Heme deficiency sensitzes yeast cells to oxidative stress induced by hydroxyurea

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Hydroxyurea (HU) has a long history of clinical and scientific interest for more than 100 years (1). Diverse therapeutic activities have been reported, which include antiviral, antibacterial, antitumor, and anti-inflammatory effects (1, 2). The therapeutic effects are generally believed to be due to the DNA damage, such as strand breaks, generated at collapsed DNA replication forks. Within the research laboratories, HU is commonly used as a cell synchronizstion agent. HU can also be cytotoxic depending on the time of exposure and the drug concentrations that are used. However, the cell killing mechanisms underlying the cytotoxic effect of HU, particularly on the non-cycling cells, are poorly understood.

The primary cellular target of HU is the enzyme ribonucleotide reductase (RNR), which is required for furnishing dNTP precursors for DNA replication and repair (3). RNR is a heterotetrameric enzyme composed of two large and two small subunits. Although the large subunits have both catalytic and allosteric regulation sites, the small subunit contains a diferenzor tyrosyl-free radical that is required for the catalysis. HU quenchens the tyrosyl radical and therefore suppresses the enzymatic activity of RNR (4, 5). Consistent with the radical scavenger mechanism, HU resistance has been observed in cells that overexpress the small subunit of RNR and in cells that express a mutant RNR (6–8). Because RNR is an essential enzyme required for cell survival, extensive research has been carried out to develop small molecule inhibitors that can be used in clinics for the treatment of cancers or other diseases (9).

When treated with HU, the proliferating cells are arrested in the S phase. The primary cellular response to the HU-induced replication stress is the activation of the DNA replication checkpoint (DRC) (10–12). The activated DRC stimulates the production of dNTPs, delays mitosis, and protects the perturbed forks from collapsing so that cells have sufficient time to accomplish the DNA replication before they divide. Consistent with its importance in genome integrity, the DRC signaling pathway is highly conserved among all eukaryotic organisms. Defects in the DRC cause aberrant mitosis and broken forks, which are likely the direct causes of cell lethality.

To understand the molecular mechanism by which the checkpoint signaling is initiated at the replication forks (13, 14), we have been searching for new DRC mutants. Because the fission yeast Schizosaccharomyces pombe is an established model for studying the cellular mechanisms that are conserved in humans, we carried out a genetic screen looking for new S. pombe mutants that are sensitive to the replication stress induced by HU. This screen has identified several mutants that are highly sensitive to chronic treatment of HU. Interestingly, these mutants are not killed by aberrant mitosis or DNA damage that are commonly observed in DRC mutants but by previously unknown mechanisms. In a previous report, we showed that HU induces cytokinesis arrest in cells carrying a hypomorphic mutation in the erg11 gene, which encodes the enzyme
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sterol-14α-demethylase required for sterol biosynthesis (15). In this study, we report the identification of a novel hem13-1 mutant in the heme biosynthesis pathway. Several lines of evidence are provided that strongly support that HU kills the mutant cells by generating oxidative stress, not by perturbed DNA replication or a DRC signaling defect. Because HU is a well established drug with multiple clinical implications, discovery of new cell-killing mechanisms may help to improve the HU-based chemotherapies or expand the therapeutic use of this clinically important drug.

Results

Identification of the novel hem13-1 mutant that is highly sensitive to HU

To understand the initiation process of the DRC signaling at perturbed replication forks (13, 14), we carried out a hus (HU-sensitive) screen in S. pombe (16) to identify new mutants that are sensitive to the replication stress induced by HU. After removal of the mutants with known mutations that are sensitive to HU by extensive genetic crossing, the newly screened hus mutants were transformed with S. pombe genomic DNA expression libraries carrying the ure4 marker. Yeast colonies grown on plates lacking uracil were replicated onto plates containing HU to screen the colonies with conferred HU resistance. The plasmids recovered from the HU-resistant yeast colonies were subjected to digestions with restriction enzymes and DNA sequencing to identify the mutated genes in the newly screened hus mutants. As a result, this screen has identified several new fission yeast mutants that are highly sensitive to HU.

We have previously reported our characterization of one of the newly screened hus mutant erg11-1 (15). Here, we report our results with the second screened mutant that carries a novel mutation in hem13 whose gene product is the predicted enzyme coproporphyrinogen III oxidase required for the biosynthesis of heme (17) (Fig. 1A). Hem13 is an oxygen-requiring enzyme that catalyzes the aerobic oxidative decarboxylation of propionate groups of rings A and B of coproporphyrinogen-III (highlighted in Fig. 1B) (18). DNA sequencing identified a single C-to-T nucleotide mutation that results in a T263I amino acid change in the enzyme (Fig. 1C). This mutant was subsequently named hem13-1 (Fig. 1D). The hem13-1 mutant also carries a cold-sensitive phenotype (Fig. 1D, lower panels), which is consistent with the essential function of the gene discovered by a previous genome-wide deletion study (19) and confirmed by our tetrad dissection (supplemental Fig. S1).

Rad3 is the sensor protein kinase of the DRC in S. pombe and the ortholog of human ATR (Ataxia telangiectasia and Rad3-related) and Saccharomyces cerevisiae Mec1. The rad3 mutant is one of the most HU-sensitive mutants that has been reported in S. pombe so far and was therefore used as a control in this study. The HU sensitivity of our newly screened hem13-1 mutant is remarkable in that it is even more sensitive than the rad3 mutant (Fig. 1D, upper panels). To confirm the mutation, we cloned the hem13 gene from both wild-type and the hem13-1 mutant and expressed on a vector in S. pombe. Although the vector carrying the wild-type hem13 rescued both HU and cold sensitivities, the vector carrying the mutant hem13 did not (Fig. 1E). Similar results were obtained when the wild-type and the mutated enzymes were expressed in cells devoid of hem13 (supplemental Fig. S2A). We then integrated the mutation at the genomic lea1 locus in hem13Δ cells (supplemental Fig. S2B) or at the hem13 genomic locus in a wild-type strain. All integrants of the mutated gene showed identical HU and cold sensitivities as the original mutant such as the one shown in Fig. 1F. In contrast, integrants of wild-type hem13 using the same method behaved like the wild-type cells (Fig. 1F). Because the HU sensitivity of hem13-1 is so remarkable, we tested the HU from different manufacturers with different batch numbers and confirmed that the HU sensitivity was indeed caused by HU and not by impurities (data not shown). Together, we have identified a novel hem13-1 mutation that dramatically sensitizes S. pombe to HU.

The hem13-1 mutant is highly sensitive to chronic HU treatment

The remarkable HU sensitivity of the hem13-1 mutant as determined by the standard spot assay shown in Fig. 1D involves a chronic drug exposure of ~3 days. To further investigate, we assessed the sensitivity of the mutant to acute HU treatment in liquid medium (16). We found that similar to the previously reported erg11-1 mutant (15), the hem13-1 mutant was relatively insensitive to acute HU treatment (Fig. 2A). Although the wild-type cells were able to survive in HU, which is consistent with the reversible S-phase arrest induced by HU, most of the rad3 cells died ~3 h or approximately 1 cell cycle time. The majority of the hem13-1 cells were able to survive in HU during the 6-h treatment. However, unlike the wild-type cells that continued to grow in HU, cell growth of the hem13-1 mutant was completely suppressed. This result suggests that unlike the DRC mutant and wild-type cells, HU may stably arrest the cell cycle of the hem13-1 mutant, which eventually leads to the cell inviability.

The hem13-1 mutant is minimally sensitive to DNA-damaging agents

Because most of the HU-sensitive DRC mutants are also sensitive to DNA damage, we then examined the sensitivity of the hem13-1 mutant to DNA damage caused by methyl methanesulfonate (MMS), camptothecin (CPT), or UV light by standard spot assay. As shown in Fig. 2B and supplemental Fig. S3A, although the rad3 mutant was highly sensitive to all three DNA-damaging agents, the hem13-1 mutant was only minimally sensitive as compared with wild-type cells. Cds1 (CHK2/Rad53) and Chk1 are the major effector kinases of the DRC and the DNA-damage checkpoint (DDC) in S. pombe, respectively. Although the cells devoid of chkl in the DDC pathway showed an intermediate sensitivity to the DNA-damaging agents as compared with rad3 cells, the cds1 mutant showed a minimal sensitivity similar to hem13-1. This suggests that the DDC signaling pathway may remain functional in the hem13-1 mutant. To provide the direct evidence, we examined the DDC signaling by monitoring the Rad3-dependent phosphorylation of Chk1 (Fig. 2C). In the presence of MMS, Chk1 was highly phosphorylated in wild-type cells, and the phosphorylation was depen-
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A

**Mitochondria**

Glycine + Succinyl coA → Aminolevulinic acid synthase → Heme

Fe

Ferrochelatase (Hem15)

Protoporphyrin IX

Protoporphyrinogen oxidase (Hem14)

**Cytosol**

Aminolevulinic Acid → Aminolevulinic acid dehydratase

Porphobilinogen → Porphobilinogen deaminase

Hydroxymethylbilane → Uroporphyrinogen III synthase

Uroporphyrinogen III → Uroporphyrinogen decarboxylase (Hem12)

Coproporphyrinogen → Coproporphyrinogen oxidase (Hem13)

B

Coproporphyrinogen oxidase

CO₂

C

T263I

D

Control 1.0 mM 2.0 mM HU

TK48 NR1826 APS19

WT rad3Δ hem13-1

30°C 22°C

TK48 APS19

WT hem13-1

E

Control 2.0 mM 3.0 mM 5.0 mM HU

30°C 22°C

TK48 APS19 APS20 APS21

WT hem13-1 + V hem13-1 + hem13 hem13-1 + hem13-1

F

30°C 22°C HU

1.0 mM 2.0 mM HU

TK48 NR1826 GBY191 APS19 APS124

WT rad3Δ cds1Δ hem13-1 hem13 hem13-1 Integrant
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Figure 2. The checkpoint independent cell killing in HU-treated hem13-1 mutant. A, minimal sensitivity of the hem13-1 cells to acute HU treatment. Wild-type TK48 strain, rad3 null mutant NR1826, and the hem13-1 mutant APS124 cells were incubated in YE6S medium containing 25 mM HU. At each time point, an aliquot of the culture was removed and diluted 1000-fold in sterile distilled H2O. The cells were spread onto YE6S plates and incubated at 30 °C for 3 days to allow cell recovery. Colonies resulting from the recovered cells were counted and are presented as the percentages of the untreated cells. Each data point represents an average number of colonies from three plates. The bars indicate standard deviation. B, the sensitivities of wild-type, the rad3, cdst, and chk1 checkpoint mutants, and the hem13-1 cells to the DNA-damaging agents MMS, CPT, and UV light were determined by standard spot assays. C, intact Chk1 phosphorylation in MMS-treated hem13-1 cells. Wild-type (LLD3427), rad3 (YM563), cdst (YM563), and hem13-1 (APS142) cells were treated with (+) or without (−) 0.01% MMS at 30 °C for 80 min. The phosphorylation of Chk1 by Rad3 was determined by standard mobility shift assay as described under “Experimental procedures.” D, the same wild-type, rad3, cdist, and hem13-1 cells were treated with (+) or without (−) 25 mM HU for 4 h at 30 °C. Chk1 phosphorylation was examined as in C.

Reduced DRC signaling in the hem13-1 mutant

Next, we examined the DRC signaling from Rad3 to MrC1 and Cds1 after HU treatment. As mentioned above, Cds1 is the major effector kinase of the DRC in S. pombe (20). Although Cds1 is directly activated by autophosphorylation of Thr236 in the activation loop, the autophosphorylation requires the phosphorylation of Thr11 by Rad3, which primes the autophosphorylation and autoactivation of the kinase (21–23). Therefore, phosphorylation of Cds1-Thr11 has been used as a reliable marker for DRC activation in S. pombe (21, 22). As shown in Fig. 3A, phosphorylation of Cds1 was significantly increased in HU-treated wild-type cells. Interestingly, Cds1 phosphorylation was reduced significantly by ~ 77% in the hem13-1 mutant cells, which suggests that the DRC signaling is seriously compromised.

To further investigate the DRC signaling defect, we examined the Rad3-dependent phosphorylation of the DRC mediator MrC1 (human Claspin). In the presence of HU, Rad3 phosphorylates two TQ motifs in S. pombe MrC1 containing Thr645 and Thr663, which function redundantly in recruiting Cds1 to be phosphorylated by Rad3 (23). As shown in Fig. 3B, MrC1-Thr645, a representative of the two TQ motifs in MrC1, was highly phosphorylated by Rad3 in HU-treated wild-type cells (compare wild type with the rad3 mutant). Under similar conditions, MrC1 phosphorylation was significantly reduced in the hem13-1 cells. Interestingly, the protein levels of MrC1 were also significantly lower, which may provide an explanation for the reduced phosphorylation of MrC1 and Cds1. Because MrC1 is specifically expressed during the S phase, it is also possible that the...
observed DRC signaling defect from Rad3 to Mrc1 and Cds1 is caused, as described below, by an alternative mechanism.

The DRC signaling is compromised indirectly by a cell-cycle defect

Next, we performed the cell cycle analysis on the mutant by flow cytometry. In the presence of HU, most of the wild-type cells were arrested at the G_{1}/S phase in ∼3–4 h and eventually finished the bulk of DNA synthesis in ∼7–8 h (Fig. 3C, left column). Under similar conditions, the hem13-1 cells were not arrested at the G_{1}/S phase during the 8-h-long HU treatment. Instead, most of them remained with a 2C DNA content, which suggests a G_{2}/M arrest (Fig. 3C, right column). In a standard HU block and release experiment, similar G_{2}/M arrest was
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Figure 3. The reduced DRC signaling in hem13-1 cells is caused indirectly by a cell cycle effect. A, phosphorylation of Cds1 was reduced in HU-treated hem13-1 cells. The cds1 tagged wild-type (Y3374), untagged (TK48), and tagged in the background of hem13-1 (APS19) cells were incubated in YE6S medium with (+) or without (−) 25 mM HU at 30 °C for 3 h or 6 h. The cells were collected for preparation of whole cell extracts as described under “Experimental procedures.” Phosphorylation of Cds1-Thr11 was assessed in immunopurified Cds1 by Western blotting using phospho-specific antibody (top panel), quantitated by ImageGauge, and shown in percentages as compared with that in HU-treated wild-type cells. The same membrane was stripped and then reprobed with anti-HA antibody for HA-tagged Cds1 (lower panel). B, phosphorylation of Mrc1-Thr645 by Rad3 in hem13-1 (top panel) and wild-type cells (bottom panel) was detected by Western blotting. C, the HU-induced cell cycle arrest in cdc10 null (NR1826) and rad3 cells (GBY191) checkpoint mutants, overexpressed RNR small subunit Suc22 did not rescue the hem13-1 null (NR1826) and rad3 mutant, regardless of the growth conditions. Consider the slower cell growth under anaerobic conditions and the issue of mitochondria are the major source of intracellular ROS. We reasoned that if broken forks directly kill the cells, the cell death should occur in both checkpoint mutants and the hem13-1 mutant, regardless of the growth conditions. Considering the slower cell growth under anaerobic conditions and the issue of Hem13-1 and the known checkpoint mutations by crossing the mutation directly, we tested the effect of overexpressed RNR small subunit Suc22 in the hem13-1 cells (25). Because one of the major functions of the DRC is to stimulate RNR and to promote dNTP production and DNA synthesis, overexpression of Suc22 can fully rescue most of the DRC mutants, including the rad3 and cds1 mutants (Fig. 3G). Interestingly, overexpression of Suc22 did not rescue the hem13-1 mutant under similar conditions. Together, these results show that the reduced DRC signaling is likely an indirect cell cycle effect that contributes minimally to the HU-induced lethality in the hem13-1 mutant.

Anerobiosis fully suppresses the HU-induced cell death

The results described above strongly suggest that it is unlikely that broken forks or the reduced DRC signaling causes the cell death in hem13-1 mutant following HU treatment. Earlier studies suggest that in addition to broken forks, HU may also kill the cells by generating oxidative stress (26–29). Treatment of human leukemia cell lines with the heme synthesis inhibitor sampangine (SMP) has been reported to generate reactive oxygen species (ROS) and to induce apoptosis (30, 31). Because of the potential defect in heme biosynthesis in hem13-1 cells, we surmised that the cell death induced by HU might be caused by accumulated ROS inside the mutant cells. To test this hypothesis, we examined HU sensitivity under anaerobic conditions because under aerobic growth conditions, mitochondria are the major source of intracellular ROS. We reasoned that if broken forks directly kill the cells, the cell death should occur in both checkpoint mutants and the hem13-1 mutant, regardless of the growth conditions. Considering the slower cell growth under anaerobic conditions and the issue of
drug decomposition, HU was used at a higher dose of 5 mM in this experiment (Fig. 4A). Sre1 is a sterol regulatory element-binding protein and a transcription factor required for the cellular response to hypoxia (32). We included the sre1Δ mutant as a control for anaerobic conditions because in the absence of molecular oxygen, the sre1Δ cells cannot survive. As shown in the top panel of Fig. 4A, although the rad3Δ and cdc1Δ checkpoint mutants showed a similar HU sensitivity under both aerobic and anaerobic conditions, the HU sensitivity of hem13-1 mutant was completely eliminated by culturing in the anaerobic environment. We also monitored the cold sensitivity of the hem13-1 mutant under the similar anaerobic conditions. The cold sensitivity was almost fully suppressed (Fig. 4A, lower panel).

The hem13-1 mutant is highly sensitive to oxidants

The results described above strongly suggest that HU treatment may increase oxidative stress inside the mutant cells, which likely causes the cell death. To further investigate, we examined whether the hem13-1 mutant is more sensitive to the oxidants H2O2 and glucose. As shown in Fig. 4B, the hem13-1 mutant is highly sensitive to both exogenous and endogenous oxidants. The same cells were also spotted on plates carrying glycerol or glycerol plus increasing concentrations of glucose as the carbon sources (lower panels). The plates were incubated for 3 days at 30 °C. The ptc4Δ mutant with a defect in cellular response to oxidative stress was used as a control. C, the ptc4Δ mutant is only minimally sensitive to HU as determined by the standard spot assay. D, the HU sensitivity of the hem13-1 cells was partially rescued by the antioxidant NAC. Wild-type, rad3Δ, cdc1Δ, and hem13-1 cells were spotted onto the plates containing HU alone or HU plus 25 mM NAC for assessing the effect of NAC on HU sensitivity.
exogenously supplied oxidant H$_2$O$_2$ (Fig. 4B, upper panel). We found that wild-type and the rad3 cells were able to survive on plates containing up to 3 mM H$_2$O$_2$ and failed to do so when the concentration was raised to 5 mM. Ptc4 is the enzyme protein phosphatase 2c that is required for cellular response induced by MAP kinase Spt1 in the presence of oxidative stress (33). The ptc4Δ mutant is highly sensitive to H$_2$O$_2$ and was therefore used as a control. We found that under the similar conditions, the hem13-1 mutant was highly sensitive to H$_2$O$_2$, and the sensitivity was even slightly higher than the ptc4 mutant.

We next examined the sensitivity of the hem13-1 cells to endogenous ROS by monitoring cell survival on plates containing glycerol. When glucose in the medium is replaced by the non-fermentable carbon source glycerol, energy is generated mainly by respiratory enzymes, which results in more ROS being produced inside the cell (34). As shown in the lower panel of Fig. 4B, wild-type and the checkpoint mutant cells were able to survive on plates containing glycerol, and the addition of glucose promoted the cell growth in a dose-dependent manner. The control ptc4 mutant was unable to survive in glycerol as expected. Similar to the ptc4 mutant, the hem13-1 mutant could not grow in glycerol, and the addition of glucose significantly improved the cell growth like the ptc4 mutant, which suggests that unlike the wild-type and checkpoint mutant cells, the hem13-1 mutant may generate more ROS or have difficulty in dealing with the increased ROS. Interestingly, the ptc4 cells were only slightly sensitive to HU under normal cell growth conditions (Fig. 4C), which suggests that unlike hem13-1 cells, HU treatment does not generate a high level of oxidative stress inside the ptc4 cells.

**Protection of hem13-1 cells by antioxidant N-acetylcysteine**

Because the HU-induced cell death in hem13-1 mutant is likely caused by oxidative stress, we then examined the effects of the antioxidant N-acetylcysteine (NAC) on the mutant (34). We reasoned that if cell death is caused by increased ROS in hem13-1 mutant, particularly the deleterious hydroxyl radical, the addition of an antioxidant should be able to promote cell survival in HU. As shown in Fig. 4D, whereas NAC had no effect on the HU sensitivity of rad3 mutant, it significantly improved cell survival in hem13-1 mutant even in the presence of 5 mM HU. Interestingly, NAC could also slightly promote the survival of cds1 mutant. An earlier report showed that inappropriate entry into mitosis in checkpoint mutants generates ROS inside the cells (35), which may provide an explanation for the improved cell survival of cds1 mutant. However, the protective effect of NAC was significantly higher on the hem13-1 cells than on the cds1 mutant.

**HU treatment increases ROS inside the hem13-1 cells**

The results described above strongly suggest that HU treatment generates oxidative stress in the hem13-1 cells. To provide the direct evidence, we measured ROS levels by staining the cells with the membrane-permeable dye 2′,7′-dichlorofluorescin diacetate (DCFDA) (36). DCFDA becomes fluorescent when oxidized by ROS. The stained cells can thus be analyzed by flow cytometry or other methods for detection and quantitation of increased ROS. To separate the dead cells, the cells were also stained with the non-permeable dye propidium iodide (PI) before flow cytometry analysis (37). Because *S. pombe* cells display a basal level of fluorescence even without the staining, we also measured the unstained cells treated with or without HU (Fig. 5A and supplemental Fig. 54). After the HU treatment, a fraction of the cell population, particularly the hem13-1 cells, were specifically stained by DCFDA (see the selected areas in Fig. 5A), which indicates that these cells may contain an increased level of ROS. Before the HU treatment, only 2.57% of the logarithmically growing wild-type cells were positively stained (numbers above the selected areas). After the HU treatment, the positively stained cells were slightly increased to 3.1%, which is consistent with a previous report that HU treatment generates a minimal oxidative stress in wild-type *S. pombe* (35). Similar to the wild-type cells, only 3.29% of the hem13-1 cells were positively stained before HU treatment. However, after HU treatment, the positively stained cells increased 3–4-fold to 11.3% (Fig. 5A, bottom panels). This result strongly suggests that HU treatment significantly increased the levels of ROS inside the mutant cells. Interestingly, unlike the wild-type cells, HU treatment also shifted the entire cell population of the hem13-1 mutant in the rightward direction. Although multiple reasons may cause this overall shift, it is possible that HU treatment may increase the ROS levels in whole cell population of the hem13-1 mutant.

**Increased protein carbonylation inside the HU-treated hem13-1 cells**

Higher levels of ROS, particularly the hydroxyl radical, can cause irreversible oxidation of macromolecules (38). We next examined protein carbonylation, which has been used as a reliable marker of oxidative damage of macromolecules (39). In the presence of elevated ROS, the side chains of lysine, arginine, proline, threonine, and other residues of the cellular proteins are oxidized to ketones and aldehydes. Protein carbonylation is an irreversible damage that often leads to the loss of functions and even cell death. The ketones and aldehydes of the damaged proteins react with 2,4-dinitrophenylhydrazine (DNPH) to form hydrazone moieties, which can then be detected by Western blotting or other methods using antibodies against DNPH-derived proteins (40, 41). We found that before the HU treatment, protein carbonylation in the hem13-1 mutant was at a level similar to or slightly higher than in wild-type or rad3 cells (Fig. 5B). After the HU treatment, protein carbonylation decreased in wild-type and rad3 cells, which suggests that the oxidative stress regulon has been activated to prevent the protein damage (29). In contrast, the protein carbonylation in hem13-1 cells was significantly increased ~3-fold after the HU treatment (compare right two lanes in Fig. 5B). Interestingly, this increase is roughly proportional to the DCFDA positively stained cells detected by flow cytometry (Fig. 5A). Together, these data clearly showed that after a 3–4-h treatment with HU, at least a fraction of the hem13-1 cell population is under a serious oxidative stress that causes irreversible macromolecular damage. Longer exposure to HU, such as in the spot assay, may generate more serious oxidative damage, which likely explains the remarkable HU sensitivity of the hem13-1 mutant shown in Fig. 1D.
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The Hem13 enzyme is homologous to coproporphyrinogen oxidase (CPOX) in humans (42). Defects in heme synthesis in humans lead to hepatic or erythropoietic porphyrias (43–45). The mutated Thr263 residue in Hem13 is conserved in CPOX and is believed to function in catalysis (42). Mutation of this residue to aspartic acid reduced the enzymatic activity of CPOX by 69%. We surmised that the hem13-1 mutation might reduce the enzymatic activity and thus cause the heme deficiency in S. pombe. To investigate this, we measured the heme levels inside the hem13-1 cells. As shown in Fig. 6A, the heme level inside the hem13-1 cells was significantly lower than in wild-type cells under normal condition. Although HU treatment reduced the heme levels inside the wild-type cells, treatment with the heme synthesis inhibitor SMP had an even more significant effect, although 4 μM SMP had only a minimal effect on the growth of wild-type cells (Fig. 7B). In contrast, HU and SMP had only a minimal effect on heme levels in the hem13-1 cells, which is probably because heme was already at a basal level required for cell growth. Heme is known to be required for ergosterol biosynthesis, and abundant ergosterol is crucial for HU resistance in S. pombe (15, 46). However, the exact mechanism by which HU decreases the heme level inside wild-type cells remains unknown.

Hem13 is an oxygen-requiring enzyme that is known to be positively regulated by low levels of heme and oxygen inside the cells (47). We monitored the protein levels of Hem13 in the mutant cells and found that it was ~2-fold higher than in wild-type cells (Fig. 6B). This result is consistent with the low heme level inside the hem13-1 cells and supports our conclusion that the hem13-1 mutation causes heme deficiency in S. pombe.

Exogenously supplied hemin fully rescues the hem13-1 mutant

Because the hem13-1 mutation causes heme deficiency, we then investigated whether the heme deficiency is directly linked to the HU and cold sensitivities of the mutant. To address this issue, we examined the effects of exogenous hemin on the mutant because hemin can functionally replace heme. The cells were preincubated with hemin for 3 days and then spotted on plates with (+) or without (−) hemin. We found that hemin could fully rescue the sensitivity of the hem13 mutant to HU, as well as the low temperature (Fig. 6C). In contrast, hemin had little or no effect on the HU sensitivity of rad3 cells. Because the hem13-1 cells were arrested by HU at G2/M, not in G1/S (Fig. 6D, left column), we then examined whether the cell cycle defect could also be chemically rescued by hemin. We found that without hemin, almost all hem13-1 cells were arrested in G2/M by HU (Fig. 6D, right column), (~25 mM HU for 4 h). The same number of cells were collected, suspended in the protein carbonylation buffer, and lysed. Cell extracts were reacted with DNP, followed by SDS-PAGE and Western blot analysis using α-DNPH antibody. The levels of protein carbonylation were quantitated and shown at the bottom as percentages relative to the untreated wild-type cells. A section of the Ponceau S-stained membrane and the Western blot using anti-PKG antibody are shown as the loading controls. The experiments were repeated two times, and similar results were obtained.

Figure 5. Higher levels of ROS and protein carbonylation inside the HU-treated hem13-1 cells. A, the increased levels of ROS were examined by analyzing the fluorescent dye DCFDA-stained cells using flow cytometry. Wild-type (TK48) and hem13-1 (APS124) cells treated with (+) or without (−) HU were incubated with (+) or without (−) DCFDA at 30 °C for 90 min as described under “Experimental procedures.” The cells were harvested, washed, and resuspended in a buffer containing PI to stain the dead cells. The samples were analyzed by flow cytometry, and the results are shown in scatter plots with the ROS signal being displayed on the horizontal FL3-A axis and the PI signal being displayed on the vertical FL1-A channel. 10,000 cells were analyzed for each sample. The DCFDA-positively stained cells were quantitated in the same selected area for all samples and are shown as percentages of all counted cells (numbers on top of the selected areas). B, wild type (TK48), rad3 (NR1826), and hem13-1 (APS124) cells were treated with (+) or without HU for 4 h. The same number of cells were collected, suspended in the protein carbonylation buffer, and lysed. Cell extracts were reacted with DNP, followed by SDS-PAGE and Western blot analysis using α-DNPH antibody. The levels of protein carbonylation were quantitated and shown at the bottom as percentages relative to the untreated wild-type cells. A section of the Ponceau S-stained membrane and the Western blot using anti-PKG antibody are shown as the loading controls. The experiments were repeated two times, and similar results were obtained.
which showed that hemin could also rescue the HU-induced cell cycle defect.

We also tested two other nonessential mutants hem12Δ and hem14Δ in the heme biosynthesis pathway (Fig. 1A) and found that both mutants were quite resistant to HU (Fig. 7A) and SMP (Fig. 7B). Under similar conditions, the hem13-1 mutant cells were highly sensitive to both HU and SMP. This result suggests that redundant enzymes may exist in the heme biosynthesis pathway and that Hem13 can be a specific drug target for suppressing cell growth.

**The SMP-treated wild-type S. pombe phenocopies the hem13-1 mutant**

The chemical rescuing effects of hemin strongly suggest that heme deficiency may sensitize the hem13-1 cells to HU-induced oxidative stress, which ultimately leads to G2/M arrest and cell death. If this is true, pretreatment of wild-type *S. pombe* with a heme synthesis inhibitor should generate the similar phenotypes of the hem13-1 mutant. To test this hypothesis, we first examined the sensitivity of wild-type, *rad3, cds1*, and *hem13-1* cells to SMP and the second heme synthesis inhibitor succinylacetone (48). We found that although *rad3, hem12*, and *hem14* mutants showed a SMP resistance similar to that of the wild-type cells, the *hem13-1* mutant was highly sensitive to SMP (Fig. 7B). The sensitivity of the *S. pombe* strains to succinylacetone was assessed using the 96-well plate method. Similar results were obtained (supplemental Fig. S5A). These results confirm the effect of the inhibitors on cell growth and are consistent with the heme deficiency in the *hem13-1* mutant shown in Fig. 6A.

We next examined the effect of SMP on cell cycle progression by flow cytometry. Dashed lines indicate the cells with a 2C DNA content. Asynchronous cells at the bottom represent the cells before the HU treatment. This experiment has been repeated multiple times, and similar results were obtained.

**Figure 6. The hem13-1 mutant can be chemically rescued by exogenously supplied hemin.** A, the heme level is significantly lower in the hem13-1 cells. Cell extracts were made from wild-type (TK48) and the hem13-1 (APS124) cells treated with 25 mM HU or with 4 μM of the heme synthesis inhibitor SMP for 4 h. The concentrations of heme in the cell extracts were measured using the hemin assay kit and normalized to the protein concentrations. The results are shown as percentages in relative to the level in untreated wild-type cells. Bar, standard deviation. *, statistically significant (p < 0.05) as determined by Student’s t test. B, the protein level of Hem13 is significantly higher in the hem13-1 cells. Untagged (TK48), HA-tagged Hem13 expressed in wild-type (APS97), and hem13-1 mutant (APS99) cells were treated with or without HU. Whole cell extracts were prepared, and the protein levels were examined by Western blotting using anti-HA antibody (top panel). The blotting signal was quantitated and shown in percentages relative to HU-untreated wild-type cells (numbers at the bottom). A section of Ponceau S-stained membrane is shown (bottom panel). C, the HU and cold sensitivity of the hem13-1 mutant can be fully rescued by exogenously supplied hemin. Wild type, *rad3*, and the hem13-1 mutant cells preincubated with (+) or without (-) 50 μg/ml of hemin for 3 days were spotted on plates with or without HU in the presence or absence of 50 μg/ml hemin. The plates were also incubated at 30 or 22 °C for 3 days and then photographed (bottom panels). D, the HU-induced cell cycle arrest at G2/M can be rescued by the exogenously supplied hemin. The hem13-1 APS124 cells were pretreated with (left panel) or without (right panel) hemin for 3 days as in B and then incubated with 25 mM HU. During the course of the HU treatment, the cells were fixed at the indicated time points for cell cycle analysis by flow cytometry. Dashed lines indicate the cells with a 2C DNA content. Asynchronous cells at the bottom represent the cells before the HU treatment. This experiment has been repeated multiple times, and similar results were obtained.
rad3 and hem13-1 cells. We then treated the wild-type cells with both HU and SMP and monitored the cell cycle progression. In the presence of SMP, wild-type cells were no longer arrested in G1/S by HU. Instead, most of them were arrested in G2/M, which is remarkably similar to the HU-treated hem13-1 cells shown in Fig. 3C. We also compared the HU sensitivity of wild-type cells in the presence or absence of SMP. The results clearly showed that SMP significantly sensitizes the cells to HU. Together, we found that wild-type S. pombe pretreated with SMP phenocopied the HU sensitivity, as well as the cell cycle defect of hem13-1 mutant, which confirms our notion that heme deficiency sensitizes S. pombe to the oxidative stress induced by HU.

**Excess of free iron inside the hem13-1 cells may contribute to the HU sensitivity**

The majority of iron taken up by yeast cells is utilized for the synthesis of heme and for the assembly of iron-sulfur clusters (49). Heme deficiency may cause an excess of labile free iron inside the cells, which is known to promote oxidative stress by Fenton chemistry (38). To investigate this possibility, we examined the effect of the membrane-permeable Fe^{2+} chelator 2,2′-bipyridine (BP). Because BP sequesters the free labile iron inside the cells (50), we hypothesized that if the free labile iron pool contributes to the HU-induced oxidative stress, treatment of the mutant cells with BP should ameliorate the HU sensitivity. As shown in Fig. 8, whereas the wild-type cells showed a slightly increased HU sensitivity in the presence of BP, the cds1 cells became significantly more sensitive to HU, which is probably caused by the sequestering of the iron required for RNR recycling (3). In contrast, although BP did not promote the cell growth of the hem13-1 mutant under normal conditions, it significantly promoted the cell survival in the presence of HU. This result suggests that an excess of free iron may exist in the HU-treated hem13-1 cells, which promote oxidative stress and thus exacerbate the cytotoxic effect of HU.

**Discussion**

HU is a well established inhibitor of RNR in various organisms. It perturbs the cellular dNTP regulation network and generates broken forks (12, 51), which are generally believed to be the direct causes of cell lethality and thus the major therapeutic effect of this agent. Earlier studies in *Escherichia coli* using systems-level analyses have revealed that prolonged treatment generates oxidative stress, which contributes to majority of the cytotoxic effect of HU (26, 52). However, whether this cell-
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killing mechanism is conserved in eukaryotes is less clear, although studies in both *S. pombe* and *S. cerevisiae* have shown that HU treatment may activate the regulons that function in redox or iron homeostasis (28, 29). In the meantime, fork collapse and aberrant mitosis induced by HU may also generate ROS in yeast cells (35), which prevents unambiguous description of the cell-killing mechanisms. In this study, we provided several lines of strong evidence that HU causes cell death of the *hem13-1* mutant by generating oxidative stress and not by fork collapse or mitotic catastrophe (12, 51). Furthermore, because HU treatment mainly generates oxidative stress in the *hem13-1* cells, the mutant may also provide a valuable tool for dissecting the cell-killing mechanisms of this clinically important drug.

First, unlike the DRC mutants, HU sensitivity of the *hem13-1* mutant cannot be rescued by overexpression of the RNR small subunit Suc22. Second, although most of the HU-treated DRC mutants die within one cell cycle time, *hem13-1* cells are only sensitive to chronic HU treatment, and this slow killing is similarly observed in HU-treated *S. cerevisiae* lacking superoxide dismutase that catalyzes the decomposition of superoxide (53). Third, unlike the DRC mutants, *hem13-1* cells are relatively insensitive to DNA damage. Fourth, the cell-killing effect of HU in the *hem13-1* mutant absolutely requires molecular oxygen. Fifth, unlike the DRC mutants and wild-type cells that show a similar resistance to endogenous and exogenous oxidants, the *hem13-1* cells are highly sensitive to both types of oxidants, and the antioxidant NAC can significantly protect the mutant cells against the HU-induced lethality. Finally, the levels of ROS and protein carbonylation are significantly increased in HU-treated *hem13-1* cells. Because irreversible oxidation of macromolecules causes cell lethality, our results clearly show that it is the oxidative stress, not the catastrophic mitosis or broken forks, that contributes to the majority of cell killing in *hem13-1* mutant.

An important question that remains to be answered is how the oxidative stress is generated inside the HU-treated *hem13-1* cells. Aerobically respiring organisms inevitably generate ROS, which include superoxide, hydrogen peroxide, and hydroxyl radical (38). One of the major sources of ROS in eukaryotes is mitochondrial electron transport chain (54). To scavenge ROS, the cells are equipped with both enzymatic and nonenzymatic defense mechanisms. Although glutathione, glutaredoxins, thioredoxins, and NADPH constitute the nonenzymatic defense systems, superoxide dismutases, catalases, and peroxidases empower the cells with the enzymatic mechanism (54). Defects in the scavenging mechanisms or increased production can all lead to ROS accumulation, which causes oxidative stress or even cell death. Because heme is absolutely required for mitochondrial electron transport chain such as the cytochrome *bc₁* complex and the cytochrome oxidase, its deficiency may perturb the normal function of electron transport chain and therefore increase the production of ROS. Although the level of ROS and protein carbonylation in *hem13-1* cells remains low under normal conditions (Fig. 5), it is possible that the ROS are already generated at a higher rate but being timely detoxified by the scavenging mechanisms. In the presence of HU, production of ROS may be further increased to a level that the scavenging mechanisms are unable to detoxify, which leads to the oxidative stress in *hem13-1* cells.

Heme synthesis and assembly of iron-sulfur clusters consume most of the iron taken up by yeast cells. A compromised heme biosynthesis may thus cause an excess of free labile iron inside the cells, which is known to promote the formation of hydroxyl radical, the most harmful ROS, by Fenton reaction. Consistent with this possibility, coproporphyrinas patients with mutations in the Hem13 ortholog CPOX have severe iron load and high levels of serum ferritin (42, 45). The partial suppression of the HU sensitivity by the Fe²⁺ chelator BP that we have observed in the *hem13-1* mutant (Fig. 8) strongly suggests that an excess of free iron inside the mutant cells may exacerbates the oxidative stress induced by HU. Hem15 is the ferrochelatase and the last enzyme in the heme biosynthesis pathway. It binds and inserts the ferrous iron into protoporphyrin IX to form the final product heme B (Fig. 1A). When overexpressed in *hem13-1* cells, this enzyme could also partially suppress the HU sensitivity (data not shown), which further supports this idea. Previous studies in both *E. coli* and yeasts have shown that HU treatment causes misregulation of iron uptake (26, 28, 29), which increases the free iron pool inside the cells available for Fenton chemistry. It is possible that misregulation of iron uptake in HU-treated *hem13-1* cells may also contribute to the excess of free iron that further fuels the oxidative stress.

On the other hand, HU may also generate oxidative stress by directly targeting the ROS-scavenging mechanisms. It has been reported previously that in addition to the primary target RNR, HU may inhibit several metalloenzymes *in vitro* and *in vivo* (55, 56). *In vivo*, HU is known to suppress the catalase in plant cells, an enzyme that catalyzes the decomposition of hydrogen peroxide as well as HU (57). Although this mechanism may offer an explanation to the HU resistance that is often observed in patients, whether the suppression of catalase contributes to the HU-induced oxidative stress remains to be investigated.

HU has been used in clinics for the treatment of sickle cell disease, HIV, and cancers (1, 55). Understanding the new cell-killing mechanisms of a well established small molecule drug with multiple clinical implications is of great importance for the development of novel therapeutics. We have recently shown that in the presence of sterol deficiency, HU causes cell lethality mainly by stably arresting *S. pombe* cells in cytokinesis (15). As we show in our next paper, when applied with the inhibitors of heme or sterol biosynthesis, the cell-killing effect of HU can be significantly enhanced in *S. pombe* and *S. cerevisiae*, as well as in the opportunistic fungal pathogen *Candida albicans*. These discoveries may therefore provide a new strategy for the treatment of fungal infections or other diseases such as cancer.
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Experimental procedures

Yeast strains and plasmids

The S. pombe strains were usually cultured at 30 °C in YE6S (0.5% yeast extract, 3% dextrose, and 6 supplements) or in synthetic EMM6S medium lacking the appropriate supplements following standard methods (58). Glycerol plates were made in YE6S medium in which 3% glycerol was used to replace the dextrose. Yeast strains, plasmids, and PCR primers used in this study are listed in supplemental Tables S1–S3, respectively. All mutations were confirmed by DNA sequencing (Retrogen, San Diego, CA). Transformation of yeast cells was carried out by electroporation (Cell-Porator; BRL Life Technologies, Inc.).

Genetic screen

Screening of new HU-sensitive (hus) mutants was carried out following a previously described method (16). Briefly, the logarithmically growing wild-type TK1 strain was harvested and suspended in 50 mM Tris-maleate buffer, pH 6.0, and treated with 375 μg/ml methylnitronitrosoguanidine in the same buffer for 90 min at 25 °C. The cells were washed twice with phosphate-buffered saline, resuspended in EMM6S medium, and saved at 4 °C. For the hus screening, the mutagenized cells were spread on YE6S plates and incubated at 30 °C. The colonies were replicated onto YE6S or YE6S plates containing HU (Sigma) to identify those that were sensitive to HU. The hus mutants were backcrossed three times to remove the bystander mutations. The resulting hus mutants were then crossed and compared with known mutants including the checkout mutants that are sensitive to HU to identify novel hus mutants.

Gene disruption and integration of the mutation

The hem13 gene was disrupted by inserting an ura4 marker between the Ncol and the HincII sites in the open reading frame (196–335 bp from the start codon; supplemental Fig. S1A). Blunt ends were generated at the Ncol site using Klenow before the ligation. The 3885-bp gene disruption fragment between BspEI and BglII sites was excised, gel-purified, and then transformed into the wild-type diploid strain YJ18 (supplemental Table S1). Colonies growing up on plates lacking uracil were screened by colony PCR using primers outside of the integration sites to confirm the correct gene disruption. Tetrad dissection was performed to confirm the essentiality of the hem13 gene (supplemental Fig. S1B). Using a similar method, the wild-type and mutant hem13 genes were integrated at the genomic locus. The plasmids containing wild-type and mutant hem13 were cut with BspEI and BglII, and the resulting 1551-bp fragments was transformed into the Δhem13::ura4/hem13 diploid strain to replace the ura4 marker. After a recovery on YES plates for 24 h, the cells were replicated onto plates containing 5-fluoroorotic acid to select for ura- colonies. The haploid strain with integrated mutation was generated by tetrad dissection.

Drug sensitivity

To test drug sensitivity by spot assay, 2 × 10^7 cells/ml of logarithmically growing S. pombe were diluted in 5-fold steps and spotted onto YE6S plates containing HU, MMS (Aldrich), CPT (Sigma), NAC (Sigma), or BP (Sigma) at the indicated concentrations. The cells spotted on YE6S plates were also treated with the indicated doses of UV light (Stratalinker 2400, Stratagene). The plates were incubated at 30 °C for 3 days and then photographed. To test the sensitivity of S. pombe to H2O2, 30% (w/w) aqueous solution of H2O2 (Alfa Aesar) was diluted to 3% solution in autoclaved H2O. Once the medium solution was cooled enough for pouring, H2O2 was added at the indicated concentrations. The plates were kept in the dark at 4 °C before the spot assay. The standard spot assay was usually carried out at least two times for each specific experiment.

To test the sensitivity of S. pombe to acute HU treatment (16), HU was added to the liquid culture in YE6S medium at a final concentration of 25 mM. At each indicated time point, 10 μl of cells were removed from the culture, diluted 1000-fold in sterile distilled H2O, and then vigorously vortexed. 100 μl of the cell suspension was spread onto the YE6S plates. The plates were incubated at 30 °C for 3 days for the cells to recover. The number of the colonies resulting from the recovered cells were counted and presented as percentages of the untreated cells. Each data point represents an average of the colonies from three plates.

To assess the cell-killing effect of succinylacetone, logarithmically growing S. pombe cells were inoculated on 96-well plates at 3000 cells/well. Succinylacetone was added to a final volume of 200 μl at the indicated concentrations. The same amounts of carriers were added as the control. The cells were incubated at 30 °C for 48 h. The plates were scanned in a plate reader at A600.

Anerobiosis

An anaerobic cell culture environment was generated using type A Bio-Bag environmental chamber (Becton Dickinson), a transparent chamber that contains a gas generator consisting of one tablet of potassium borohydride and sodium bicarbonate, an ampule of hydrochloric acid, a catalyst cup containing palладium catalyst, and an indicator containing an ampule of resazurin. To test the drug sensitivity under anaerobic conditions, serial dilution of cells were spotted on YE6S or YE6S plates containing HU. The plates and all of the components mentioned above were put into the Bio-Bag. After the bag was properly heat-sealed, the generator was activated. The oxygen level was monitored using the oxygen reduction indicator by following the manufacturer’s instructions.

Detection of protein carbonylation

Protein carbonylation was detected by in vitro derivatization of oxidized proteins with DNPH (Sigma) (40, 41). Yeast cells were seeded into fresh medium at 0.1 A₆₀₀ₙₘ/ml and incubated at 30 °C for approximately two doubling times. The culture was split into two tubes, with one being exposed to 25 mM HU for 4 h and the other to water alone. Similar numbers of cells were harvested and resuspended in carbonylation buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.05% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 0.16 mg/ml aprotinin) and lysed using the glass beads method. After clarification by centrifugation, the protein concentration was determined with the Pierce BCA protein assay kit (ThermoFisher Scientific).
Scientific) and then adjusted to 10 mg/ml with the carboxylytion buffer. 10 µg of total proteins were denatured by heating at 100 °C for 3 min in the presence of 6% sodium dodecyl sulfate. The reaction was started by adding 10 mM DNPH in 10% trifluoroacetic acid in an equal volume of the protein sample and then incubated for 10 min at room temperature. The reaction was stopped by addition of one volume of 2 M Tris base containing 10% glycerol and 15% 2-mercaptoethanol. The samples were separated by 10% SDS-PAGE and then transferred to a nitrocellulose membrane. Carboxylated proteins were detected by Western blotting using the polyclonal anti-2,4-dinitrophenyl antibody (Sigma). Protein kinase G (PKG) in the same blot was detected using rabbit polyclonal anti-PKG2 antibody (Sigma) as a loading control.

Western blotting

The phospho-specific antibodies against phosphorylated Mrc1-Thr645 and Cds1-Thr11 were generated in rabbits by Bethyl Laboratories. The specificities of the antibodies were verified as described in previous studies (15, 23, 59). The Rad3-dependent phosphorylation of Chk1 was assessed using the mobility-shift method developed by the Russell lab using the mouse monoclonal anti-HA 12CA5 antibody (Sigma) (60). The horseradish peroxidase-linked goat anti-mouse (catalog no. 31430) or rabbit (catalog no. 31460) secondary antibodies were purchased from Thermo Scientific. The immunoblotting signal was detected by electrochemiluminescence and photographed with an Image Reader LAS-3000 (Fuji Film). Intensities of the bands were quantitated with ImageGauge (Fuji Film).

Flow cytometry

Logarithmically growing cells were collected and fixed in 1 ml of ice-cold 70% ethanol for 6 h or overnight at 4 °C. The fixed cells were treated with 0.1 mg/ml RNase A in 50 mM sodium citrate at 37 °C for 6 h or overnight and then stained with 4 µg/ml PI. The stained cells were analyzed using an Accuri C6 flow cytometer following the standard protocol. The collected data were examined using the FCS Express 4Flow software. The FL2-A channel was used for all DNA histograms unless otherwise indicated.

ROS staining assay

Logarithmically growing S. pombe cells were treated for 3 h with 25 mM HU at 30 °C. After the HU treatment, the ROS indicator dye DCFDA was added to 10 µg/ml, and the cells were cultured for an additional 90 min. The cells were then harvested, washed twice in citrate buffer (50 mM sodium citrate, pH 7.0), and resuspended in the same buffer with or without 4 µg/ml PI. The cells were analyzed by flow cytometry as described above.

Measuring heme levels in S. pombe

An overnight culture of S. pombe cells was seeded into fresh medium at 0.1 A600/ml and incubated at 30 °C for ~6 h. The culture was split into three tubes, with one being exposed to 25 mM HU for 4 h, one to 4 µM SMP for 4 h, and the third treated with dimethyl sulfoxide as the carrier control. An equal number of cells from each culture were collected, washed once with ice-cold water, washed once with phosphate buffer saline, and then lysed. A 2-µl portion of the cell lysate was used to measure the amount of heme by using the hemin assay kit (BioVision) according to the manufacturer’s instructions. The protein concentration of each cell lysate was measured using the Pierce BCA protein assay kit (ThermoFisher Scientific). Three independent experiments were performed for each culture, and the results were averaged and are shown as percentages of the heme level in the untreated wild-type cells. Statistical differences were assessed by Student’s t-test.

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