

The N-end rule pathway is a sensor of heme

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The conjugation of arginine, by arginyl-transferase, to N-terminal aspartate, glutamate or oxidized cysteine is a part of the N-end rule pathway of protein degradation. We report that arginyl-transferase of either the mouse or the yeast *Saccharomyces cerevisiae* is inhibited by heme (Fe³⁺-heme). Furthermore, we show that heme inhibits arginyl-transferase through a redox mechanism that involves the formation of disulfide between the enzyme's Cys-71 and Cys-72 residues. Remarkably, heme also induces the proteasome-dependent degradation of arginyl-transferase *in vivo*, thus acting as both a "stoichiometric" and "catalytic" down-regulator of the N-end rule pathway. In addition, heme was found to interact with the yeast and mouse E3 ubiquitin ligases of the N-end rule pathway. One of substrate-binding sites of the yeast N-end rule's ubiquitin ligase UBR1 targets CUP9, a transcriptional repressor. This site of UBR1 is autoinhibited but can be allosterically activated by peptides that bear destabilizing N-terminal residues and interact with two other substrate-binding sites of UBR1. We show that heme does not directly occlude the substrate-binding sites of UBR1 but blocks the activation of its CUP9-binding site by dipeptides. The N-end rule pathway, a known sensor of short peptides, nitric oxide, and oxygen, is now a sensor of heme as well. One function of the N-end rule pathway may be to coordinate the activities of small effectors, both reacting to and controlling the redox dynamics of heme, oxygen, nitric oxide, thiols, and other compounds, in part through conditional degradation of specific transcription factors and G protein regulators.

arginylation | ATE1 | ubiquitin | UBR1

The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue. The ubiquitin (Ub)-dependent N-end rule pathway recognizes several kinds of degradation signals (degrons), including a set called N-degrons (Fig. 1A) (1–6). One essential determinant of N-degron is a protein's destabilizing N-terminal residue. The N-end rule has a hierarchic structure (Fig. 1A). In eukaryotes, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation, to yield the secondary destabilizing residues Asp and Glu (1, 6). The activity of N-terminal Asp and Glu requires their conjugation to Arg, by ATE1-encoded isoforms of Arg-tRNA-protein transferase (arginyl-transferase or R-transferase) (2–4, 7). Arg is one of the primary destabilizing N-terminal residues, which are recognized by E3 Ub ligases of the N-end rule pathway, called N-recognins (Fig. 1A) (6, 8, 9). In eukaryotes that produce nitric oxide (NO), the set of destabilizing residues contains Cys as well (Fig. 1A) (3). The arginylation of N-terminal Cys must be preceded by its oxidation to Cys-sulfinate or Cys-sulfonate, a process that requires NO *in vivo* (3). The N-end rule pathway is thus a sensor of NO, through the ability of this pathway to destroy a subset of proteins with N-terminal Cys, at rates controlled by NO, and by oxygen as well (Fig. 1A) (3, 4).

The functions of the N-end rule pathway include regulation of signaling by transmembrane receptors, through the NO/O₂-dependent degradation of G protein regulators RGS4, RGS5 and RGS16; regulation of import of short peptides, through degradation of the import's repressor CUP9; fidelity of chromosome segregation, through degradation of a separase-produced cohesin fragment; regulation of apoptosis, through degradation of a caspase-processed inhibitor of apoptosis; regulation of the HIV

replication cycle, through degradation of HIV integrase; a multitude of processes mediated by the transcription factor c-FOS, a conditional substrate of the N-end rule pathway; as well as regulation of meiosis, neurogenesis, pancreatic functions, cardiovascular development, and leaf senescence in plants (refs. 3–8 and 10–12, and references therein).

Heme is an iron-containing protoporphyrin IX. Two major species of heme are ferrous (Fe²⁺) heme and its ferric (Fe³⁺) counterpart, called hemin. Intracellular proteins whose functions depend on their binding to heme include hemoglobins, cytochrome oxidases, NO synthases, catalases, and cGMP cyclases, as well as specific kinases, transcription factors, ion channels, and regulators of iron metabolism (refs. 13–23 and references therein). A major aspect of heme is its ability to interact with physiologically relevant gases such as O₂, NO and carbon monoxide (CO). Several hemoproteins, including cytochrome oxidase and NO synthases, contain heme (Fe³⁺-heme) rather than Fe²⁺-heme as an essential prosthetic group (15). Although the bulk of intracellular heme is sequestered in major hemoproteins such as hemoglobin and cytochrome oxidase, there is also a pool of "exchangeable" heme. *In vivo* regulation of this pool, including redox-mediated transitions between Fe²⁺-heme and hemin, is not well understood, and is likely to involve heme chaperones.

We now show that mammalian and yeast R-transferases (ATE1s) (Fig. 1A) are heme-binding enzymes whose arginylation activity is inhibited by heme, through the redox-based formation of disulfide between vicinal Cys-71 and Cys-72 of mouse ATE1. Heme was also found to interact with yeast and mouse Ub ligases of the N-end rule pathway (Fig. 1A). The binding of heme to *S. cerevisiae* UBR1 is shown to block the activation of one of its substrate-binding sites. Remarkably, heme was also discovered to induce the proteasome-dependent degradation of R-transferase *in vivo*, thus acting as both a "stoichiometric" and "catalytic" down-regulator of the N-end rule pathway.

Results and Discussion

Heme Binds to R-Transferase. ATE1^{-/-} mice, which lack arginylation, died as embryos with cardiovascular defects (7). The levels of both heme and embryonic ζ -globin chain were reduced in ATE1^{-/-} embryos [supporting information (SI) Fig. 6A and C]. Conversely, the activity of heme oxygenase, which catabolizes heme (23), was higher in ATE1^{-/-} embryos (SI Fig. 6B). Both metazoan and yeast R-transferases contain Heme Regulatory Motifs (HRMs), which occur in a subset of heme-binding proteins and contain the characteristic sequence Cys-Pro (13, 17, 23). C⁷¹C⁷²P and HSC⁴¹¹P, two of the four HRM-like motifs in mouse ATE1, are conserved in ATE1s of distant species (Fig. 1B–D). Gel filtration of heme (Fe³⁺-heme) and purified ATE1, as well as hemin-agarose affinity

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The authors declare no conflict of interest.

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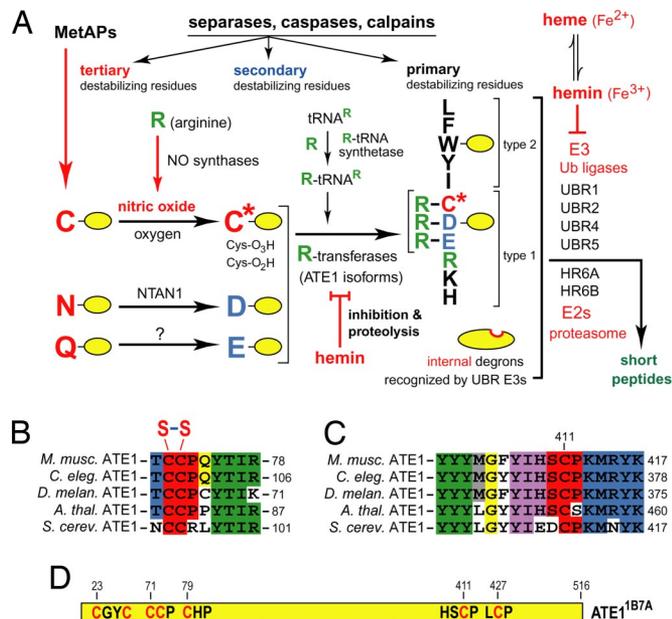


Fig. 1. Heme-mediated regulation of the N-end rule pathway. (A) The mammalian N-end rule pathway. N-terminal residues are indicated by single-letter abbreviations for amino acids. Yellow ovals denote the rest of a protein substrate. A notation, upward of “hemin” in the middle of diagram, is a “down-regulation” sign that denotes, specifically, a down-regulation of a target macromolecule that is mediated, at least in part, by target’s degradation. MetAPs, methionine aminopeptidases. C* denotes oxidized Cys, either Cys-sulfinate or Cys-sulfonate, produced in reactions mediated by nitric oxide (NO), oxygen (O₂) and their derivatives. Oxidized N-terminal Cys is arginylated by ATE1-encoded isoforms of R-transferase (2–4). Type-1 and type-2 primary destabilizing N-terminal residues are recognized by the pathway’s E3 Ub ligases, called N-recognins (9). Through their other substrate-binding sites, these E3s also recognize internal (non-N-terminal) degrons in other substrates of the N-end rule pathway, denoted by a larger oval. (B and C) Heme regulatory motifs (HRMs) in R-transferases. Numbers refer to position of the last residue of a shown sequence in the corresponding protein. A disulfide between Cys-71 and Cys-72 refers to hemin-mediated inhibition of ATE1. (D) The mouse ATE1^{1B7A} isoform (2), with locations of significant Cys-containing motifs.

chromatography with mouse cell extracts indicated the binding of hemin to mouse ATE1^{1B7A} and ATE1^{1A7A}, two isoforms of mouse R-transferase (Fig. 2C and SI Fig. 6E and G). [“ATE1” is used interchangeably with “R-transferase” (2).] Gel filtration-based spectroscopic assays (16–18) revealed the characteristic Soret band at 372 nm, with a shoulder at 423 nm, indicating a complex of hemin and R-transferase, as distinguished from free hemin (Fig. 2C). Negative controls included proteins such as lysozyme, which does not bind to hemin (Fig. 2C). The results of tryptophan fluorescence quenching assay suggested that ATE1^{1B7A} contains a hemin-binding site with the apparent K_d of $\approx 0.15 \mu\text{M}$, and possibly a second hemin-binding site as well, with the apparent K_d of $\approx 3.2 \mu\text{M}$ (Fig. 2D and SI Fig. 7A–C). The corresponding K_d values for ATE1_{C411A}, the C⁴¹¹ → A⁴¹¹ mutant in the C-terminal HRM motif (Fig. 1C), were $\approx 1.2 \mu\text{M}$ and $\approx 13 \mu\text{M}$, respectively (Fig. 2D), suggesting the relevance of this HRM, as well as the presence of more than one hemin-binding site in ATE1, in agreement with analyses of several other ATE1^{1B7A} mutants (SI Figs. 6E and 8).

Hemin Inhibits Arginylation by R-Transferase. Hemin at 5 and 10 μM inhibited either the mouse or yeast R-transferases by more than 90% and 95%, respectively, as measured by the ¹⁴C-Arg-based *in vitro* arginylation assay (2, 3) (Fig. 2). Neither FeCl₃, nor protoporphyrin IX (heme without iron), nor Zn²⁺-protoporphyrin had this effect (SI Fig. 9A). Another control showed that Arg-tRNA

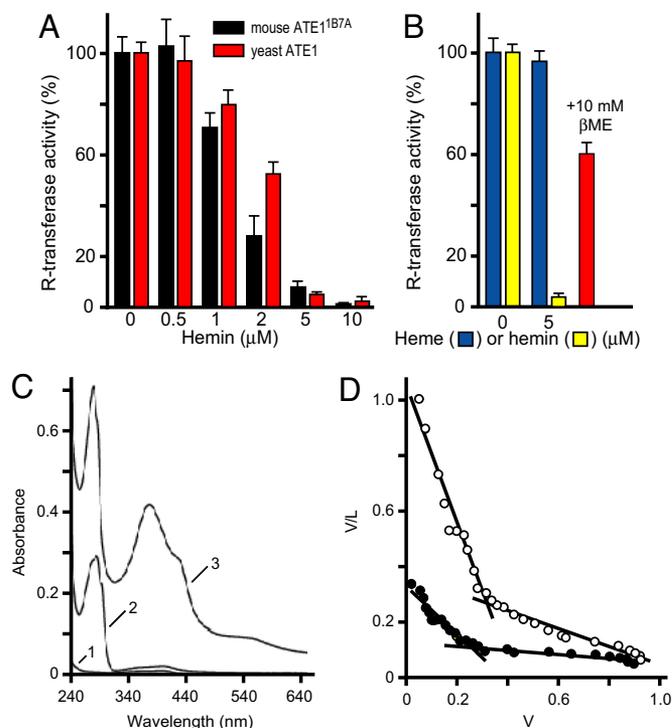


Fig. 2. Heme binds to arginyl-transferase and inhibits its activity. (A) Hemin-mediated inhibition of arginylation by mouse ATE1^{1B7A} (black bars) and *S. cerevisiae* ATE1 (red bars). (B) Comparison of mouse ATE1^{1B7A} inhibition by Fe²⁺-heme (blue bars) and Fe³⁺-heme (hemin) (yellow bars). Red bar, a partial rescue of hemin-inhibited ATE1 by 10 mM 2-mercaptoethanol. (C) Hemin was added either to buffer alone (curve 1), or to lysozyme, which does not bind to hemin (curve 2), or to purified ATE1^{1B7A} (curve 3), followed by gel filtration (to remove unbound hemin) and spectroscopic measurements. (D) Determination of hemin’s affinity for the purified wild-type ATE1^{1B7A} (open circles) and ATE1_{C411A} mutant (filled circles) (see SI Fig. 7A–C for unprocessed data).

synthetase was not significantly inhibited by hemin (SI Fig. 9B). Arginylation assay was also carried out under argon, with the reduction of hemin to Fe²⁺-heme by dithionite (17). At 5 μM , Fe²⁺-heme inhibited mouse ATE1 by <5%, whereas the inhibition by hemin (at the same concentration) exceeded 90% (Fig. 2B). Thus, in contrast to hemin, Fe²⁺-heme may not inhibit ATE1 at all, a finding whose significance becomes clear below. The inhibition of ATE1 by hemin did not involve a *trans*-acting oxidizing compound such as H₂O₂, because the inhibition was unaltered by catalase (SI Fig. 7A and B). A thiol-reducing compound such as 2-mercaptoethanol could rescue >60% of the activity of hemin-treated ATE1 (Fig. 2B). Together, these results suggested that the binding of hemin to ATE1 may lead to a redox-mediated modification of its critical Cys residue(s).

Hemin-Induced Disulfide in R-Transferase. Hemin-ATE1 interactions did not result in formation of a dimer or oligomer of ATE1 (data not shown). We are not aware of precedents for a hemin-induced intramolecular disulfide that alters activity of an enzyme. However, in non-heme contexts the activity-altering intramolecular S—S bonds were demonstrated with several cytosolic and nuclear proteins (ref. 24 and references therein). To determine whether hemin-ATE1 interactions result in an intramolecular disulfide, we used selective labeling of thiols with fluorescein-5-maleimide (FM) (Fig. 3C and SI Fig. 10C–F). At most, trace amounts of FM-conjugated ATE1 were present in the sample that had not been treated with hemin (SI Fig. 10C–F, lane 2). In contrast, hemin-treated ATE1 contained significant levels of FM (SI Fig. 10C and D, lane 1, and E, lanes 3 and 4). To identify specific disulfide-

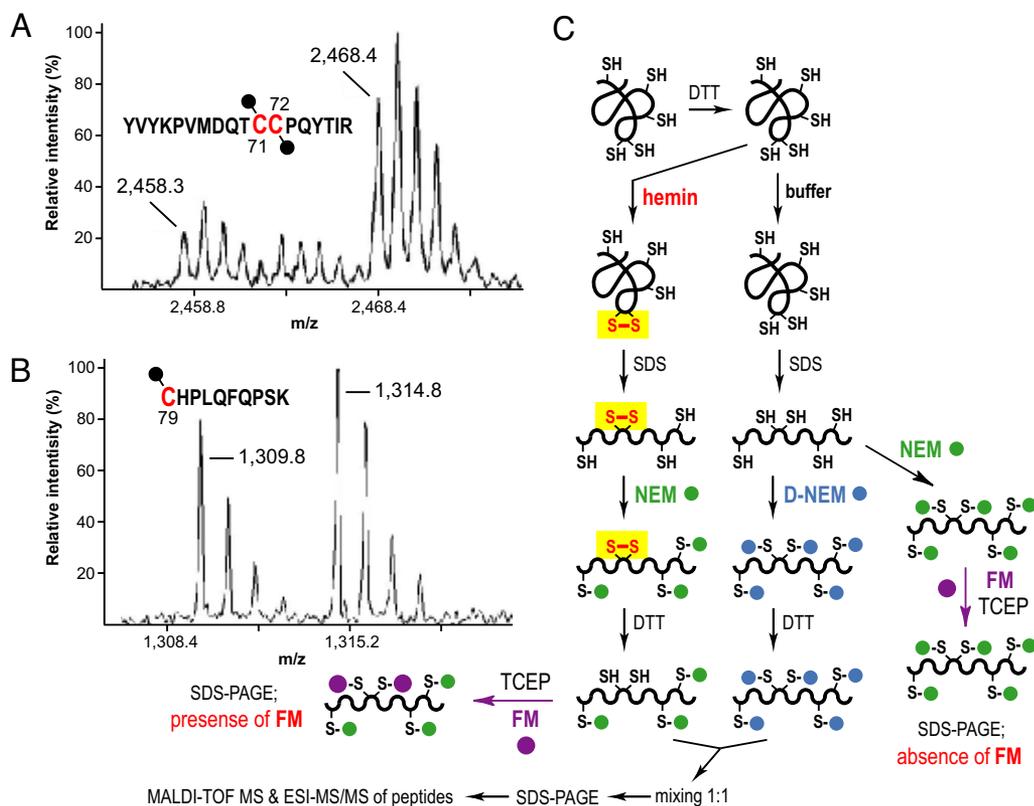


Fig. 3. Detection and mapping of hemin-induced disulfide in arginyltransferase. (A and B) mass spectrometric (MS) analysis of specific tryptic peptides from wild-type ATE1^{1B7A} that had been either treated or untreated with hemin, and thereafter processed as described in C and *Hemin-Induced Disulfide in R-Transferase*. Black circles denote NEM/D-NEM moieties conjugated to Cys residues. (C) The logic and design of experiments described in A and B and in SI Fig. 10 C–F. D-NEM, deuterated NEM. SDS-mediated denaturation of a test protein is denoted by the transition from “folded” polypeptide chains to “wavy” ones.

forming Cys residues of ATE1, the hemin-exposed and control ATE1 proteins were treated, respectively, with ¹H-NEM (NEM), i.e., protium (¹H)-containing NEM, and with ²H₅(D₅)-NEM (D-NEM), i.e., NEM containing nonexchangeable deuterium (²H). The two ATE1 samples were mixed at 1:1 molar ratio and purified by SDS/PAGE, and ATE1’s tryptic peptides were analyzed by MALDI-TOF MS and ESI-MS/MS (Fig. 3). NEM-conjugated versus D-NEM-conjugated species of a peptide such as C⁷⁹(H/D-NEM)HPLQFQPSK were present at close to 1:1 ratio (Fig. 3B). In contrast, the peptide YVYKPVMDQTC⁷¹(H/D-NEM)C⁷²(H/D-NEM)PQYTIR, which encompassed the only HRM sequence of ATE1 with vicinal Cys residues (Fig. 1B–D), contained much more of the heavier, D-NEM-containing species (Fig. 3A), as would be expected, given the logic of mapping procedure (Fig. 3C), if hemin-treated ATE1 contained the C⁷¹–C⁷² S–S bond before the second disulfide-reducing treatment (Figs. 1B and 3C). To verify this conclusion in a different way, we used the double-mutant ATE1^{C71A,C72A} (SI Fig. 8). If the C⁷¹–C⁷² disulfide (Fig. 1B) was, in fact, the single (or nearly so) hemin-induced S–S bond in wild-type ATE1, it would follow that FM treatment (Fig. 3C) would not result in a hemin-specific labeling of ATE1^{C71A,C72A}, which lacked both C⁷¹ and C⁷². This prediction was confirmed (SI Fig. 6H and I). Thus, the binding of hemin to ATE1 results in the formation of S–S bond between the vicinal C⁷¹ and C⁷² residues (Figs. 1B and 3).

ATE1^{C71A,C72A} (SI Fig. 8) virtually lacked arginylation activity, whereas ATE1^{C71S}, the C⁷¹ → S⁷¹ mutant, retained 7% of activity (SI Fig. 6D and F). The C⁷¹/C⁷² requirement for the activity of R-transferase is in agreement with the finding that the hemin-induced disulfide between C⁷¹ and C⁷² abrogates ATE1 activity (Figs. 1B, 2A and B, and 3), at least in part because a disulfide between vicinal Cys residues is a strained 8-member ring. The HRM sequence HSC⁴¹¹P (Fig. 1C and D) is likely to encompass a hemin-binding site of ATE1, and may be spatially proximal, in the folded enzyme, to its N-terminal domain that contains both the disulfide-forming region C⁷¹C⁷²P (Fig. 1B) and the thioredoxin-like motif C²³GYC²⁶ (SI Fig. 8A). In this arrangement, bound hemin

would be close to the region where hemin induces the S–S bond. Together, our results indicate a redox mechanism of ATE1 inhibition by hemin (SI Fig. 9D). In this mechanism, the intramolecular C⁷¹–C⁷² disulfide is produced through a spatially localized oxidation of C⁷¹–C⁷² by the ATE1-bound hemin (Fe³⁺-heme), in conjunction with a coupled reduction of hemin to Fe²⁺-heme (SI Fig. 9D). This model, which remains to be definitively verified, also explains the observed difference between hemin (Fe³⁺-heme) and Fe²⁺-heme in regard to inhibition of ATE1 (Fig. 2B).

Hemin Induces Degradation of R-Transferase *in Vivo*. A mouse cell line, termed 3T3^{off}ATE1^{tap}, expressed ATE1^{1B7A} with C-terminal TAP tag (see SI Text). Cells were adapted to growth in a serum-free medium and thereafter treated for 10 h with 7 μM hemin. Remarkably, both the endogenous (untagged) ATE1 and ATE1^{tap} were strongly decreased by hemin (Fig. 4F), through the induced degradation of ATE1, as could be shown directly by a pulse–chase assay (Fig. 4I and J). This effect could also be observed by measuring the arginylation activity of R-transferase in cell extracts (Fig. 4E and G). Hemin was previously shown to induce the *in vivo* degradation of Bach1, a heme-binding transcription factor (22), and of IRP2, a heme-binding translational regulator (17). A test with MG132, a proteasome inhibitor, indicated that hemin-mediated ATE1 degradation was proteasome-dependent, and also suggested that ATE1 was a substrate of the Ub-proteasome system (albeit to a lower extent) even in the absence of added hemin (Fig. 4H).

We also constructed 21 mutants of ATE1^{1B7A} (SI Fig. 8C–E) and characterized their sensitivity to hemin *in vitro*, focusing on mutants that retained at least 90% of R-transferase activity (SI Fig. 8C–E). ATE1 mutants with alterations in the HRM-like motif C⁷⁹HP were as sensitive to inhibition by hemin as was wild-type ATE1 (SI Fig. 11A). In contrast, ATE1 mutants that contained the C⁴¹¹ → A⁴¹¹ mutation in the HRM motif HSC⁴¹¹P (Fig. 1C), exhibited both a significantly lower affinity for hemin and higher resistance to inhibition by hemin (Fig. 2D and SI Fig. 11A). To explore these results *in vivo*, we constructed a cell line, termed

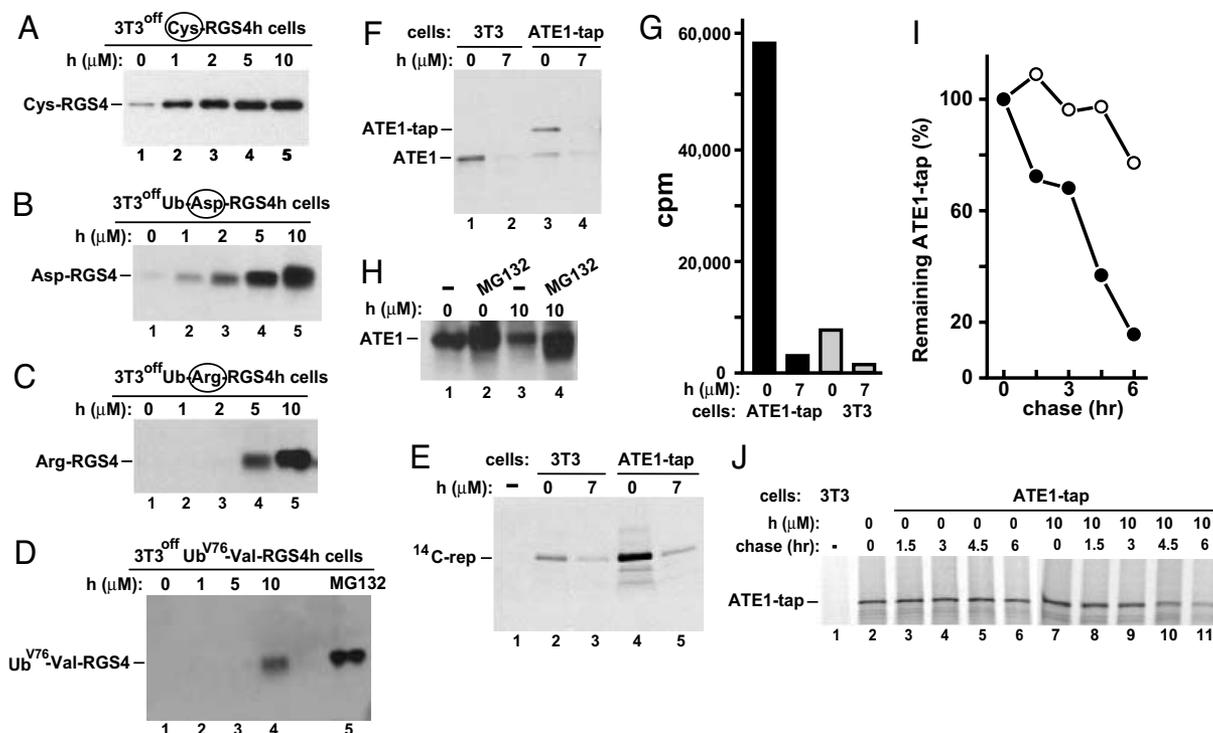


Fig. 4. Hemin induces degradation of arginyl-transferase *in vivo*. (A) A mouse cell line expressing His₆-tagged Cys-RGS4 (Cys-RGS4h) (3), was exposed for 10 h to hemin (denoted as “h”) in the medium, followed by SDS/PAGE and immunoblotting of cell extracts with anti-RGS4 antibody. (B) Same but a cell line expressing Asp-RGS4h (derived from Ub-Asp-RGS4h). (C) Same but a cell line expressing Arg-Asp-RGS4h (derived from Ub-Arg-Asp-RGS4h). (D) Same but a cell line expressing Ub^{G76V}-Val-RGS4h. Lane 5, same as lane 1 but in the presence of 5 μ M MG132 (proteasome inhibitor). (E) Comparison of ¹⁴C-arginylation of the α -lactalbumin reporter (denoted as “¹⁴C-rep”) in extracts from NIH 3T3 cells (lanes 2 and 3) or 3T3^{off}ATE1^{1B7A}tap cells (lanes 4 and 5), that had been either untreated or treated for 10 h with 7 μ M hemin in the growth medium. Lane 1, no extract added. (F) Relative amounts (determined by immunoblotting with antibody to mouse ATE1) of either untagged ATE1 (lanes 1 and 2) or both ATE1 and ATE1^{1B7A}tap (lanes 3 and 4), in either NIH 3T3 or 3T3^{off}ATE1^{1B7A}tap cells, respectively, after incubations with hemin in the medium. (G) Quantitation, by autoradiography of ¹⁴C-lactalbumin, of the data in E. (H) Lanes 1 and 2, ATE1 in NIH 3T3 cells, detected by immunoblotting in the absence of hemin treatment and either the absence or presence of 5 μ M MG132. Lanes 3 and 4, same but with hemin at 10 μ M for 10 h in the growth medium. (I) Quantitation of ATE1^{1B7A}tap pulse-chase data that are shown in J. Open circles, no added hemin. Filled circles, 10 μ M hemin was added at the end of a 30-min pulse with [³⁵S]methionine (time 0). (J) Pulse-chase assay, with cells stably expressing ATE1^{1B7A}tap in the absence or presence of added hemin. ATE1^{1B7A}tap was precipitated by immobilized IgG, which bound to the TAP tag of ATE1^{1B7A}tap. Lane 1, same as lanes 2–11 but with NIH 3T3 cells, which lack ATE1^{1B7A}tap.

3T3^{off}ATE1_{C411A,P412A}tap, that expressed ATE1_{C411A,P412A}tap, a double mutant at the HRM motif HSC^{411P} (Fig. 1C). In agreement with the *in vitro* findings (Fig. 2D and SI Fig. 11A), the *in vivo* down-regulation of wild-type ATE1 by hemin was much stronger than that of ATE1_{C411A,P412A} (SI Fig. 11B). (The actual difference must be even higher than the observed one, because 3T3^{off}ATE1_{C411A,P412A}tap cells expressed not only ATE1_{C411A,P412A}tap but also, at lower levels, the endogenous, hemin-sensitive ATE1.) Thus, the down-regulation of ATE1 by hemin *in vivo* (Fig. 4E–J) is mediated primarily (possibly solely) by the binding of hemin to ATE1.

Other constructed mouse cell lines expressed, through the Ub fusion technique (1, 2), two RGS4-based reporters: Asp-RGS4h (ATE1-dependent N-end rule substrate) and Arg-Asp-RGS4h (ATE1-independent N-end rule substrate) (Fig. 1A), with “h” denoting His₆. Another cell line expressed Ub^{G76V}-Val-RGS4h, a reporter with a “nonremovable” Ub moiety that is targeted for degradation by a non-N-end rule pathway called the UFD pathway (25). The levels of Cys-RGS4h and Asp-RGS4h, whose *in vivo* degradation requires arginylation, were strongly increased by hemin, with a significant increase already at 1 μ M hemin (Figs. 1A and 4A and B). This increase was mediated largely, possibly entirely, by the binding of hemin to ATE1, as indicated by the above result with ATE1_{C411A,P412A} (SI Fig. 11B) and also, independently, by the finding that the levels of RGS16 (a G protein regulator naturally expressed in fibroblasts) were strongly increased by hemin in wild-type

fibroblasts but not in ATE1^{-/-} fibroblasts (SI Fig. 11C). [RGS4, RGS5 and RGS16 are physiological N-end rule substrates that bear N-terminal Cys (3, 4).] Thus, while increasing the rate of *in vivo* degradation of ATE1 (Fig. 4E–J), hemin decreases the rate of degradation of a physiological ATE1 substrate such as RGS16 (SI Fig. 11C), at least in part through a decrease in the *in vivo* level of ATE1 (R-transferase).

Interestingly, Arg-Asp-RGS4h, the ATE1-independent N-end rule substrate (Fig. 1A), was detectably stabilized only at 5 μ M and higher levels of hemin, in contrast to Cys-RGS4h and Asp-RGS4h (Fig. 4A and B, compare C). This pattern suggests a differential, fine-tuned response to heme by specific parts of the N-end rule pathway (Fig. 1A), a finding to be explored. Ub^{G76V}-Val-RGS4h, a reporter substrate of a non-N-end rule pathway (25), was detectably stabilized only at 10 μ M hemin (Fig. 4D, compare A and B). Thus, hemin can inhibit not only the N-end rule pathway, but also (at higher concentrations) other Ub-dependent pathways as well (Figs. 1A and 4A–C). More than 25 years ago, the addition of hemin (at levels an order of magnitude higher than above) to an extract from reticulocytes was shown to inhibit degradation of some proteins in the extract (ref. 26 and references therein).

Hemin Blocks the Activation of N-End Rule’s Ubiquitin Ligase. Extracts from mouse 3T3 cells were subjected to hemin-agarose chromatography and immunoblotting for UBR1 (SI Fig. 7D). Both UBR1 and UBR2 were found to bind to hemin, with UBR2 examined by

a similar assay, employing an extract from *S. cerevisiae* that expressed mouse UBR2 tagged with the flag epitope (SI Fig. 7C, lane 1). Several otherwise dissimilar mouse E3s, termed UBR4-UBR7, have in common the UBR domain (which is also present in UBR1 and UBR2) and have been shown to behave, in binding assays, as N-recognins (9). These E3s also contain HRM motifs. One such N-recognin, the 300-kDa mouse UBR5 (EDD/hHYD), was examined and found to interact with hemin (data not shown), similarly to mouse UBR1 and UBR2. Two substrate-binding sites of yeast UBR1, the sole N-recognin of *S. cerevisiae*, bind, respectively, to the type-1 (basic) and type-2 (bulky hydrophobic) destabilizing N-terminal residues of either protein-sized substrates or short peptides (Fig. 1A) (1, 8, 10). The third binding site of UBR1 targets (through an internal degron) the transcriptional repressor CUP9, which controls a regulon that includes PTR2, the importer of short peptides (8). Peptides bearing type-1 or type-2 N-terminal residues (Fig. 1A) interact with the corresponding sites of UBR1, and allosterically activate its (autoinhibited) third site, which targets CUP9. Through this positive feedback, mediated by the conditional degradation of CUP9, *S. cerevisiae* can sense the presence of extracellular peptides and react by accelerating their uptake (8, 10). The activation, by dipeptides, of the UBR1-CUP9 interaction can be reconstituted *in vitro* (Fig. 5A and SI Fig. 10G and H) (8). Whereas ^fUBR1 did not bind to CUP9 in the presence of Ala-Leu and Ala-Arg (dipeptides with a stabilizing N-terminal residue), the binding of CUP9 was activated in the presence of Arg-Ala and Leu-Ala, bearing type-1/2 N-terminal residues (Figs. 1A and 5A, lane 2 vs. 3) (8).

Remarkably, the addition of hemin inhibited the binding of UBR1 to CUP9 irrespective of the presence of binding-activating dipeptides. The inhibition was essentially complete at 50 μ M hemin (Fig. 5 and SI Fig. 10G). Previous work (8) has shown that the autoinhibition of CUP9-binding site of UBR1 (its default state, in the absence of type-1/2 dipeptides) requires full-size UBR1, in that UBR1¹⁻¹¹⁴⁰, its N-terminal half, could bind to CUP9 in either the presence or absence of dipeptides (8). We found that, whereas hemin inhibited the dipeptide-mediated activation of CUP9 binding by full-length UBR1, hemin had no effect, even at 0.1 mM, on the binding of CUP9 to C-terminally truncated UBR1¹⁻¹¹⁴⁰ (Fig. 5A and SI Fig. 10G and H). [Both full-length UBR1 and UBR1¹⁻¹¹⁴⁰ have affinity for hemin, similarly to mouse UBR2 (SI Fig. 10C).] Hemin did not affect the ability of full-length UBR1 to recognize destabilizing N-terminal residues (SI Fig. 10I). Thus, hemin does not directly occlude the substrate-binding sites of UBR1, but blocks a conformational transition (8) that underlies the allosteric activation (mediated by type-1/2 dipeptides) of the CUP9-binding site of UBR1 (Fig. 5B and C). Hemin did not appear to have an *in vivo* proteolytic effect on mouse UBR1 (data not shown).

Concluding Remarks

We discovered that the *ATE1*-encoded R-transferase, which mediates the arginylation branch of the N-end rule pathway (Fig. 1A), is a heme-binding enzyme in both fungi and mammals. The arginylation activity of ATE1s was inhibited by low-micromolar levels of hemin (Fe³⁺-heme), through a redox mechanism that produces disulfide between vicinal Cys-71 and Cys-72 of mouse ATE1. Remarkably, hemin was also found to induce the proteasome-dependent degradation of R-transferase *in vivo*, thus acting as both a “stoichiometric” and “catalytic” down-regulator of the N-end rule pathway. The mechanism through which hemin up-regulates the *in vivo* degradation of ATE1 remains to be analyzed. The above “dual” repression of R-transferase by hemin greatly increased the *in vivo* levels of N-end rule substrates such as RGS4 and RGS16 (Fig. 4A and SI Fig. 11C), which control the signaling by G proteins. Hemin also binds to the yeast and mouse Ub ligases of the N-end rule pathway (Fig. 1A). The binding of hemin to *S. cerevisiae* UBR1 blocks the activation of one of its substrate-binding sites (Fig. 5B and C). The N-end rule pathway, a known sensor of

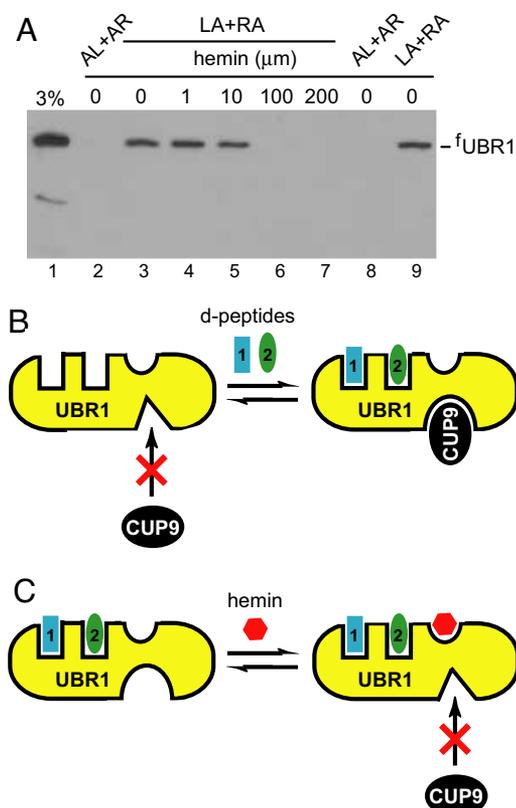


Fig. 5. Hemin inhibits activation of CUP9 binding by the UBR1 ubiquitin ligase. (A) Equal amounts of extract from *S. cerevisiae* expressing ^fUBR1 were incubated with glutathione-Sepharose beads preloaded with GST-CUP9, in either the presence or absence of indicated dipeptides (at 1 mM each) (8), and either in the absence or presence of hemin, at indicated levels. The bound proteins were eluted, fractionated by SDS/PAGE and immunoblotted with anti-flag antibody. Lane 1, input sample (3% of extract's amount used in GST-pulldowns). Lane 2, ^fUBR1 and GST-CUP9 in the presence of Ala-Leu and Ala-Arg (note the absence of interaction between UBR1 and CUP9). Lane 3, same but in the presence of Leu-Ala and Arg-Ala. Lanes 4–7, same as lane 3 but in the presence of 1, 10, 100, or 200 μ M hemin. Lanes 8 and 9, same as lanes 2 and 3, respectively, but in the absence of 1 mM NaOH (a control, because 1 mM NaOH was present in other samples (lanes 2–7), owing to the NaOH-containing hemin's stock solution). (B and C) A diagram (based in part on the present findings) of interactions amongst UBR1, dipeptides with type-1 or -2 destabilizing N-terminal residues, and the CUP9 transcriptional repressor. See the main text.

short peptides, NO and oxygen (2–4, 8, 10), and a regulator of biological processes cited in Introduction, is now a sensor of heme as well. One function of this proteolytic circuit may be to coordinate the redox dynamics of heme, NO, oxygen, thiols, and other small effectors, by sensing them through components of the N-end rule pathway, and by acting to alter the levels, activity or spatiotemporal gradients of these compounds, in part through the conditional degradation of transcriptional regulators such as CUP9 and c-FOS (8, 10, 12) or regulators of G proteins such as RGS4, RGS5 and RGS16 (3, 4).

The inhibition of R-transferase by Fe³⁺-heme (hemin) is a property of this enzyme that is conserved from fungi to mammals. What physiology of the heme-ATE1 connection underlied selective pressures that led to the emergence and retention of this property over vast phylogenetic distances? Given the cardiovascular defects of *ATE1*^{-/-} embryos (7), their low levels of embryonic globin and heme, high levels of heme oxygenase (SI Fig. 6A–C), and their perturbed hematopoiesis (J. Sheng, R.-G.H., and A.V., unpublished data), the N-end rule pathway is likely to play a role in the control

of heme synthesis, transport, and/or catabolism. RGS4 is a down-regulator of tubulogenesis, a process that underlies the development and homeostasis of blood vessels and other tubular structures such as those of the mammary gland, kidney and the lung (27). Heme-mediated inhibition of the N-end rule pathway (Fig. 1A) and the resulting increase in the levels of N-end rule substrates such as RGS4 and RGS16 (Fig. 4A–C and SI Fig. 11C) would be expected to inhibit tubulogenesis. Both RGS4 and RGS16 block the signaling by VEGF, whereas RGS5 (another physiological N-end rule substrate) was implicated in the control of vessel remodeling during neovascularization (27, 28). Thus, in addition to the discovery of a link between heme and R-transferases (Fig. 1A), one physiological insight of the present work is that the N-end rule pathway is a mediator of heme's effects on tubulogenesis. The sensor–effector link between the N-end rule pathway and heme may be relevant not only to normal conditions but also to perturbations of heme homeostasis, for example upon a “spontaneous” or wound-induced hemorrhage, or in a hemorrhage-prone setting of a growing tumor. Our identification of the G protein regulators RGS4, RGS5, and RGS16 as NO-dependent N-end rule substrates (3), and the present discovery that the N-end rule pathway is a sensor of both heme and NO (Fig. 1A) adds a new dimension to the involvement of NO in heme-mediated processes (16).

Over the last decade, it became clear that reactive oxygen species (ROS) such as H_2O_2 , if they are present at “signaling” (i.e., sufficiently low) levels, can act as regulators of circuits that underlie not only stress responses but also other functions, including the cell cycle, transcription and differentiation (reviewed in ref. 24). ROS-mediated signaling utilizes cysteine-containing protein sensors, some of which contain heme as well. A direct connection, through the N-end rule pathway, between heme and protein degradation (Fig. 1A) may also play a role in the multifaceted regulation by “signaling” levels of H_2O_2 and other ROS compounds. This aspect of our findings remains to be explored.

In contrast to O_2 and CO, which bind only to Fe^{2+} -heme, NO can interact with either Fe^{3+} -heme (hemin) or Fe^{2+} -heme. The latter is apparently inactive as an efficacious inhibitor of ATE1 (Fig. 2B). Moreover, the hemin moiety bound to NO usually undergoes rapid autoreduction to Fe^{2+} -heme. Thus, in addition to its role in converting proteins with N-terminal Cys into “arginylatable” N-end rule substrates (3) (Fig. 1A), NO may also counteract the hemin-

mediated inhibition of ATE1. In other words, besides making possible (through oxidation of N-terminal Cys) the arginylation of Cys-bearing ATE1 substrates (3), NO may also up-regulate the targeting of all ATE1 substrates, including those with N-terminal Asp or Glu (Fig. 1A), by protecting ATE1 (R-transferase) from down-regulation by hemin.

It is likely that the set of arginylation-dependent N-end rule substrates is much larger than the three RGS proteins above. For example, yet another such substrate is a separase-produced fragment of mammalian cohesin, whose stabilization in mouse *ATE1*^{-/-} cells (which lack arginylation) results in chromosome instability (J. Zhou, J. Sheng, R. G. H., and A.V., unpublished data). The simultaneous sensitivity of the N-end rule pathway to heme, NO, oxygen, short peptides, and redox may underlie the ability of cells to integrate the often divergent effects of small compounds to produce functionally adaptive outputs.

Methods Summary

For descriptions of materials and methods, see *SI Text*, *SI Figs. 6–11*, *SI Tables 1 and 2*, and associated references. Mouse *ATE1*^{187A} R-transferase (2), *S. cerevisiae* ATE1, and *ATE1*^{187A} mutants (*SI Fig. 8*), were produced and purified as described in ref. 3 and *SI Text*. Spectroscopic assays were performed as described (16–18). Measurements of heme oxygenase, heme, and embryonic ζ -globin chain were carried with extracts from wild-type and *ATE1*^{-/-} mouse embryos. Arginylation assays were carried out with ¹⁴C-Arg, the α -lactalbumin reporter, and purified *S. cerevisiae* or mouse R-transferase (or, alternatively, with extracts from mouse cells either treated or untreated with hemin) (2, 3), as described in *SI Text*. Tryptophan fluorescence quenching assay and the mapping of disulfide in ATE1 are also described in *SI Text*. For methods used to characterize heme interactions with UBR proteins, see refs. 8 and 9 and *SI Text*. For construction of mouse cell lines and their use, see *SI Text*.

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