Heme oxygenase-2 (HO2) and -1 (HO1) catalyze heme degradation to biliverdin, CO, and iron, forming an essential link in the heme metabolism network. Tight regulation of the cellular levels and catalytic activities of HO1 and HO2 is important for maintaining heme homeostasis. HO1 expression is transcriptionally regulated; however, HO2 expression is constitutive. How the cellular levels and activity of HO2 are regulated remains unclear. Here, we elucidate the mechanism of post-translational regulation of cellular HO2 levels by heme. We find that, under heme-deficient conditions, HO2 is destabilized and targeted for degradation, suggesting that heme plays a direct role in HO2 regulation. HO2 has three heme binding sites: one at its catalytic site and the others at its two heme regulatory motifs (HRMs). We report that, in contrast to other HRM-containing proteins, the cellular protein level and degradation rate of HO2 are independent of heme binding to the HRMs. Rather, under heme deficiency, loss of heme binding to the catalytic site destabilizes HO2. Consistently, an HO2 catalytic site variant that is unable to bind heme exhibits a constant low protein level and an enhanced protein degradation rate compared with the WT HO2. Finally, HO2 is degraded by the lysosome through chaperone-mediated autophagy, distinct from other HRM-containing proteins and HO1, which are degraded by the proteasome. These results reveal a novel aspect of HO2 regulation and deepen our understanding of HO2’s role in maintaining heme homeostasis, paving the way for future investigation into HO2’s pathophysiologic role in heme deficiency response.

Heme is a commonly used prosthetic group in a myriad of biochemical processes. High levels (>1 μM) of free heme are seen in many pathologic processes and diseases, such as cardiovascular diseases (1). The cytotoxicity of heme arises from the overproduction and accumulation of reactive oxygen species, leading to oxidative stress and tissue damage (2, 3). Heme oxygenase (HO) is the only known mammalian enzyme that catalyzes the degradation of heme, thus maintaining systemic heme homeostasis. Furthermore, HO converts heme to biliverdin (further reduced to bilirubin) and CO, which play essential roles in cytoprotection against oxidative stress (4–7). Therefore, understanding the regulation of HO activity is important for human health.

In mammals, two major isoforms of HO have been identified: HO1 and HO2. These two enzymes share high sequence homology and have very similar structures (4, 5, 8), especially for regions around the catalytic site where heme binds and is metabolized. The catalytic site binds heme with high affinity through a conserved histidine (His-45 in HO2, His-25 in HO1) as the proximal ligand and an aquo (HxO) ligand H-bonded to Gly-159 (Gly-139 in HO1) as the distal ligand (Fig. 1A). Consistent with their high homology, these enzymes possess very similar heme degradation activities (8).

Despite these similarities, HO1 exists in a tissue-specific and inducible manner, with its expression stimulated at the transcriptional level by various conditions, such as infection, excess heme, and oxidative stress (4). In contrast, HO2 is constitutively expressed and highly concentrated in brain, testes, and neural tissues (4). HO2 is considered to provide the baseline heme detoxification activity under normal conditions and to be cytoprotective under stressed conditions, when or where HO1 is not available, such as ischemic stroke (9, 10). As of now, little is known about how HO2 is regulated to maintain its proper heme detoxification and cytoprotective functions.

Notably, the biggest difference between those two enzymes exists in the region C-terminal to the catalytic core, where HO1 harbors a PEST domain that is usually associated with proteasomal degradation of short-lived proteins (11, 12). HO2, however, possesses two heme regulatory motifs (HRMs) in this corresponding region (Fig. 1A). The HRM is a conserved sequence, featured by a Cys-Pro dipeptide core with Cys serving as the heme ligand (13). In HO2, there are two HRMs, centered at Cys-265 and Cys-282, each of which binds heme under reducing conditions; yet in an oxidizing environment, they form a disulfide bond and are unable to bind heme. Previous in cellulo and in vitro studies showed that this thiol-disulfide redox switch is 60–70% reduced under normal physiological conditions and exhibits a midpoint potential of −200 mV, which is within the range of ambient cellular redox potential (14). Moreover, the binding affinities for heme of the two HRMs match well with the cellular regulatory heme pool level (14–17). Thus, this redox-gated heme binding appears to be physiologically relevant.

Several key proteins within the heme metabolism network are subject to HRM-mediated heme-dependent regulation of their expression, degradation, and activity (18). For instance, heme binding to the HRMs of Bach1, a transcriptional repressor of the HO1 gene, disrupts its DNA binding activity (19) and triggers the ubiquitination and proteasomal degradation of Bach1 protein, leading to HO1 induction (20). In
another case, the cellular level of δ-aminolevulinic acid synthase 1 (ALAS1), the rate-limiting enzyme in heme biosynthesis, is also mediated by heme-HRM interaction through a negative feedback mechanism (21). A recent survey of HRM-containing proteins demonstrated that the most common HRM function is to promote protein degradation upon heme binding (18). It has been speculated that heme binding to the HRM may serve as a molecular signature for recognition by an E3 ubiquitin ligase so as to target those HRM-containing proteins for proteasomal degradation (20, 22). Therefore, we hypothesized that heme binding to the two HRMs in HO2 modulates HO2 protein degradation.

To understand how heme regulates HO2 protein stability and degradation, we constructed HO2 mutants with various heme binding abilities at each of the three heme binding sites (Fig. 1A). Our work shows that, unlike other HRM-containing proteins, the HRMs in HO2 do not mediate protein degradation. In addition, HO2 is degraded by the lysosome through chaperone-mediated autophagy instead of the ubiquitin-proteasome pathway used for other HRM-containing proteins (18) or the endoplasmic reticulum (ER)-associated protein degradation (ERAD) pathway used for HO1 (12). Rather, we find an unexpected regulatory function of the catalytic site of HO2 beyond its enzymatic activity: the cellular protein level of HO2 is controlled by heme occupancy at the catalytic site. Under the condition of heme deficiency, heme is absent from HO2’s catalytic site, resulting in a lower protein level. This low-protein-level phenomenon is well-mimicked by an HO2 variant that cannot accommodate heme at the catalytic site by default. Our study reveals important insight into how this housekeeping HO is regulated to maintain heme homeostasis and adds one potential link in cellular response against heme deficiency.

**Results**

**HO2 stability and degradation is independent of heme binding to the HRMs**

Given that the most common role of HRMs is to promote protein degradation upon binding heme, we first asked whether the two HRMs in HO2 also control protein stability and degradation. Earlier, we showed that substituting Cys-265 or Cys-282 with Ala abolishes heme binding (light purple), weaker heme binding (blue and yellow), and tighter heme binding mutations (red). A, a representative Western blotting demonstrating steady-state expression levels of HO2 in HEK293 cells transiently transfected with transfection reagent only (Crl, control) or the corresponding expression vector for WT-HO2 (WT) and HRM mutants (C265A, C282A, or C265A/C282A) for 24 h. The samples were probed with anti-HO2 antibody (top panel) or anti-actin antibody as a loading control (bottom panel). C, the intensities of the bands in (B) were quantified by densitometry. Data represent mean ± S.D. (error bars) of n = 4 replicates for the Crl group and n = 6 replicates for all other groups. D, HEK293 cells transfected with WT-HO2 (top experiment) or the C265A/C282A mutant (bottom experiment) were treated with CHX (35 μg/ml) for the indicated time before collection. Cell lysates were probed by Western blotting with anti-HO2 antibody or anti-actin antibody. E, the intensities of the bands in (D) were quantified by densitometry, and the mean intensities ± S.D. (error bars) of three independent experiments were plotted. Data were fitted into a one-phase decay curve using GraphPad Prism 8 to calculate protein t1/2 for WT-HO2 (black) and C265A/C282A mutant (green), respectively.

Figure 1. Western blotting analysis indicates that lack of heme binding to HRMs does not affect HO2 protein stability and degradation. A, a diagram of HO2 sequence with all variants used in this article indicated as follows: HRM mutations (green); catalytic site mutations: loss of heme binding (light purple), weaker heme binding (blue and yellow), and tighter heme binding mutations (red). B, a representative Western blotting demonstrating steady-state expression levels of HO2 in HEK293 cells transiently transfected with transfection reagent only (Crl, control) or the corresponding expression vector for WT-HO2 (WT) and HRM mutants (C265A, C282A, or C265A/C282A) for 24 h. The samples were probed with anti-HO2 antibody (top panel) or anti-actin antibody as a loading control (bottom panel). C, the intensities of the bands in (B) were quantified by densitometry. Data represent mean ± S.D. (error bars) of n = 4 replicates for the Crl group and n = 6 replicates for all other groups. D, HEK293 cells transfected with WT-HO2 (top experiment) or the C265A/C282A mutant (bottom experiment) were treated with CHX (35 μg/ml) for the indicated time before collection. Cell lysates were probed by Western blotting with anti-HO2 antibody or anti-actin antibody. E, the intensities of the bands in (D) were quantified by densitometry, and the mean intensities ± S.D. (error bars) of three independent experiments were plotted. Data were fitted into a one-phase decay curve using GraphPad Prism 8 to calculate protein t1/2 for WT-HO2 (black) and C265A/C282A mutant (green), respectively.
respectively (15, 16). Therefore, we introduced mutations, i.e., C265A, C282A, and C265A/C282A, in the HRMs of HO2 to disrupt heme binding, anticipating that the mutations would lead to HO2 stabilization. The three variants and WT HO2 were constructed in a mammalian expression vector to dissect their properties under a cellular environment. HEK293 cells were transfected to overexpress the WT and variants of HO2, and their steady-state expression levels were studied. When probing HO2 protein levels with a rabbit polyclonal anti-HO2 antibody, we observed two bands corresponding to HO2 in cell extracts, with the higher molecular weight one assigned to full-length HO2 (Fig. 1B). The lower molecular weight band has been often observed with high cellular HO2 levels and may result from an N-terminal truncated species of HO2 (Fig. S7) (11, 14, 23). Because both bands correspond to HO2, they both have been taken into account when quantifying their intensities.

Extracts from nontransfected cells yielded very low levels of endogenous protein (Fig. 1B, Ctrl lane). In contrast to what has been observed with ALAS1 or Bach1, in which a loss-of-function mutation in the HRM leads to a higher cellular level of protein (19, 21), all three HRM variants of HO2 maintained a steady-state expression level very similar to that of WT protein (Fig. 1, B and C). Furthermore, we compared the \( t_{1/2} \) of the C265A/C282A variant to that of WT protein using a cycloheximide (CHX) chase assay (12, 24). In cells treated with CHX to repress protein synthesis, the HO2 protein level in total cell extracts decreased exponentially with increasing chase time from 0 to 24 h (Fig. 1, D and E), whereas in the control group treated with vehicle DMSO, HO2 protein levels were stable (Fig. S1). Fitting the data to a one-phase decay equation yielded a degradation rate of the C265A/C282A variant similar to that of the WT protein (Fig. 1E).

To ensure that the long hours of CHX treatment did not lead to cell death and compromise our conclusions, we analyzed cell viability at different time points post CHX treatment (Fig. S2). Over the entire time course of the experiment, the viable cell percentage remained at levels (>95%) similar to those at the beginning.

It is curious that HO2 protein degradation levels plateau at around 50%, a phenomenon that is not unique to HO2 (25, 26). We propose that, because HO2 possesses critical cytoprotective functions, an unknown factor(s) is induced to protect it from further degradation when a threshold low cellular concentration is reached. When fitting the degradation data to the one-phase decay curve, we defined the degradation span to the plateau instead of zero to ensure accurate half-life calculation. Together, these results suggest that heme binding to the HRMs does not significantly impact the stability or degradation rate of HO2.

**HO2 protein level decreases in response to lowered cellular heme concentration**

We next examined how HO2 protein level responds to changes in cellular heme concentration. We first quantified total cellular heme in HEK293 cells grown in a basal culture medium. With single cell volume estimated as \( 3 \times 10^{-12} \) liters (27), the cellular total heme concentration was calculated to be \( 3.16 \pm 0.03 \) \( \mu \)M. However, the regulatory heme pool, which has been reported to be around 20–340 \( \mu \)M (17, 28, 29), is far lower than total heme. Considering the affinity of the HRMs for heme (15), and the limited availability of the cellular regulatory heme pool (17), the HRMs may not be fully heme-replete under regular culture conditions.

Addition of the heme biogenesis inhibitor succinylacetone (SA) to the heme-depleted (HD) medium for 72 h depletes the regulatory heme pool (17) and decreases total cellular heme by
Heme binding at the catalytic site controls HO2 degradation

Nearly 25% (Fig. 2A). Supplementing with hemin after SA treatment replenishes cellular heme, and depending on the dose of hemin added, the cellular heme increases by up to 3-fold (Fig. 2A). Whereas the degradation of other HRM-containing proteins, such as ALAS1 or Bach1, are accelerated by hemin treatment (20, 21), we did not observe any effect of increasing the cellular heme content on HO2 expression (Fig. 2B). This result reinforces our conclusion that heme binding to the HRMs of HO2 does not regulate protein stability or degradation. Quite surprisingly, however, we observed a decrease in HO2 steady-state protein level in response to SA treatment, indicating that HO2 protein is destabilized under heme-deficient condition (Fig. 2, B and C). Therefore, we set out to understand how heme levels influence HO2 stability and postulated that lack of heme binding to the catalytic site, but not the HRMs, may be responsible for the observed destabilization of HO2.

Lack of heme occupancy at the catalytic site leads to HO2 degradation

Because of its high affinity for heme ($K_d \approx 3.6$ nM) (30), in a regular cellular environment where the regulatory heme pool is around 20–340 nM (31), the catalytic site of WT HO2 would most likely be heme-replete. To explore the role of heme binding to the catalytic site in regulating HO2 stability, we generated a catalytic site loss-of-function mutant, H45W/G159W, for expression in HEK293 cells. In vitro experiments have confirmed that in H45W/G159W variant, there is no detectible heme binding at the catalytic site and that the overall structure and heme binding to the HRMs are unperturbed (32). As expected, no changes were observed in steady-state expression levels of the H45W/G159W mutant upon cellular heme level variation (Fig. 3, A and B). Rather, it exhibited a consistently lower protein level when compared with its WT counterpart (Fig. 3E and Fig. S1A).

Because the H45W/G159W variant cannot bind heme, it is also necessarily catalytically inactive. Therefore, to dissect whether the enzymatic activity contributes to the regulation of HO2 stability, we introduced another HO2 variant, H45A, in which the catalytic core still accommodates heme but lacks catalytic activity (Fig. S4 and Table S1) (33). Similar to the WT protein, the H45A variant showed a decreased steady-state expression level when grown in the presence of SA, which lowers cellular heme level (Fig. 3, C and D). This result suggests that it is the heme occupancy at the catalytic site, not enzymatic activity, that controls HO2 stability.

To further validate that the heme-dependent HO2 destabilization is contributed by lack of heme binding to the catalytic site only, we probed steady-state HO2 levels when HRMs were present or absent (fulfilled with C265A/C282A) in H45A and H45W/G159W mutants. A decrease in steady-state HO2 protein level was only observed when the catalytic core was in the “apo” state, i.e. harboring H45W/G159W mutations, regardless of whether the HRMs were present or absent (Fig. 3E). To ensure that the observed lower steady-state level of the H45W/G159W variant indeed results from an enhanced protein degradation, its protein $t_{1/2}$ was measured. We found the $t_{1/2}$ of the H45W/G159W variant (1.5 h) to be $\sim 6$-fold shorter than that of WT HO2 (8.6 h) (Fig. 3, F and G). To better characterize this fast-degraded variant, we collected more data time points between 0 and 3 h post CHX treatment and observed a 12-fold decrease in the $t_{1/2}$ (~0.7 h) of the H45W/G159W variant (Fig. S5).

Even though a single point-mutation of H45A does not disturb the structure of the catalytic core (Fig. S3), it still eliminates a strong heme-iron axial ligand. Therefore, the majority of the heme accommodated by the H45A variant is only weakly associated with the protein (Fig. S4). To better distinguish the effect of heme binding versus heme metabolism on HO2 stability, we considered other variants in which heme affinity to the catalytic site is not as markedly decreased as H45A. We measured heme off-rates of these variants; heme off-rates are a reflection of heme binding affinity ($K_d$) because it has been shown by our group and others that heme on-rates of many hemoproteins, despite structural differences, are very similar (15, 30, 34). The G163D variant is catalytically inactive (Fig. S4 and Table S1) but, unlike the H45A variant, exhibits a very similar affinity for heme at the catalytic site as WT HO2 (Table S1).

The marked effect of heme occupancy at the catalytic site on HO2 stability also led us to explore whether increasing heme affinity of the catalytic site would further stabilize HO2 protein, leading to a higher steady-state HO2 level than WT. Therefore, the G163H variant, also catalytically inactive (Table S1), however with a 100-fold higher heme affinity of the catalytic site (32), was tested. Surprisingly, both the G163D and G163H variants showed similar steady-state protein levels as WT, and the G163H variant has a similar $t_{1/2}$ as WT HO2 (Fig. 4, A–D). Our results thus suggest that the WT protein, and even the H45A variant, already is sufficiently heme-replete in a regular cellular environment, so that no further stabilization can be achieved by increasing the heme affinity of the catalytic site. The similarity of the stability of G163H variant and WT HO2 also suggests that the cellular protein degradation machinery does not respond to the transient millisecond-to-second time-frame degradation and rebinding of heme at the active site during catalysis. Instead, it reacts to the slow global changes in the regulatory heme pool (Fig. 2B and Fig. 3C). We therefore postulate that the only time we would be able to observe a decrease in protein stability with G163H is when the regulatory heme pool drops into the pM level.

Immunofluorescence microscopy reveals that heme binding to the catalytic site or the HRMs does not affect HO2 subcellular localization

Both HO isoforms were reported to be residents of the ER as type II membrane proteins, anchored by their single C-terminal hydrophobic helix (11, 35). Because heme markedly affects HO2 stability, it is important to examine whether the introduced mutations or heme binding to the catalytic site or the HRMs altered HO2 subcellular localization. We attempted to visualize endogenous HO2; however, immunostaining using anti-HO2 antibody was insufficiently sensitive to detect HO2 when using the same exposure as for the overexpressed protein (Fig. 5, panel I). When using higher exposure, we observed foci among whole cells which likely reflects nonspecific binding of
the antibody (Fig. S6). Therefore, we used anti-FLAG antibodies to visualize FLAG-tagged HO2 expressed in HEK293 cells. Calnexin, an ER-integrated chaperone that assists in the proper folding and quality control of glycoproteins, was used as the ER marker (36, 37), whereas nuclei were shown by 4′,6-diamidino-2-phenylindole (DAPI) staining. To ensure that the N-terminal FLAG-tag does not alter HO2 properties, we analyzed the steady-state expression levels of a FLAG-tagged version of WT HO2 and the two heme binding site mutants, C265A/C282A (HRMs) and H45W/G159W (catalytic site). Their expression levels matched those of untagged HO2 (Fig. S7, A and B). Notably, only one band is observed by immunoblotting with anti-HO2 antibodies, and it corresponds to full-length HO2. We reason that because the untagged HO2 and FLAG-tagged HO2 are in different expression vectors (pcDNA3.1 and pQCXIP, respectively), this may affect how HO2 is processed in the cells. Indeed, the FLAG-tag itself does not affect HO2 properties, because when we put the FLAG-tagged HO2 into pcDNA3.1 vector, we observed the same expression pattern as untagged-HO2 in pcDNA3.1 (Fig. S7, C and D). Therefore, observation from using FLAG-tagged HO2 applies to untagged HO2 as well. WT HO2 colocalized with calnexin and also exhibited a

Figure 3. Binding of heme to the catalytic site stabilizes cellular HO2 protein. A and C, HEK293 cells cultured in HD media were transfected with HO2-H45W/G159W (A) or H45A (C), then treated with 0.5 mM SA for 48 h. Hemin (0, 10, or 50 μM) was then supplied to the cultures as indicated for 24 h before sample collection. Representative Western blots for samples probed with anti-HO2 antibody (top panel) or anti-actin antibody as a loading control (bottom panel) are shown. B and D, the intensities of the bands in (A) and (C) were quantified by densitometry and shown as the mean ± S.D. (error bars) of n = 3 replicates.

**p < 0.01; E, a representative Western blotting demonstrating steady-state expression levels of HO2 in HEK293 cells transiently transfected with the corresponding expression vector for WT HO2, catalytic site mutants (H45A or H45W/G159W), and catalytic site mutations combined with HRM mutations (H45A/C265A/C282A or H45W/G159W/C265A/C282A). The samples were probed with anti-HO2 antibody (top panel) or anti-actin antibody as a loading control (bottom panel). The intensities of the bands were quantified by densitometry. Data represent mean ± S.D. (error bars) of n = 3 independent experiments. *p < 0.05; **p < 0.01 versus lane 1. F, HEK293 cells transfected with H45W/G159W variant were treated with CHX (35 μg/ml) for the indicated time before collection. Cell lysates were probed by Western blotting with anti-HO2 antibody (top panel) or anti-actin antibody as a loading control (bottom panel). A representative Western blotting for WT HO2 degradation is shown in Fig. 1D. G, the intensities of the bands in (F) were quantified by densitometry, and the means ± S.D. (error bars) of n = 3 independent experiments were plotted. Data were fitted into a one-phase decay curve using GraphPad Prism 8 to calculate protein t1/2 for H45W/G159W (light gray) compared with WT-HO2 (block), as shown in Fig. 1, D and E.
Heme binding at the catalytic site controls HO2 degradation

Figure 4. Tighter heme binding to the catalytic site does not further stabilize HO2 protein. A, a representative Western blotting demonstrating steady-state expression levels of HO2 in HEK293 cells transiently transfected with transfection reagent only (Crl, control) or the corresponding expression vector for WT HO2 and catalytic core mutants (G163D or G163H). The samples were probed with anti-HO2 antibody (top panel) or anti-actin antibody as a loading control (bottom panel). B, the intensities of the bands in (A) were quantified by densitometry. Data represent mean ± S.D. (error bars) of n = 3 independent experiments. ***p < 0.001 versus WT; n.s. (not significant), p > 0.05 versus WT. C, HEK293 cells transfected with the tighter binding mutant G163H were treated with CHX (35 μg/ml) for the indicated time before collection. Cell lysates were probed by Western blotting with anti-HO2 antibody (top panel) or anti-actin antibody as a loading control (bottom panel). A representative Western blotting for WT HO2 degradation has been shown in Fig. 1D. D, the intensities of the bands in (C) were quantified by densitometry, and the mean intensities ± S.D. (error bars) of n = 3 independent experiments were plotted. Data were fitted into a one-phase decay curve using GraphPad Prism 8 to calculate protein $t_{1/2}$ for G163H (red), which is compared with WT-HO2 (black), as shown in Fig. 1, D and E.

Figure 5. HO2 and its variants localize to the ER. HEK293 cells cultured on coverslips were transfected with transfection reagent only (Endogenous HO2) or with corresponding expression vectors for FLAG-tagged HO2 and its variants as indicated. Endogenous HO2 was stained with anti-HO2 antibody, and FLAG-tagged HO2 was probed with anti-FLAG antibody. Anti-calnexin antibody was used to visualize calnexin as a marker for ER, and DAPI was used to show the position of nuclei. Merge is the merged images of calnexin channel and HO2 channel. All images are in the same scale as indicated with the scale bar.
clear pattern surrounding the nuclei (Fig. 5, panel 2). Loss of heme binding at either the HRMs or the catalytic site did not alter HO2's subcellular localization, suggesting that association of HO2 with the ER is heme-independent (Fig. 5, panels 3 and 4). The results also validate that the mutations introduced in the catalytic site and that HRMs did not alter HO2's association with the ER membrane.

**Unlike HO1, HO2 is not a substrate of the ERAD pathway**

HO1 has been demonstrated to be an ERAD substrate, tagged by poly-ubiquitin and retro-translocated to the proteasome for degradation (12). Based on the high similarity of HO1 and HO2 in structure, catalytic activity, and localization, we predicted that HO2 is also degraded through the ubiquitin-proteasome pathway. However, in the presence or absence of CHX, incubation with the proteasome inhibitor MG-132 did not increase levels of either endogenous (Fig. S8) or transiently overexpressed HO2 (Fig. 6, A and B). Furthermore, FLAG-HO2 was immunoprecipitated from HEK293 cell extracts treated with or without MG-132, then detected with a poly-ubiquitin specific antibody. In the whole cell lysate, accumulation of poly-ubiquitin was observed upon MG-132 treatment, indicating an effective blockage of proteasomal degradation (Fig. 6C, left). In contrast to the whole cell lysate, ubiquitinated HO2 and overall poly-ubiquitin accumulation were not detected in the immunoprecipitants, nor did we observe FLAG-HO2 accumulation with MG-132 treatment (Fig. 6C, right). These combined results indicate that the ubiquitin-proteasome machinery is not required for HO2 degradation, making HO2 distinct from other HRM-containing proteins and HO1 in their degradation pathways (18, 21, 24, 38).

**HO2 is degraded by lysosomes through chaperone-mediated autophagy**

Another major protein degradation pathway in mammals is the autophagy-lysosomal pathway. Therefore, we assessed...
cellular HO2 level in the presence and absence of CHX and each of three different lysosomal inhibitors, bafilomycin (BAF), chloroquine (CQ), and NH4Cl. Each inhibitor significantly attenuated HO2 degradation (Fig. 7A), strongly suggesting that HO2 is degraded by the lysosome.

We next asked whether heme binding to the catalytic site or to the HRMs affects the HO2 degradation pathway. HEK293 cells expressing C265A/C282A or H45W/G159W variants were subject to the protein degradation assay with or without the aforementioned three lysosomal inhibitors. Each inhibitor blocked lysosomal degradation, leading to protein accumulation of both variants (Fig. 7, B and C). These results indicate that heme binding to the catalytic site controls the rate of HO2 protein degradation but not the degradation pathway.

The three lysosome inhibitors described above repress both macroautophagy- and chaperone-mediated autophagy (CMA)-mediated lysosomal degradation. Therefore, to further assess which mode of lysosomal degradation HO2 is subject to, we tested steady-state levels of HO2 in the presence of 3-methyladenine (3-MA), an inhibitor that only blocks macroautophagy (39), or 6-aminonicotinamide (6-AN), which specifically activates CMA (40). The addition of 3-MA did not affect the steady-state level of WT HO2, whereas treatment with 6-AN caused a significant decrease in the HO2 level (Fig. 8, A and B). Thus, we conclude that CMA mediates HO2 protein degradation.

Discussion

It has been unclear how HO2 is regulated to maintain cellular heme homeostasis. In this study, we investigated how the cellular protein level of HO2 is controlled post-translationally by

Figure 7. HO2 degradation is blocked by lysosomal inhibitors. A–C, HEK293 cells transfected with expression vector for WT-HO2 (A), C265A/C282A mutant (B), or H45W/G159W mutant (C) treated with or without CHX (35 μg/ml) in combination with or without BAF (10 nM), CQ (50 μM), or NH4Cl (10 mM) as indicated for 12 h before collection. HO2 protein levels were quantified by Western blotting analysis and densitometry. Representative Western blots of WT, C265A/C282A, and H45W/G159W are shown in (A, left panel), (B, upper panel), and (C, upper panel). Data represent mean ± S.D. (error bars) of n = 3 independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 8. HO2 is degraded by CMA. A, HEK293 cells expressing FLAG-HO2-WT were treated with 5 mM 3-MA for 12 h or 50 mM 6-AN for 24 h before collection. A representative Western blotting of samples probed with anti-HO2 antibody (top panel) or anti-actin antibody as a loading control (bottom panel) is shown. B, HO2 protein levels were quantified by densitometry. Data represent mean ± S.D. (error bars) of n = 3 independent experiments for ± 3-MA groups and n = 6 for ± 6-AN groups. ***p < 0.001.
Heme binding at the catalytic site controls HO2 degradation

The possession of two additional heme binding sites at its HRMs is the most intriguing feature that distinguishes HO2 from HO1. The most commonly reported HRM function is to promote target protein ubiquitination and proteasomal degradation upon heme binding (18). However, as shown here, heme binding to the HRMs of HO2 does not affect either its steady-state expression level or its protein degradation rate. Furthermore, addition of excess cellular heme does not promote HO2 degradation. Although these properties contrast with those of other HRM-containing proteins, they are consistent with HO2’s role as a heme detoxification enzyme; it would be counterproductive for heme to reduce HO2 level and hence heme metabolic activity. In light of HRM’s function in HO2, we recently proposed a heme transfer model in which HO2 readily transfers HRM-bound Fe3+-heme to the catalytic site for degradation to facilitate turnover and equilibrates heme between the HRMs and the catalytic core to maintain heme homeostasis through heme-dependent conformational changes in the protein (32). This model defines a novel function for the HRM while maintaining consistency with a conformational change model proposed for another HRM-containing protein, Bach1, in which heme induces local conformational changes that alter interactions with its binding partners (41). It also integrates the thermodynamic properties of these three heme binding sites in HO2, with the catalytic site exhibiting the highest affinity for heme and the HRMs binding it more weakly (15).

The most surprising finding described here, based on our characterization of several catalytic site variants of HO2 (Fig. 1A), is a previously unrecognized regulatory function of the active site beyond catalysis. Based on our comparison of the steady-state expression levels and degradation rates of these variants versus the WT protein, we propose that, when heme is absent from the catalytic site, likely under the condition of heme deficiency, HO2 is destabilized and more subject to degradation (Fig. 3). This proposal agrees with HO2’s function in maintenance of heme homeostasis, because when the cellular heme concentration decreases below its normal physiological level, less heme oxygenase activity is required. Consistently, it has been described that in neuronal cells where HO2 is highly abundant, heme deficiency leads to undesired iron accumulation (42), which can potentially be attenuated by decreasing cellular HO2 level and activity. Given that HO1 is upregulated in response to excess cellular heme, we propose that HO2 helps to restore heme homeostasis by decreasing its own protein level in response to cellular heme deficiency. These proposals offer a rationale for why our cells express two HO isoforms: HO1, whose expression levels are highly responsive to excess heme, reacts to cellular injury and stress, whereas HO2, though possessing similar catalytic activity as HO1, exhibits a dual role of heme degradation (under physiological conditions) and of restoring regulatory heme pool (under conditions of heme deficiency). A similar situation where two heme oxygenase isoforms differentially regulated by heme has been observed in the Isd heme-iron acquisition system of Staphylococcus aureus (43). The Isd system contains two heme oxygenases, IsdI and IsdG, which share high structural and sequence homology and possess similar catalytic activities; however, they are differentially regulated by heme and iron (44–46). Whereas the stability and degradation of IsdI is not affected by heme, IsdG is destabilized when heme is absent from the catalytic site (44); furthermore, a catalytically inactive mutant of IsdG is as stable as the WT protein in the presence of heme (43), similar to what we describe here for WT HO2 and the inactive H45A variant.

It is very interesting that it is the absence of heme binding to the active site (mimicked by H45W/G159W), not the absence of catalytic activity (mimicked by H45A), that triggers HO2 protein degradation. This finding clearly shows that the degradation signal is not triggered by changes in the levels of the products (CO, Fe, biliverdin) of heme degradation. Our recent hydrogen-deuterium exchange MS (HDX-MS) studies demonstrated that in the H45W/G159W variant, the tryptophan mutations themselves led to minimal to no structural perturbations and that the only observed differences in HDX-MS related to heme binding to the catalytic core were specific to the distal and proximal helices, which form the heme binding pocket (47). Furthermore, the CD spectra of WT HO2 and the H45W/G159W variant were nearly indistinguishable (32). In a search for potential signals for HO2 degradation in the absence of heme, we identified a KFERQ-like motif, which has been classified as a recognition motif by CMA. In the process of CMA, the KFERQ-like motif in the target protein is exposed and bound by the heat shock cognate protein of 70 kDa (HSC70), followed by unfolding and translocation of the protein substrate across the lysosomal membrane by lysosome-associated membrane protein type 2A (LAMP 2A) (48). In HO2, one KFERQ-like motif, 52QFVKD57, absent in HO1, is found at the proximal helix, which harbors the heme ligand His-45 at the HO2 catalytic site. According to our HDX-MS analysis, compared with an apo active site, decreased deuterium incorporation at the proximal helix was observed when heme was bound at the catalytic site (47). This heme-dependent protection of the KFERQ-like motif could explain how the apo active site of HO2 is differentially recognized by the lysosomal degradation machinery, leading to more rapid protein degradation. Another potential signal for HO2 lysosomal degradation is a di-leucine motif (32DLSELL37), which usually serves as a sorting sequence for target protein delivery to the lysosomes (49). This di-leucine motif, not conserved in HO1, also locates at the proximal helix and undergoes similar heme-dependent protection as the aforementioned KFERQ-like motif. Future studies of concrete functions of the di-leucine motif and KFERQ-like motif in HO2 degradation will be important.

It is also intriguing that HO1 and HO2 are targeted by different protein degradation machinery. CMA has recently been shown to be involved in the execution of ferroptosis, a necrosis related to iron overload (50). It is possible that defects in heme biosynthesis that lead to cellular iron overload induce ferroptosis (42). Therefore, CMA is already activated when a rapid HO2 degradation is required, providing a convenient pathway. Future study of understanding whether heme deficiency indeed activates CMA and therefore enhances HO2 degradation will be necessary and valuable.

Even though stabilization of an enzyme by substrate binding is not an uncommon feature, in the case of HO2 it is surprising because the stabilization occurs at concentrations two orders of magnitude lower than the Michaelis constant (K_m). Because
HO2 degradation is modulated only by heme binding but not by heme metabolism at the catalytic site, it is worth considering the relative $K_m$ (0.40 μM) (51) and $K_d$ values for heme (3.6 nM for the catalytic site) (30) in relation to the total (~3.2 μM) and regulatory (20–340 nM) heme levels found in mammalian cells (31). The agreement of the $K_d$ value with the regulatory heme pool suggests that the degradation signal of HO2 is related to changes in the regulatory, not the total, heme pool. Consistently, HO2 overexpression in mammalian cells does not alter total heme level but only shifts the regulatory heme pool.3 Thus, degradation of the housekeeping heme detoxification enzyme HO2 is likely to be linked to the larger network of regulatory heme trafficking within the cell. The detailed mechanism of how this heme-dependent HO2 degradation helps to restore cellular heme homeostasis under heme deficiency is open for investigation.

**Experimental procedures**

**Materials**

Cycloheximide (CHX), MG-132, bafilomycin (BAF), 3-methyladenine (3-MA), 6-ammoniocotinamide (6-AN), and poly-lysine were purchased from Sigma-Aldrich. Chloroquine and NH4Cl were from Thermo Fisher Scientific. FBS, DMEM, Opti-MEM, Lipofectamine LTX and PLUS reagent, and a transfection reagent with DAPI were from Invitrogen. A rabbit polyclonal heme oxygenase 2 antibody (ab90515) was mounting reagent with DAPI were from Invitrogen. A mouse monoclonal M2-FLAG antibody polyclonal actin antibody (A2066), and a rabbit polyclonal calnexin antibody (MA3-027) was from Invitrogen. A mouse monoclonal ubiquitin antibody (P4D1) (sc-8017) was from Santa Cruz Biotechnology. An anti-FLAG M2 magnetic bead (M8823) was purchased from Sigma-Aldrich. Goat anti-mouse-Alexa 568 antibody and goat anti-rabbit-Alexa 488 antibody (Molecular Probes) were generously provided by Dr. Phyllis I. Hanson (University of Michigan).

**Plasmid constructs and mutagenesis**

Full-length human HO2 cDNA was subcloned into a pcDNA3.1 (+) vector using Hind III and Xho I restriction sites (14). The FLAG-tagged WT-HO2 construct was generously provided by Dr. Stephen P. Goff (Columbia University). HO2 point mutations were generated by either site-directed mutagenesis with the Quick-Change protocol (Agilent Technologies) or the Q5 site-directed mutagenesis kit (NEB E0554S).

**Cell culture conditions**

HEK293 cells were obtained from ATCC and cultured in basal media (1× DMEM containing 10% FBS (v/v) and 1× penicillin-streptomycin). The cells were reseeded at 24–48 h prior to transfection (with lipofectamine LTX and PLUS reagent in Opti-MEM) and various treatments. For transient transfection experiments, HEK293 cells were transfected with plasmids expressing HO2 or its variants using the Lipofectamine LTX transfection reagent according to the manufacturer’s instructions. After 24 h, the cells were subjected to various treatments as indicated (24).

**Protein degradation assay and degradation pathway assignment**

The protein stability and degradation rate was determined by the CHX degradation assay, as described before with slight modifications (24). Briefly, for the protein $t_{1/2}$ assessment, HEK293 cells were transfected with different constructs for expressing HO2 and its variants. 24 h after the transfection, the cells were treated with 35 μg/ml of CHX for the indicated time before collection. For degradation pathway assignment, 24 h after transfection, the cells were treated with indicated inhibitors (MG-132 at 20 μM, BAF at 10 nM, chloroquine at 50 μM, or NH4Cl at 10 mM) for 12 h before harvesting (12). For studying the involvement of different types of autophagy in HO2 degradation, HEK293 cells were transfected to express WT-HO2. 24 h after transfection, the cells were treated with 3-MA or vehicle media for 12 h before harvesting to probe the effect of macroautophagy on HO2 degradation. For testing chaperone-mediated autophagy, the cells were treated with 6-AN or vehicle DMSO for 24 h before collection (52). Cell pellets were lysed with RIPA buffer (Thermo Fisher Scientific) containing 2× protease inhibitor mixture (Roche) and centrifuged at 12,000 rpm for 10 min at 4°C to get rid of cell debris. The supernatant was extracted to a clean tube and protein concentrations were determined by bicinchoninic acid protein assay (Pierce BCA protein assay kit). The samples were analyzed by Western blotting.

**Protein assays, SDS-PAGE, and Western blotting analysis**

All protein concentrations were analyzed by the BCA assay before being further processed. The same amount of total protein from each sample was loaded onto the SDS gel. SDS-PAGE was performed with Laemmli buffers (53) and 4–20% gradient gels (Bio-Rad), and protein bands were visualized with Coomassie Brilliant Blue (Bio-Rad) solution or transferred to PVDF membranes (Bio-Rad). Transfer was performed in transfer buffer (25 mM Tris, 192 mM glycine, and 0.025% SDS (w/v)) at 4°C, 90 V for 1.5 h. The membranes were blocked in 5% (w/v) non-fat milk, 0.1% Tween 20 (v/v) prior to incubation with primary antibodies overnight at 4°C. All primary antibodies were diluted in TBST containing 0.1% sodium azide (w/v) and 0.1% Tween 20 (v/v) prior to incubation with primary antibodies. After removal of the primary antibody, the membranes were washed with 1× TBST for 5× 5 min and incubated with HRP-conjugated secondary antibodies against the species that primary antibodies were produced in. All secondary antibody was diluted with 5% (w/v) blotting-grade blocker in TBST with a dilution range from 1:50,000 to 1:30,000. Membranes were washed for 5× 5 min with TBST again before being developed with SuperSignal West Femto enhanced chemiluminescent substrate for HRP

3 Personal communication with Professor Amit R. Reddi.
Heme binding at the catalytic site controls HO2 degradation

Protein expression, purification, and analysis

A truncated, soluble form of HO2 spanning residues 1–248 (the core of the protein) was expressed from the pET28a vector in BL21(DE) cells (Life Technologies) and purified by nickel-nitrioltriacetic acid–agarose affinity chromatography (Qiagen) as described previously (15). The N-terminal six-His tag was removed by treatment with thrombin prior to use (15). The H64Y/V68F myoglobin was purified as described previously (30 against 50 mM Tris buffer, pH 8.0/50 mM KCl prior to use, and for the WT protein. All proteins were extensively dialyzed (Agilent Technologies). All variants were purified as described by site-directed mutagenesis with the QuikChange protocol to introduce the mutation, and the H45A variant was constructed Q5 site-directed mutagenesis kit (New England Biolabs) to introduce the mutation, and the H45A variant was constructed by site-directed mutagenesis with the QuikChange protocol (Agilent Technologies). All variants were purified as described for the WT protein. All proteins were extensively dialyzed against 50 mM Tris buffer, pH 8.0/50 mM KCl prior to use, and protein concentrations were determined by Bradford assay.

A heme stock was freshly prepared in 50 mM Tris buffer, pH 8.0/50 mM KCl with 15% DMSO (v/v) and 0.1 M NaOH. The stock was passed through a 0.22-μm filter to remove insoluble matter, and the concentration was determined by using an ε385 of 58.4 mM⁻¹ cm⁻¹ (54). WT HO2 and variants (3 μM) were incubated with 2.5 μM heme for 1 h, and then absorbance spectra were measured at 20 °C in 50 mM Tris buffer, pH 8.0/50 mM KCl on a Shimadzu UV-2600 UV–vis spectrophotometer. The heme-bound proteins were subsequently mixed with apo-H64Y/V68F myoglobin (30 μM), and the absorbance at 600 nm over time was recorded. Rates were determined by fits of the data to a double exponential equation using GraphPad Prism 8. H64Y/V68F-myoglobin (green heme) was purified and converted to the apo-form as described previously (30) using an expression vector generously supplied by J. Olson (Rice University, Houston, TX).

Steady-state activity of HO2 was measured as described previously (55) with minor modifications. Briefly, a 200-μl reaction containing 0.1 μM HO2, 15 μM heme, 1 μM biliverdin reductase, 0.25 mg/ml BSA, 10 units of catalase, and 10 μM cytochrome P450 reductase in 50 mM Tris, pH 8.0, and 50 mM KCl was incubated for 2 min at 37 °C. The reaction was initiated by the addition of 1 μl of 100 mM NADPH. Activity was monitored by following the increase in absorbance at 468 nm because of bilirubin formation. Using a difference extinction coefficient of 43.5 mM⁻¹ cm⁻¹, activity was calculated as nmol bilirubin formed/min/mg HO2. Cytochrome P450 reductase and biliverdin reductase were purified as described previously (55).

CD spectroscopy

CD spectra of the HO2 (1–248) protein samples were acquired in phosphate buffer (50 mM potassium phosphate, pH 7.4) at a concentration of 5 μM on a J-1500 CD Spectrometer (JASCO). Each spectrum was acquired with five scans in a cuvette with a path length of 0.1 cm. Data were analyzed using DichroWeb server and secondary structures were estimated by fitting data with CONTIN database.

HO2 immunoprecipitation

HEK293 cells were transfected to express FLAG-tagged HO2 or its variants for 24 h before harvesting for immunoprecipitation assay (56). The cells were lysed in Celllytic M Cell Lysis Reagent (Sigma-Aldrich, C2978) on ice for 20 min with mild agitation. The lysate was centrifuged for 12 min at 12,000 rpm and 4 °C to clear out cell debris. Immunoprecipitation was performed as the manufacturer instructed. Briefly, the supernatant was mixed with Anti-FLAG M2 magnetic bead (M8823) and the mixture was incubated at 4 °C for 4 h. After removing the flow-through, the magnetic bead was washed with lysis reagent once and TBST twice, 5 min for each wash at 4 °C. Protein complexes were eluted in SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), and 0.004% bromophenol blue (w/v)) with boiling for 10 min. Eluted samples were subjected to electrophoresis and Western blotting detection.

Cellular heme concentration modulation

Modulation of intracellular heme concentration was performed as described before (17) with modifications. Briefly, the cells cultured with regular media were collected from confluent master plates and reseeded into 35-mm dishes with 1:10 dilution in fresh basal media on day 0. On day 2, the media were replaced with HD media (1X DMEM + 10% HD FBS (v/v)) and the cells were transfected with 1 μg of plasmids for expressing HO2 or its variants in Opti-MEM with 0.5 mM SA. On day 4, the media were replaced with fresh HD media containing 0.5 mM SA and supplemented with 0, 10, or 50 μM hemin. After 24 h of media exchange, the cells were harvested for further analysis.

Oxalic acid assay for total heme content

After modulating intracellular heme concentration, the heme level in soluble cell extracts was determined by iron extraction with a supersaturated oxalic acid solution and measurement of the resulting protoporphyrin fluorescence as described previously (24, 57) with slight modifications. Briefly, the cells were lysed in Celllytic M Cell Lysis Reagent (Sigma-Aldrich, C2978) containing 2X protease inhibitor mixture (Roche); protein concentrations were determined by BCA assay. Cell extracts were diluted at 1:20 in supersaturated oxalic acid in sealed glass cryo vials, mixed, and then heated at 121 °C for 30 min in an autoclave. A parallel set of samples was left at room temperature. The heated samples were cooled down and transferred to a black 96-well plate. The fluorescence emission spectrum of protoporphyrin was recorded from 550–700 nm with excitation at 400 nm in a Tecan Safire microplate reader. A standard curve was generated with hemin prepared in DMSO. Heme concentration (nmol/mg protein) was quantified after subtraction of background emission from nonheme protoporphyrin in the unheated samples from the intensity of the heated samples at 662 nm.
Flow cytometry

HEK293 cells were treated with CHX (35 μg/ml) for the desired time and harvested for analysis as described under “Protein degradation assay and degradation pathway assignment.” The cells were stained with Live-or-Dye NucFix™ Red reagent (Biotium, 32010) and fixed with paraformaldehyde following manufacturer’s instruction. Flow cytometric measurements were performed using a Beckman Coulter MoFlo Astrios Cell Sorter. NucFix Red was excited with a laser at 488 nm and measured using a 576/21-nm bandpass filter. Data evaluation was conducted using DeNovo Software’s FCS Express Flow Cytometry Analysis software. The number of cells measured per experiment was set to 10,000. Fixed cells that do not contain NucFix Red were used as a negative control for fluorescence.

Immunofluorescence microscopy

HEK293 cells were cultured on poly-lysine–coated coverslips for 24 h and transfected with plasmids expressing FLAG-tagged H2O2 and its variants. 24 h after transfection, the cells were washed with PBS twice and fixed with 4% paraformaldehyde (w/v) for 10 min at room temperature. Immunofluorescence samples were then washed with PBS twice more before being permeabilized with 1× PBS containing 0.2% Triton X-100 (v/v) for 10 min and then washed with PBS containing 0.1% (v/v) Tween 20 (PBST) for 3× 5 min. Permeabilized samples were transferred to a new 12-well plate and blocked with blocking buffer (PBST + 3% BSA (w/v)) for 1 h. The coverslips were washed thoroughly with PBST for 3× 5 min and incubated with primary antibody diluted in PBST containing 1% BSA (w/v) for 1 h. After removing primary antibody, the coverslips were washed again with PBST for 3× 5 min and incubated with secondary antibody for 1 h. The coverslips were washed with PBST before being mounted onto microscope slides. Sample images were acquired on an Olympus IX81 microscope with a Yokogawa CSU-W1 spinning disk confocal scanner unit, an Olympus PlanApo 60 × 1.4 NA objective, and a Hamamatsu ImagEMX2-1K EM-CCD digital camera. DAPI and secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568 were excited with 405-nm (Omicron), 488-nm (Omicron), and 561-nm (Coherent) solid-state lasers paired with Chroma ET460/50m, ET525/50m, and ET605/52m emission filters, 561-nm (Coherent) solid-state lasers paired with Chroma ET460/50m, ET525/50m, and ET605/52m emission filters, respectively. Images were acquired with MetaMorph 7.10.2.240 and prepared in FIJI software. Primary antibodies were applied as follows: HO2 (1:200), calnexin (1:200), and FLAG (1:100). Secondary antibodies were applied as follows: Goat anti mouse-Alexa 568 (1:1,000) and Goat anti rabbit-Alexa 488 (1:1,000). The mounting reagent contains DAPI.

Statistical analysis

Data values of at least three independent experiments were analyzed by a two-tailed unpaired student’s t test. For comparison among more than two groups, one-way analysis of variance test was used. Significance was established at p < 0.05. Error bars represent ± S.D.

Data availability

All data are contained within this article or in the supporting information.

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Abbreviations—The abbreviations used are: 6-AN, 6-aminonicotinamide; ALAS1, d-aminolevulinic acid synthase 1; BAF, bafilomycin; CHX, cycloheximide; CMA, chaperone-mediated autophagy; ERAD, endoplasmic reticulum-associated degradation; HD, heme-depleted; HDX-MS, hydrogen-deuterium exchange mass spectrometry; HO, heme oxygenase; HRM, heme regulatory motif 3-MA, 3-methyladenine; SA, succinylacetone; ER, endoplasmic reticulum; DAPI, 4,6-diamidino-2-phenylindole; TBST, Tris-buffered saline with Tween; PBST, PBS containing 0.1% Tween 20.

References
1. Ayer, A., Zariou, A., Agarwal, A., and Stocker, R. (2016) Heme oxygenases in cardiovascular health and disease. Physiol. Rev. 96, 1449–1508 CrossRef Medline
2. Sawicki, K. T., Chang, H. C., and Ardehali, H. (2015) Role of heme in cardiovascular physiology and disease. J. Am. Heart Assoc. 4, e001138 CrossRef Medline
3. Olmez, I., and Ozyurt, H. (2012) Reactive oxygen species and ischemic cerebrovascular disease. Neurochem. Int. 60, 208–212 CrossRef Medline
4. Maines, M. D. (1997) The heme oxygenase system: A regulator of second messenger gases. Annu. Rev. Pharmacol. Toxicol. 37, 517–554 CrossRef Medline
5. Maines, M. D. (2005) The heme oxygenase system: Update 2005. Antioxid. Redox Signal. 7, 1761–1766 CrossRef Medline
6. Adler, V., Yin, Z. M., Tew, K. D., and Ronai, Z. (1999) Role of redox potential and reactive oxygen species in stress signaling. Oncogene 18, 6104–6111 CrossRef Medline
22. Yamanaka, K., Ishikawa, H., Megumi, Y., Tokunaga, F., Kanie, M., Rouault, T. A., Morishima, I., Minato, N., Ishimori, K., and Iwai, K. (2003) Identification of the ubiquitin-protein ligase that recognizes oxidized IRP2. J. Biol. Chem. 278, 37624–37631. CrossRef Medline

23. Linnenbaum, M., Busker, M., Kraehling, J. R., and Behrends, S. (2012) Heme oxygenase-2. J. Biol. Chem. 287, 1783–1793. CrossRef Medline

24. Carter, E. L., Gupta, N., and Ragsdale, S. W. (2016) High affinity heme binding to a heme regulatory motif on the nuclear receptor Rev-Erb B leads to its degradation and indirectly regulates its interaction with nuclear receptor corepressor. J. Biol. Chem. 291, 2196–2222. CrossRef Medline

25. Guha, A., Ahuja, D., Das Mandal, S., Parasar, B., Deysi, K., Roy, D., Sharma, V., Willard, B., Ghosh, A., and Ray, P. S. (2019) Integrated regulation of hmr by translation repression and protein degradation determines pulsatile expression of p53 under DNA damage. iScience 15, 342–359. CrossRef Medline

26. Huang, E. Y., To, M., Tran, E., Dionisio, L. T. A., Cho, H. J., Baney, K. L. M., Pataki, C. I., and Oltzmann, J. A. (2018) A VCP inhibitor substrate trapping approach (VISTA) enables proteomic profiling of endogenous ERAD substrates. Mol. Biol. Cell 29, 1021–1030. CrossRef Medline

27. Mateus, A., Matsson, P., and Artursson, P. (2013) Rapid measurement of intracellular unbound drug concentrations. Mol. Pharm. 10, 2467–2478. CrossRef Medline

28. Song, Y. Q., Yang, M. Y., Wegner, S. V., Zhao, J. Y., Zhu, R. F., Wu, Y., He, C., and Chen, P. R. (2015) A genetically encoded FRET sensor for intracellular heme. ACS Chem. Biol. 10, 1610–1615. CrossRef Medline

29. Atamna, H., Brahambhatt, M., Atamna, W., Shanower, G. A., and Dhahbi, J. M. (2015) ApoHRP-based assay to measure intracellular regulatory heme. Metallomics 7, 309–321. CrossRef Medline

30. Carter, E. L., Ramirez, Y., and Ragsdale, S. W. (2017) The heme-regulatory motif of nuclear receptor Rev-ErbB is a key mediator of heme and redox signaling in circadian rhythm maintenance and metabolism. J. Biol. Chem. 292, 11280–11299. CrossRef Medline

31. Donegan, R. K., Moore, C. M., Hanna, D. A., and Reddi, A. R. (2016) Handling heme: The mechanisms underlying the movement of heme within and between cells. Free Radic. Biol. Med. 133, 88–100. CrossRef Medline

32. Fleischhacker, A. S., Gunawan, A. L., Kochert, B. A., Liu, L., Wales, T. E., Borowy, M. C., Engen, J. R., and Ragsdale, S. W. (2020) The heme regulatory motifs of heme oxygenase-2 contribute to the transfer of heme to the catalytic site for degradation. J. Biol. Chem. 295, 5177–5191. CrossRef Medline

33. Ishikawa, K., Matera, K. M., Zhou, H., Fuji, H., Sato, M., Yoshimura, T., Ikeda-Saito, M., and Yoshida, T. (1998) Identification of histidine 45 as the axial heme iron ligand of heme oxygenase-2. J. Biol. Chem. 273, 4317–4322. CrossRef Medline

34. Owens, C. P., Du, J., Dawson, J. H., and Goulding, C. W. (2012) Characterization of heme ligation properties of Rv0203, a secreted heme binding protein involved in Mycobacterium tuberculosis heme uptake. Biochemistry 51, 1518–1531. CrossRef Medline

35. Shibahara, S., Müller, R., Taguchi, H., and Yoshida, T. (1985) Cloning and expression of cdna for rat heme oxygenase. Proc. Natl. Acad. Sci. U. S. A. 82, 7865–7869. CrossRef Medline

36. de la Vega, M., Burrows, J. F., McFarlane, C., Govender, U., Scott, C. J., and Reddi, A. R. (2016) Heme oxygenase-2. J. Biol. Chem. 291, 1531–1536. CrossRef Medline

37. Ou, W. J., Cameron, P. H., Thomas, D. Y., and Bergeron, J. J. M. (1993) Association of folding intermediates of glycopolypeptides with calnexin during protein maturation. Nature 364, 771–776. CrossRef Medline

38. Shen, J., Sheng, X. P., Chang, Z. N., Wu, Q., Wang, S., Xuan, Z. L., Li, D., Wu, Y. L., Shang, Y. J., Kong, X. T., Yu, L., Li, L., Ruan, K. C., Hu, H. Y., Huang, Y., et al. (2014) Iron metabolism regulates p53 signaling through direct heme-p53 interaction and modulation of p53 localization, stability, and function. Cell Rep. 7, 180–193. CrossRef Medline

39. Seglin, P. O., and Gordon, P. B. (1982) 3-methyladenine: specific inhibitor of autophagic lysosomal protein degradation in isolated rat hepatocytes. Proc. Natl. Acad. Sci. U. S. A. 79, 1889–1892. CrossRef Medline

40. Finn, P. E., Mesieres, N. T., Vine, M., and Dice, J. F. (2005) Effects of small molecules on chaperone-mediated autophagy. Autophagy 1, 141–145. CrossRef Medline

41. Hira, S., Tomita, T., Matsui, T., Igarashi, K., and Ikeda-Saito, M. (2007) Bach1, a heme-dependent transcription factor, reveals presence of multiple heme binding sites with distinct coordination structure. IUBMB Life 59, 542–551. CrossRef Medline
42. Atamna, H., Killilea, D. W., Killilea, A. N., and Ames, B. N. (2002) Heme deficiency may be a factor in the mitochondrial and neuronal decay of aging. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 14807–14812 CrossRef Medline
43. Reniere, M. L., Haley, K. P., and Skaar, E. P. (2011) The flexible loop of *Staphylococcus aureus* IsdG is required for its degradation in the absence of heme. *Biochemistry* **50**, 6730–6737 CrossRef Medline
44. Reniere, M. L., and Skaar, E. P. (2008) *Staphylococcus aureus* haem oxygenases are differentially regulated by iron and haem. *Mol. Microbiol.* **69**, 1304–1315 CrossRef Medline
45. Skaar, E. P., Gaspar, A. H., and Schneewind, O. (2004) IsdG and IsdI, heme-degrading enzymes in the cytoplasm of *Staphylococcus aureus*. *J. Biol. Chem.* **279**, 436–443 CrossRef Medline
46. Lee, W. C., Reniere, M. L., Skaar, E. P., and Murphy, M. E. P. (2008) Rufiling of metalloporphyrins bound to IsdG and IsdI, two heme-degrading enzymes in *Staphylococcus aureus*. *J. Biol. Chem.* **283**, 30957–30963 CrossRef Medline
47. Kochert, B. A., Fleischhacker, A. S., Wales, T. E., Becker, D. F., Engen, J. R., and Ragsdale, S. W. (2019) Dynamic and structural differences between heme oxygenase-1 and-2 are due to differences in their c-terminal regions. *J. Biol. Chem.* **294**, 8259–8272 CrossRef Medline
48. Vembar, S. S., and Brodsky, J. L. (2008) One step at a time: Endoplasmic reticulum-associated degradation. *Nat. Rev. Mol. Cell Biol.* **9**, 944–957 CrossRef Medline
49. Vergarajauregui, S., and Puertollano, R. (2006) Two di-leucine motifs regulate trafficking of mucolipin-1 to lysosomes. *Traffic* **7**, 337–353 CrossRef Medline
50. Wu, Z. M., Geng, Y., Lu, X. J., Shi, Y. Y., Wu, G. W., Zhang, M. M., Shan, B., Pan, H. L., and Yuan, J. Y. (2019) Chaperone-mediated autophagy is involved in the execution of ferroptosis. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 2996–3005 CrossRef Medline
51. Traksel, G. M., Kutty, R. K., and Maines, M. D. (1986) Purification and characterization of the major constitutive form of testicular heme oxygenase - the noninducible isoform. *J. Biol. Chem.* **261**, 1131–1137 Medline
52. Hubbi, M. E., Hu, H., Kshitz, Ahmed, I., Levchenko, A., and Semenza, G. L. (2013) Chaperone-mediated autophagy targets hypoxia-inducible factor-1α (HIF-1α) for lysosomal degradation. *J. Biol. Chem.* **288**, 10703–10714 CrossRef Medline
53. Laemmli, U. K. (1970) Cleavage of structural proteins during assembly of head of bacteriophage-t4. *Nature* **227**, 680–685 CrossRef Medline
54. Dawson, R. M. C. (1986) *Data for biochemical research*, 3rd edition, Clarendon Press, New York
55. Spencer, A. L. M., Bagai, I., Becker, D. F., Zuiderweg, E. R. P., and Ragsdale, S. W. (2014) Protein/protein interactions in the mammalian heme degradation pathway heme oxygenase-2, cytochrome P450 reductase, and biliverdin reductase. *J. Biol. Chem.* **289**, 29836–29858 CrossRef Medline
56. Zhu, Y., Luo, S., Sabo, Y., Wang, C., Tong, L., and Goff, S. P. (2017) Heme oxygenase 2 binds myristate to regulate retrovirus assembly and TLR4 signaling. *Cell Host Microbe* **21**, 220–230 CrossRef Medline
57. Morrison, G. R. (1965) Fluorometric microdetermination of heme protein. *Anal. Chem.* **37**, 1124–1126 CrossRef Medline