, 100 units/ml catalase, 10 \(\mu\)M L-arginine, 5 \(\mu\)M Oxy(PEG)Hb, and 80 \(\mu\)g of lysate or 0.1 \(\mu\)m purified iNOS protein as a positive control. Activity of iNOS in the cell lysates was assessed by measuring the conversion of OxyHb to MetHb by monitoring increases in absorbance at 401 nm. Absorbance was read on a SpectraMax M2e (Molecular Devices) plate reader at 401 nm for 10 min, and the initial rate was used to determine \(V_{\text{max}}\).

**Western blot analysis**

SDS-PAGE and Western blot analysis were performed using standard procedures with antibodies against HA for HA–GAPDH (Sigma), iNOS (Cell Signaling), and \(\beta\)-actin (Ori-
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gene). Horseradish peroxidase-linked secondary antibodies (Santa Cruz Biotechnology) were used to visualize the proteins using a Western Lightning Plus-ECL kit from PerkinElmer Life Sciences.

**Yeast strains, transformations, and growth conditions**

*S. cerevisiae* strains used in this study were derived from BY4741 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0). The tdlh3Δ::KanMX4 strain was obtained from the yeast gene deletion collection (ThermoFisher Scientific). Yeast transformations were performed by the lithium acetate procedure (46). Strains were maintained at 30 °C on either enriched yeast extract (1%)–peptone (2%)–based medium supplemented with 2% glucose (YPD) or synthetic complete medium (SC) supplemented with 2% glucose and the appropriate amino acids to maintain selection (8). Cells cultured on solid media plates were done so with YPD or SC media supplemented with 2% agar (8). Selection for yeast strains containing the KanMX4 marker was done with YPD agar plates supplemented with G418 (200 μg/mL) (8). WT cells treated with the heme synthesis inhibitor, SA, and hem1Δ cells were cultured in YPD or SC media supplemented with 50 μg/mL ALA or 15 mg/mL ergosterol and 0.5% Tween 80 (YPDE or SCE, respectively) (8, 47). All liquid cultures were maintained at 30 °C and shaken at 220 rpm.

**Yeast plasmids**

Yeast plasmids expressing the cytosolic heme sensor, HS1-M7A, which was subcloned into pRS415 and driven by the GPD promoter, and the Hop1 reporter plasmid in which EGFP is driven by the CYC1 promoter were previously described (8). The yeast Tdh3 expression plasmid was generated by amplifying TDH3 from yeast genomic DNA and sub-cloning it into the SpeI/BamHI sites of p416-GPD (48) to generate plasmid pAR1031. The yeast Tdh3-H51A mutation was generated by QuikChange mutagenesis (Agilent Technologies) using pAR1031 as a template, generating pAR1035. The yeast hGAPDH and hGAPDH-H53A expression plasmids were generated by amplifying hGAPDH from pGex4T2-GST-hGAPDH or pGexX2T2-GST-hGAPDH-H53A and sub-cloning it into the SpeI/BamHI sites of p416-GPD (48) to generate plasmids pAR1035 and pAR1036, respectively.

**Labile heme measurements using a fluorescent heme sensor**

WT or tdlh3Δ cells co-expressing HS1-M7A and the indicated GAPDH variant or empty vector (p416-GPD) (48) were cultured in SCE/LEU/URA to an *A*₆₀₀ nm of 1.0 (2 × 10⁷ cells/ml). Cells were washed in ultrapure water and resuspended in phosphate-buffered saline (PBS) solution to give a concentration of 1 × 10⁷ cells/ml, prior to recording EGFP (excitation 488 nm and emission 510 nm) and mKATE2 (excitation 588 nm and emission 620 nm) fluorescence in a Synergy H1 hybrid multimode microplate reader (BioTek). Labile heme concentration was calculated using Equation 1,

\[
[heme] = K_p \times \frac{R_{\text{expt}} - R_{\text{min}}}{R_{\text{max}} - R_{\text{expt}}} \frac{F_{\text{mKATE2}}}{F_{\text{mKATE2}}} \quad \text{(Eq. 1)}
\]

where *K*ₚ is the HS1-M7A–ferrous heme dissociation constant at pH 7.0, 25 nM; *R*ₚ is the EGFP/mKATE2 fluorescence ratio under any given condition; *R*ₚ is the EGFP/mKATE2 fluorescence ratio when 0% of the sensor is bound to heme; *R*ₚ is the EGFP/mKATE2 fluorescence ratio when 100% of the sensor is bound to heme; *F*ₚ is the mKATE2 emission intensity when 0% of the sensor is bound to heme, and *F*ₚ is the mKATE2 emission intensity when 100% of the sensor is bound to heme (8).

Determination of *R*ₚ and *F*ₚ involves recording EGFP and mKATE2 fluorescence after digitonin permeabilization of cells and incubation with 50 μM heme (8). Briefly, 1 × 10⁸ cells/ml of cells are resuspended in PBS with 200 μg/mL digitonin, 1 mM ascorbate, and 50 μM hemin chloride. After a 30-min incubation at 30 °C, cells were harvested, washed, and resuspended in PBS buffer prior to recording of fluorescence. Determination of *R*ₚ and *F*ₚ involves recording EGFP and mKATE2 fluorescence from parallel cultures treated with the heme biosynthesis inhibitor succinylacetone (8, 49).

**Hap1 activity**

WT or tdlh3Δ cells co-expressing p415–CYC1–EGFP or EGFP driven by the Hop1p-regulated CYC1 promoter (8) and the indicated GAPDH variant or empty vector (p416–GPD) (42) were cultured in 10 ml of SCE/LEU/URA medium for 15 h to 1 × 10⁷ cells/ml. Cells were resuspended in PBS to a concentration of 1 × 10⁸ cells/ml, and 100 μl was used to measure EGFP fluorescence (excitation 488 nm and emission 510 nm). As a positive and negative control, WT and hem1Δ cells were cultured for each experiment. Background autofluorescence of cells not expressing EGFP was recorded and subtracted from the EGFP-expressing strains.

**References**

1. Ponka, P. (1999) Cell biology of heme. *Am. J. Med. Sci.* 318, 241–256

2. Mense, S. M., and Zhang, L. (2006) Heme: a versatile signaling molecule controlling the activities of diverse regulators ranging from transcription factors to MAP kinases. *Cell Res.* 16, 681–692

3. Adachi, Y., Umeda, M., Kawaoe, A., Satoh, T., Ohkawa, Y., Kitaïjima, S., Izawa, S., Sagami, I., and Takei, S. (2017) The novel heme-dependent inducible protein, SRRD regulates heme biosynthesis and circadian rhythms. *Arch. Biochem. Biophys.* 631, 19–29

4. Harvey, J. W., and Beutler, E. (1982) Binding of heme by glutathione S-transferase: a possible role of the erythrocyte enzyme. *Blood* 60, 1227–1230

5. Iwahara, S., Satoh, H., Song, D. X., Webb, J., Burlingame, A. L., Nagae, Y., and Muller-Eberhard, U. (1995) Purification, characterization, and clon-
ing of a heme-binding protein (23 kDa) in rat liver cytosol. Biochemistry 34, 13398–13406 CrossRef Medline

6. Taketani, S., Adachi, Y., Kohno, H., Ikehara, S., Tokunaga, R., and Ishii, T. (1998) Molecular characterization of a newly identified heme-binding protein induced during differentiation of urine erythroleukemia cells. J. Biol. Chem. 273, 31388–31394 CrossRef Medline

7. Vincent, S. H., and Muller-Eberhard, U. (1985) A protein of the Z class of liver cytosolic proteins in the rat that preferentially binds heme. J. Biol. Chem. 260, 14521–14528 Medline

8. Hanna, D. A., Harvey, R. M., Martinez-Guzman, O., Yuan, X., Chandrasekharan, B., Raju, G., Outten, F. W., Hamza, I., and Reddi, A. R. (2016) Heme dynamics and trafficking factors revealed by genetically encoded fluorescent heme sensors. Proc. Natl. Acad. Sci. U.S.A. 113, 7539–7544 CrossRef Medline

9. Chakravarti, R., Aulak, K. S., Fox, P. L., and Stuehr, D. J. (2010) GAPDH regulates cellular heme insertion into inducible nitric-oxide synthase. Proc. Natl. Acad. Sci. U.S.A. 107, 18004–18009 CrossRef Medline

10. Severance, S., and Hamza, I. (2009) Trafficking of heme and porphyrins in metazoa. Chem. Rev. 109, 4596–4616 CrossRef Medline

11. Yuan, X., Fleming, M. D., and Hamza, I. (2013) Heme transport and erythropoiesis. Curr. Opin. Chem. Biol. 17, 204–211 CrossRef Medline

12. Hannibal, L., Collins, D., Brassard, J., Chakravarti, R., Vempati, R., Dorlet, P., Santolini, J., Dawson, J. H., and Stuehr, D. J. (2012) Heme binding properties of glyceraldehyde-3-phosphate dehydrogenase. Biochemistry 51, 8514–8529 CrossRef Medline

13. Shedlofsky, S. I., Sinclair, P. R., Bonkovsky, H. L., Healey, J. F., Swim, A. T., and Robinson, J. M. (1987) Haem synthesis from exogenous 5-aminolaevulinate in cultured chick-embryo hepatocytes. Effects of inducers of cytokromes P-450. Biochem. J. 248, 229–236 CrossRef Medline

14. Huang, Y., Zhang, P., Yang, Z., Wang, P., Li, H., and Gao, Z. (2017) Interaction of glyceraldehyde-3-phosphate dehydrogenase and heme: the relevance of its biological function. Arch. Biochem. Biophys. 619, 54–61 CrossRef Medline

15. Chibrado, D., Marro, S., Mercurio, S., Giorgi, C., Petrolli, S., Vinchi, F., Fiorito, V., Fagonee, S., Camporeale, A., Turco, E., Merlo, G. R., Silengo, L., Altruda, F., Pinton, P., and Tolosano, E. (2012) The mitochondrial heme exporter FLYCR1 mediates erythroid differentiation. J. Clin. Invest. 122, 4569–4579 CrossRef Medline

16. Sun, F., Cheng, Y., and Chen, C. (2015) Regulation of heme biosynthesis and transport in metazoa. Sci. China Life Sci. 58, 757–764 CrossRef Medline

17. Khan, A. A., and Quigley, J. G. (2011) Control of intracellular heme levels: heme transporters and heme oxygenases. Biochim. Biophys. Acta 1813, 668–682 CrossRef Medline

18. Hara, M. R., Agrawal, N., Kim, S. F., Cascio, M. B., Fujimuro, M., Ozeki, Y., Takahashi, M., Cheah, J. H., Tankou, S. K., Hester, L. D., Ferris, C. D., Hayward, S. D., Snyder, S. H., and Sawa, A. (2005) S-Nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. Nat. Cell Biol. 7, 665–674 CrossRef Medline

19. Sen, N., Hara, M. R., Kornberg, M. D., Cascio, M. B., Bae, B. I., Shahani, N., Thomas, B., Dawson, T. M., Dawson, V. L., Snyder, S. H., and Sawa, A. (2008) Nitric oxide-induced nuclear GAPDH activates p300/CPB and mediates apoptosis. Nat. Cell Biol. 10, 866–873 CrossRef Medline

20. Hannibal, L., and Stuehr, D. J. (2013) Handbook of Porphyrin Science, Vol. 30, pp. 55–102, World Scientific Publishing Co., Singapore

21. Ghosh, A., Stach, J. P., Papapetropoulos, A., and Stuehr, D. J. (2014) Nitric oxide and heat shock protein 90 activate soluble guanylate cyclase by driving rapid change in its subunit interactions and heme content. J. Biol. Chem. 289, 15259–15271 CrossRef Medline

22. Waheed, S. M., Ghosh, A., Chakravarti, R., Biswas, A., Haque, M. M., Panda, K., and Stuehr, D. J. (2010) Nitric oxide blocks cellular heme insertion into a broad range of hemeoxygenases. Free Radic. Biol. Med. 48, 1548–1558 CrossRef Medline

23. Chakravarti, R., Gupta, K., Majors, A., Ruple, L., Aronica, M., and Stuehr, D. J. (2015) Novel insights in mammalian catalse heme maturation: effect of NO and thioredoxin-I. Free Radic. Biol. med. 82, 105–113 CrossRef Medline

24. Ghosh, A., Garee, G., Sweeny, E. A., Nakamura, Y., and Stuehr, D. J. (2018) Hsp90 chaperones hemoglobin maturation in erythroid and nonerythroid cells. Proc. Natl. Acad. Sci. U.S.A. 115, E1117–E1126 CrossRef Medline

25. Anandakrishnan, R., Aguilar, B., and Onufriev, A. V. (2012) Heme binding of GAPDH is a chaperone that controls bioavailable heme. J. Biol. Chem. 287, 13398–13406 CrossRef Medline

26. Maier, J. A., Martinez, C., Kasavajhala, K., Wickstrom, L., Hauser, K. E., and Simmerling, C. (2015) f3145b: improving the accuracy of protein side chain and backbone parameters from f99SB. J. Chem. Theory Comput. 11, 3696–3713 CrossRef Medline

27. Republican simulations of NaCl association in polarizable water. J. Chem. Phys. 100, 3757–3766 CrossRef Medline

28. Walker, R. C., de Souza, M. M., Mercier, I. P., Gould, I. R., and Klug, D. R. (2002) Large and fast relaxations inside a protein: calculation and measurement of reorganization energies in alcohol dehydrogenase. J. Phys. Chem. B 106, 11658–11665 CrossRef Medline

29. Rape, D., Martin, M. A., Bidon-Chenal, A., Nadra, A., Luoque, F. J., and Estrin, D. A. (2009) High pressure reveals structural determinants for globin hexacoordination: neuroglobin and myoglobin cases. Proteins 75, 885–894 CrossRef Medline

30. Nadra, A. D., Martin, M. A., Pesce, A., Bolognesi, M., and Estrin, D. A. (2008) Exploring the molecular basis of heme coordination in human neuroglobin. Proteins 71, 695–705 CrossRef Medline

31. Berendsen, H. J. C., Postma, J. P. M., Vanguesterne, W. F., Dinola, A., and Haak, J. R. (1984) Molecular-dynamics with coupling to an external bath. J. Chem. Phys. 81, 3684–3690 CrossRef Medline

32. Ryckaert, J. P., Ciccotti, G., and Berendsen, H. J. C. (1977) Numerical integration of cartesian equations of motion of a system with constraints-molecular-dynamics of N-alkanes. J. Comput. Phys. 23, 327–341 CrossRef Medline

33. Darden, T., York, D., and Pedersen, L. (1993) Particle Mesh Ewald-an N. J. Chem. Theory Comput. 9, 10089–10092 CrossRef Medline

34. Salomon-Ferrer, R., Götz, A. W., Poole, D., LeGrand, S., and Walker, R. C. (2013) Routine microsecond molecular dynamics simulations with AMBER on GPUs. 2. Explicit solvent particle mesh Ewald. J. Chem. Theory Comput. 9, 3878–3888 CrossRef Medline

35. Case, D. A., Cheatham, T. E., III, Darden, T. A., Duke, R. E., Giese, T. J., Luo, L., Merz, K. M.,대안, K., Nguyen, N. V., Wang, J. W., and Woods, R. C. (2013) PTRAJ and CPPTRAJ: software for processing and analysis of molecular dynamics trajectory data. J. Chem. Theory Comput. 9, 3084–3095 CrossRef Medline

36. Ritz, K. R., Swartzlander, R., Sadikot, T. N., Berridge, M. V., and Smith, A. (2007) Interaction of heme and heme-hemopexin with an extracellular oxidant system used to measure cell growth-associated plasma membrane electron transport. Biochim. Biophys. Acta 1767, 1107–1117 CrossRef Medline

37. Stover, P. J., Chen, L. H., Suh, J. R., Stover, D. M., Keyomarsi, K., and Shae, B. (1997) Molecular cloning, characterization, and regulation of the hu-
GAPDH is a chaperone that controls bioavailable heme

man mitochondrial serine hydroxymethyltransferase gene. J. Biol. Chem. 272, 1842–1848

45. Narkewicz, M. R., Sauls, S. D., Tjoa, S. S., Teng, C., and Fennessey, P. V. (1996) Evidence for intracellular partitioning of serine and glycine metabolism in Chinese hamster ovary cells. Biochem. J. 313, 991–996

46. Gietz, R. D., and Schiestl, R. H. (1991) Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. Yeast 7, 253–263

47. Ness, F., Achstetter, T., Duport, C., Karst, F., Spagnoli, R., and Degryse, E. (1998) Sterol uptake in Saccharomyces cerevisiae heme auxotrophic mutants is affected by ergosterol and oleate but not by palmitoleate or by sterol esterification. J. Bacteriol. 180, 1913–1919

48. Mumberg, D., Müller, R., and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156, 119–122

49. Ebert, P. S., Hess, R. A., Frykholm, B. C., and Tschudy, D. P. (1979) Succinylacetone, a potent inhibitor of heme biosynthesis: effect on cell growth, heme content and δ-aminolevulinic acid dehydratase activity of malignant murine erythroleukemia cells. Biochem. Biophys. Res. Commun. 88, 1382–1390