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The N-end rule pathway as a nitric oxide sensor controlling the levels of multiple regulators

Rong-Gui Hu[1]*, Jun Sheng[1]*, Xin Qi[2], Zhenming Xu[1]†, Terry T. Takahashi[2] & Alexander Varshavsky[1]

The conjugation of arginine to proteins is a part of the N-end rule pathway of protein degradation. Three amino (N)-terminal residues—aspartate, glutamate and cysteine—are arginylated by *ATE1*-encoded arginyl-transferases. Here we report that oxidation of N-terminal cysteine is essential for its arginylation. The *in vivo* oxidation of N-terminal cysteine, before its arginylation, is shown to require nitric oxide. We reconstituted this process *in vitro* as well. The levels of regulatory proteins bearing N-terminal cysteine, such as RGS4, RGS5 and RGS16, are greatly increased in mouse *ATE1*$^{-/-}$ embryos, which lack arginylation. Stabilization of these proteins, the first physiological substrates of mammalian N-end rule pathway, may underlie cardiovascular defects in *ATE1*$^{-/-}$ embryos. Our findings identify the N-end rule pathway as a new nitric oxide sensor that functions through its ability to destroy specific regulatory proteins bearing N-terminal cysteine, at rates controlled by nitric oxide and apparently by oxygen as well.

The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue[1–4]. A ubiquitin-dependent pathway, called the N-end rule pathway, recognizes degradation signals (degrons) that include the signals called N-degrons (Fig. 1a). An N-degron consists of a protein's destabilizing N-terminal residue and an internal Lys residue. The latter is the site of formation of a polyubiquitin chain[5–8]. The N-end rule has a hierarchic structure. N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their deamidation, by N-terminal amidohydrolases[9,10], to yield the secondary destabilizing residues Asp and Glu. The activity of N-terminal Asp and Glu requires their conjugation, by *ATE1*-encoded isoforms of Arg-tRNA-protein transferase (R-transferase), to Arg, one of the primary destabilizing residues[11,12]. The latter are recognized by E3 ubiquitin ligases of the N-end rule pathway[2,4,13,14]. In mammals, destabilizing N-terminal residues that function through their arginylation are not only Asp and Glu but also Cys (Fig. 1a), which is a stabilizing (unarginylated) residue in the yeast *Saccharomyces cerevisiae*[11,12,15]. Known functions of the N-end rule pathway include the control of peptide import (through conditional degradation of the import's repressor)[13,16], the regulation of apoptosis (through degradation of a caspase-processed inhibitor of apoptosis)[3,17] and the fidelity of chromosome segregation (through degradation of a conditionally produced cohesin's fragment)[18], as well as the regulation of meiosis, cardiovascular development in animals and leaf senescence in plants (refs 4, 12 and references therein).

Nitric oxide (NO) is produced in eukaryotes largely by NO synthases. This compound and its derivatives have a function, as either stressors or regulators, in a vast range of processes, including cardiovascular homeostasis, immunity, neurotransmission, ion conductance, glycolysis and apoptosis (reviewed in refs 19–26). Biological effects of NO are mediated by its covalent modifications of proteins, either of their prosthetic groups or amino-acid residues, particularly Cys and Tyr. The reactivity of these residues towards NO is modulated by their sequence contexts[20–23,27–29]. NO converts Cys residues to *S*-nitrosothiols, a process that can involve oxygen or its derivatives. *S*-Nitrosylation modulates protein functions either directly or after additional (often oxygen-dependent) transformations that yield oxidized Cys, such as Cys-sulphinic acid ($CysO_2H$) or Cys-sulphonic acid ($CysO_3H$) (refs 20–22).

An N-degron is produced from pre-N-degron through a proteolytic cleavage. Methionine aminopeptidases (MetAPs) remove Met from the N terminus of a newly formed protein if, and only if, the residue at position 2, to be made N-terminal after cleavage, has a small enough side chain[2]. Consequently, of the 13 destabilizing residues of the mammalian N-end rule (Fig. 1a), only Cys can be made N-terminal by MetAPs (Fig. 1b). (Any destabilizing residue, including Cys, can be made N-terminal through internal cleavages of proteins by other proteases, such as separases, caspases and calpains[3,17,18] (Fig. 1a).) Our previous work showed that Cys at position 2 of the RGS4 protein arginylated *in vivo* was $CysO_3H$, rather than Cys, suggesting that the oxidation of N-terminal Cys might precede arginylation, and might be required for it[12].

We now show that oxidation of N-terminal Cys is essential for its arginylation. We also discovered that the oxidation of a protein's N-terminal Cys *in vivo*, before its arginylation, requires NO. This explains why N-terminal Cys is a destabilizing residue in mammalian cells[2,12,15], which produce NO, but stabilizing in yeast[5], which lack NO synthases. We reconstituted the NO-dependent arginylation of N-terminal Cys in an *in vitro* system as well. This process requires a basic residue at position 2 of a substrate. The levels of regulatory proteins with this N-terminal motif (Cys-(basic residue)), such as RGS4, RGS5 and RGS16, are greatly increased in mouse *ATE1*$^{-/-}$ embryos, which lack arginylation. RGS4, RGS5 and RGS16 are the first physiological substrates of the mammalian N-end rule pathway. Given the involvement of these proteins in cardiovascular

[1]Division of Biology, and [2]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125, USA. †Present address: Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697-4120, USA.
*These authors contributed equally to this work.

homeostasis[30,31] and tubulogenesis[32], their stabilization might underlie the previously observed abnormal angiogenesis and heart defects in *ATE1*$^{-/-}$ embryos[12]. A mammalian genome encodes approx. 30 proteins, including RGS4, RGS5 and RGS16, that contain the Met-Cys-(basic residue) N-terminal motif, which acts as a MetAP-cleaved, NO-dependent, arginylation-mediated, Cys-containing pre-N-degron. Taken together, our results identify the arginylation branch of the N-end rule pathway as a new sensor of NO in mammalian cells that functions through its ability to destroy specific regulatory proteins bearing N-terminal Cys, at rates controlled by NO and apparently by oxygen as well.

Oxidation of N-terminal cysteine is required for its arginylation

We wished to determine whether the presence of $CysO_3H$ (instead of Cys) at position 2 of the RGS4 protein arginylated *in vivo*[12] reflected the requirement for oxidation of Cys before its arginylation, as

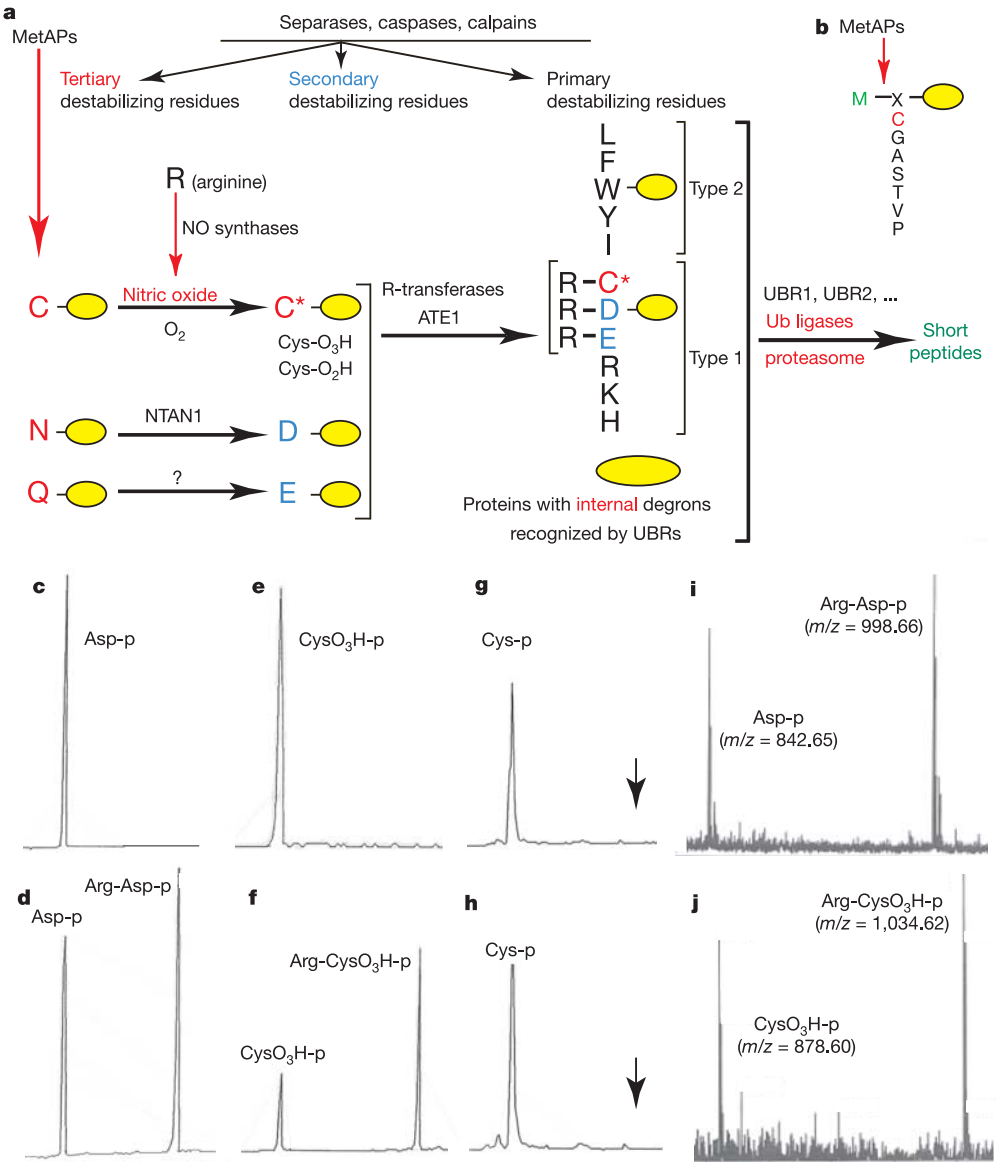

Figure 1 | N-terminal cysteine must be oxidized before its arginylation. **a**, The mammalian N-end rule pathway. N-terminal residues are indicated by single-letter abbreviations for amino acids. Yellow ovals denote the rest of a protein substrate. The 'cysteine' (Cys) sector, in the upper left corner, describes the main discovery of this work: the NO-mediated oxidation of N-terminal Cys, with subsequent arginylation of oxidized Cys by *ATE1*-encoded isoforms of R-transferase. C* denotes oxidized Cys, either Cys-sulphinic acid ($CysO_2H$) or Cys-sulphonic acid ($CysO_3H$). Type 1 and type 2 primary destabilizing N-terminal residues are recognized by E3 ubiquitin (Ub) ligases of the N-end rule pathway, including UBR1 and UBR2. Through their other substrate-binding sites these E3 ubiquitin ligases also recognize internal (non-N-terminal) degrons in other substrates of the N-end rule pathway, denoted by a larger yellow oval. **b**, MetAPs remove Met from the N terminus of a polypeptide if the residue at position 2 belongs to the set of residues shown. **c–j**, N-terminal Cys must be oxidized before its arginylation. Three eight-residue peptides are denoted as X-p; their N-terminal residues (X) were either Asp, Cys or $CysO_3H$. X-p was incubated with mouse ATE1-1 R-transferase at pH 7.5 in the presence of ATP, *S. cerevisiae* Arg-tRNA synthetase and tRNAs, followed by analyses of peptide products, either by capillary electrophoresis (CE) (**c–h**) or by MALDI–TOF MS (**i**, **j**). The *x* and *y* axes (not shown) in CE patterns correspond, respectively, to the time of elution from CE column and A_{200}. **c**, **d**, Arginylation assay with Asp-p, for 0 min (**c**) and 60 min (**d**), followed by CE. **e**, **f**, As in **c** and **d** but with $CysO_3H$-p. **g**, **h**, As in **c** and **d** but with Cys-p. Vertical arrows in **g** and **h** indicate the electrophoretic position of the (separately run) marker Arg-Cys-p, a chemically synthesized arginylated Cys-p. **i**, MALDI–TOF MS of the sample in **d**. **j**, MALDI–TOF MS of the sample in **f**. The molecular masses in **i** and **j** are of ionized $[+H^+]$ derivatives of the molecules indicated on these panels in their un-ionized form.

distinguished from oxidation of Cys after its arginylation. Three eight-residue XHGSGAWL peptides (where X represents Asp, Cys or $CysO_3H$) were incubated with mouse ATE1-1 R-transferase[11] in the presence of ATP, *S. cerevisiae* Arg-tRNA synthetase and tRNAs. Both the Asp-peptide and the $CysO_3H$-peptide were efficiently arginylated, whereas the Cys-peptide was not arginylated, as determined by capillary electrophoresis (compare Fig. 1c–f with Fig. 1g, h), and confirmed, with regard to the identities of products, by matrix-assisted laser desorption ionization–timeofflight (MALDI–TOF) MS (Fig. 1i, j). The same results were obtained with *S. cerevisiae* ATE1 R-transferase[33], a strong 'sequelogue'[34] of mammalian R-transferases[11] (Supplementary Fig. S1). We conclude that the oxidation of N-terminal Cys is essential for its arginylation (Fig. 1c–j).

Increased levels of RGS proteins in *ATE1*$^{-/-}$ embryos

RGS4 is a GTPase-activating protein (GAP) for specific Gα subunits of G proteins and is a member of the family of RGS proteins that downregulate signalling by G proteins[30–32,35–37]. Our earlier work showed that RGS4 (which begins, as a nascent protein, with Met-Cys) was a substrate of the N-end rule pathway in reticulocyte extract[38]. The levels of endogenous RGS4 were also increased by proteasome inhibitors[37] but the pathway of RGS4 degradation *in vivo* remained to be identified.

We compared the levels of endogenous RGS4 between 12.5-day-old (E12.5) wild-type mouse embryos and *ATE1*$^{-/-}$ embryos, which lacked R-transferases and therefore lacked arginylation[12]. The level of RGS4 in E12.5 *ATE1*$^{-/-}$ embryos was strikingly higher than in E12.5 wild-type embryos (Fig. 2a). Similar results were obtained in pairwise comparisons of RGS4 in specific tissues from E14.5 wild-type and *ATE1*$^{-/-}$ embryos (Fig. 2b). Two other members of the RGS family, RGS5 and RGS16, also bear N-terminal Cys. Experiments analogous to those with RGS4 revealed strong increases in RGS5 and RGS16 levels in *ATE1*$^{-/-}$ embryos (Fig. 2c–f). These protein patterns (Fig. 2a–f) were not caused by increased levels of the corresponding

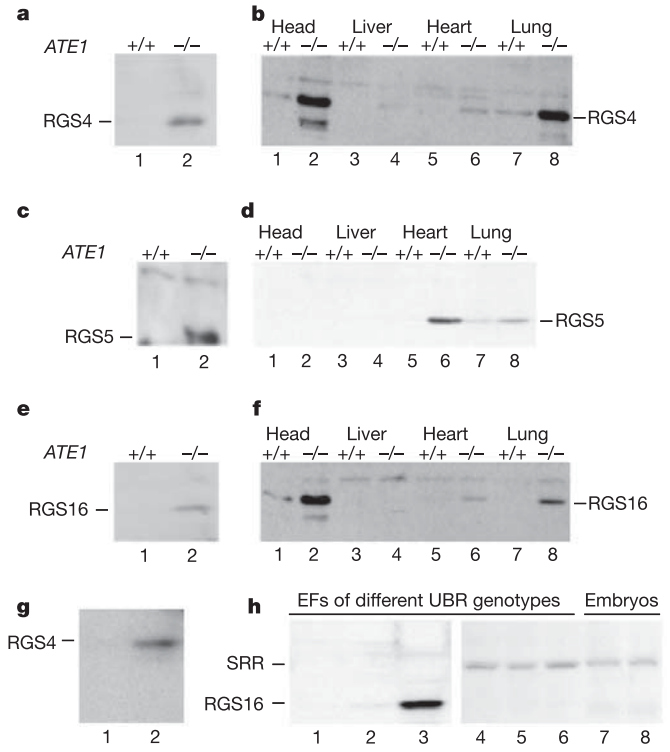

Figure 2 | Strongly increased levels of RGS4, RGS5 and RGS16 proteins in *ATE1*$^{-/-}$ embryos. **a**, Lanes 1 and 2, equal amounts of total protein in extracts from wild-type (+/+) and *ATE1*$^{-/-}$ E12.5 embryos were fractionated by 12% SDS–PAGE, followed by immunoblotting with anti-RGS4 antibody. **b**, As in **a** but with extracts from the indicated tissues of wild-type and *ATE1*$^{-/-}$ E14.5 embryos. **c**, As in **a** but with anti-RGS5 antibody and E14.5 embryos. **d**, As in **b** but with anti-RGS5 antibody. **e**, As in **a** but with anti-RGS16 antibody. **f**, As in **b** but with anti-RGS16 antibody. **g**, 3T3^{toff}RGS4$_{fh}$ cells expressing RGS4$_{fh}$ were grown in the presence of either ambient air (lane 1) or low (0.5%) oxygen concentration (lane 2). Equal amounts of total protein in extracts were subjected to immunoblotting with antibody against RGS4. **h**, Lanes 1–3, equal amounts of total protein in extracts from wild-type (lane 1), [*UBR1*$^{-/-}$*UBR2*$^{-/-}$] (lane 2) and UBR1/2^{dnR2} (lane 3) cell lines were subjected to immunoblotting with antibody against RGS16. Lanes 4–6, as in lanes 1–3, respectively, but immunoblotting with antibody against serine racemase (SRR). Lanes 7, 8, as in lanes 4–6 but with extracts from wild-type (lane 7) and *ATE1*$^{-/-}$ (lane 8) E12.5 embryos. The bands of 23K RGS4 (apparent molecular mass ~28 kDa), 20K RGS5 (apparent molecular mass ~21 kDa), 23K RGS16 (apparent molecular mass ~25 kDa) and 37K SRR are indicated in **a**–**h**.

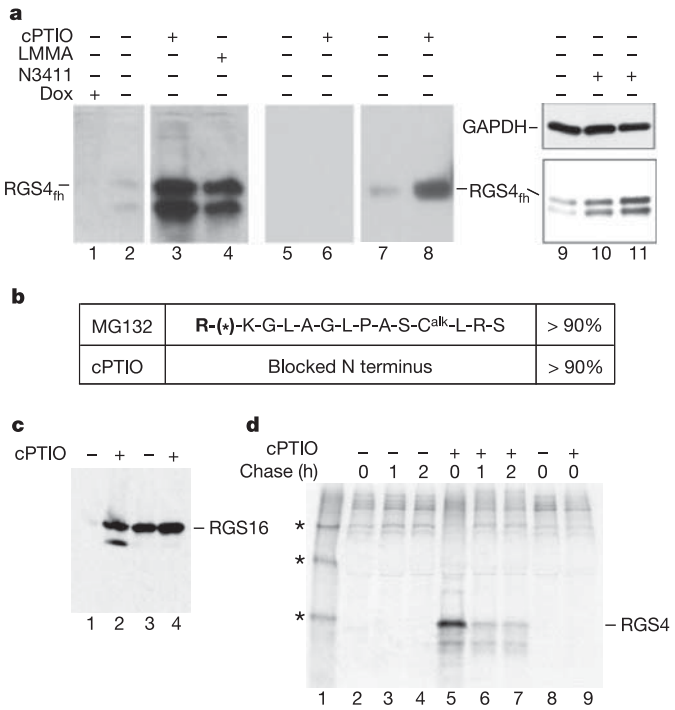

Figure 3 | Decreasing NO concentration *in vivo* stabilizes RGS4 and RGS16. **a**, 3T3^{toff}RGS4$_{fh}$ cells, expressing RGS4$_{fh}$, were either untreated or treated with compounds that decrease the levels of intracellular NO, followed by immunoblotting with antibody against RGS4. Lanes 7–8 and 9–11 show two sets of experiments independent of those in lanes 1–4, with independently grown 3T3^{toff}RGS4$_{fh}$ cells. The concentrations of N3411 used in lanes 10 and 11 were 0.5 and 1 mM, respectively. Also indicated is the band of 37-kDa glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a loading control, detected on the same membrane with antibody against GAPDH. **b**, Determination, through Edman degradation, of the N-terminal sequence of RGS4$_{fh}$ isolated from 3T3^{toff}RGS4$_{fh}$ cells that had been treated as described in the panel and in the text. C^{alk}, alkylated Cys residue. The asterisk after the N-terminal, post-translationally conjugated Arg (R) residue indicates a 'sequenceable' but unidentified residue, at the position of oxidized Cys residue in RGS4, in contrast to alkylated (identifiable) Cys (C^{alk}) at position 12. **c**, Lanes 1 and 2, immunoblotting, with antibody against RGS16, of extracts from NIH-3T3 cells that were either untreated (lane 1) or treated with cPTIO (lane 2). Lanes 3 and 4, as in lanes 1 and 2 but with *ATE1*$^{-/-}$ EF cells[12] lacking arginylation. **d**, Lane 1, ^{14}C-labelled protein markers, of molecular masses 66, 45 and 30 kDa (asterisks). Lanes 2–4, RGS4$_{fh}$-expressing 3T3^{toff}RGS4$_{fh}$ cells were labelled for 10 min with ^{35}S-methionine/cysteine and chased for 1 and 2 h, followed by immunoprecipitation of extracts with antibody against RGS4, then by SDS–PAGE and autoradiography. Lanes 5–7, as in lanes 2–4 but with cPTIO-treated 3T3^{toff}RGS4$_{fh}$ cells. Lanes 8 and 9, as in lanes 2 and 5, respectively, but with 3T3^{toff} (RGS4$_{fh}$-lacking) cells.

messenger RNAs, as indicated by both complementary DNA microarray comparisons (J.S., R.G.H., S. Choi., M. Simon and A.V., unpublished observations) and northern analyses (Supplementary Fig. S2).

To address the above issues differently, we employed mouse cells termed UBR1/2^{dnR2}. These [*UBR1*$^{-/-}$*UBR2*$^{-/-}$] cells, lacking two of several E3 ubiquitin ligases of the N-end rule pathway[4,14], stably express UBR2^{1041}, the N-terminal fragment of mouse UBR2 that functions as a dominant-negative inhibitor of the (residual) N-end rule pathway in parental [*UBR1*$^{-/-}$*UBR2*$^{-/-}$] cells (J.S., Z. Xia, Y. T. Kwon and A.V., unpublished observations). The level of endogenous RGS16 was negligible in wild-type cells, barely detectable in [*UBR1*$^{-/-}$*UBR2*$^{-/-}$] cells and much higher in UBR1/2^{dnR2} cells (Fig. 2h, lanes 1–3). These results indicated yet again, in a setting independent from that of *ATE1*$^{-/-}$ cells, that RGS16 was a substrate of the N-end rule pathway *in vivo*.

Decreasing NO *in vivo* stabilizes RGS4, RGS5 and RGS16

To examine the possibility that the oxidation of N-terminal Cys might involve NO, we constructed a mouse cell line, termed 3T3^{toff}RGS4$_{fh}$, that expressed RGS4-Flag-His$_6$ (RGS4$_{fh}$) from a doxycycline-repressible promoter. Treatment of 3T3^{toff}RGS4$_{fh}$ cells with N^G-monomethyl-L-arginine (LMMA), an inhibitor of NO synthases (NOSs), markedly increased the level of RGS4 *in vivo* (Fig. 3a, compare lanes 2 and 4). When 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a cell-penetrating NO scavenger, was used to decrease NO levels, the increase in RGS4 was even more striking (Fig. 3a, compare lanes 2 and 3, and lanes 7 and 8). In the experiments of Fig. 3a, lanes 1–4 and 9–11, anti-RGS4 antibody detected two bands: the upper band's position was that expected for RGS4$_{fh}$; the lower was apparently a proteolytic fragment of RGS4$_{fh}$, because changes in its levels paralleled those of full-length RGS4$_{fh}$. The lower band was not observed in an otherwise identical but independent experiment of Fig. 3a, lanes 7 and 8. In addition, the same anti-RGS4 antibody did not detect RGS4 in parental 3T3^{toff} cells (Fig. 3a, lanes 5 and 6), and detected one RGS4 band in *ATE1*$^{-/-}$ embryos (Fig. 2a).

Reverse-transcriptase-mediated polymerase chain reaction showed that 3T3^{toff}RGS4$_{fh}$ cells expressed *NOS1* (neuronal NOS) mRNA but little if any *NOS2* (inducible NOS) and *NOS3* (endothelial NOS) mRNAs (Supplementary Fig. S3). Given these findings, we also used an NOS inhibitor (N3411) that is highly selective for NOS1. In agreement with other NO results (Fig. 3a, lanes 1–8), and despite the near-confinement of inhibition by N3411 to NOS1, this inhibitor substantially increased the levels of RGS4 (Fig. 3a, lanes 9–11). We also examined whether RGS4 was elevated in *NOS1*$^{-/-}$ adult mice[24]. Despite the presence of NOS2 and NOS3 in these mice, RGS4 was strongly increased in the *NOS1*$^{-/-}$ lung, relative to the wild-type lung, and to a smaller extent in other tissues as well (Supplementary Fig. S4).

To determine whether a decrease in NO concentration could affect the arginylation of RGS4 *in vivo*, we used N-terminal (Edman) sequencing. RGS4$_{fh}$ purified from control 3T3^{toff}RGS4$_{fh}$ cells was completely or nearly completely (more than 90%) arginylated *in vivo* (Fig. 3b). In contrast, most RGS4$_{fh}$ from cells that had been treated with cPTIO could not be sequenced by Edman degradation (Fig. 3b). The identity of the blocking modification of RGS4$_{fh}$ remains to be determined. Thus, RGS4$_{fh}$ from cells with decreased NO was largely unarginylated (Fig. 3b) and therefore not a target of the N-end rule pathway, in agreement with other results (Fig. 3a). In a pulse–chase assay, a negligible amount of ^{35}S-labelled RGS4$_{fh}$ was present at the end of a 10-min pulse in untreated 3T3^{toff}RGS4$_{fh}$ cells (Fig. 3d, lanes 2–4). In contrast, a strongly labelled band of RGS4$_{fh}$ was observed with cPTIO-treated cells at the end of a 10-min pulse (Fig. 3d, compare lanes 2 and 5). Although stabilized in cPTIO-treated cells, RGS4$_{fh}$ remained partly unstable even in the presence of cPTIO (Fig. 3d, lanes 5–7), which is consistent with the incomplete elimination of NO by cPTIO (refs 21, 22 and references therein).

RGS16 (but not RGS4) was naturally expressed in NIH-3T3 cells and in EF cell lines. Anti-RGS16 antibody detected trace amounts of RGS16 in untreated 3T3 cells but a much higher level of RGS16 after treatment with cPTIO (Fig. 3c, compare lanes 1 and 2). Crucially, this effect of cPTIO was nearly absent when the same experiment was performed with *ATE1*$^{-/-}$ EF cells[12], which lacked arginylation (Fig. 3c, compare lanes 3 and 4), yielding independent evidence that the degradation of RGS16 requires both NO and arginylation. We also found that the level of RGS4 was much higher in 3T3^{toff}RGS4$_{fh}$ cells grown under conditions of low (0.5%) oxygen concentration (Fig. 2g; see Discussion).

NO-dependent arginylation of RGS4 *in vitro*

Cys-RGS4, and also the otherwise identical Asp-RGS4 and Val-RGS4 (Fig. 1a), were produced in *Escherichia coli* with the ubiquitin fusion technique[2]. X-RGS4 proteins were incubated with mouse ATE1-1 R-transferase in the presence of ATP, [^{3}H]arginine, tRNA and other components of the analogous assay with eight-residue peptides (Fig. 1c–j), except that arginylation of X-RGS4 was detected by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and fluorography. In addition to untreated controls, X-RGS4 proteins were also preincubated with one of two NO donors, either diglutathionyl-dinitroso-iron (DNIC-(GSH)$_2$, a physiologically relevant NO carrier[39,40]) or diethylenetriamine/nitric oxide adduct (DETA-NO). The results indicated an almost complete dependence of the arginylation of Cys-RGS4 *in vitro* on its previous exposure to a donor of NO (Fig. 4, compare lane 4 with lanes 5 and 6). (Dissolved oxygen and other gases were at levels that are normally present in buffers.) In contrast, Asp-RGS4 was efficiently arginylated irrespective of pretreatment with NO, and Val-RGS4 was not arginylated (Fig. 4). Thus, the NO dependence of *in vivo* arginylation of proteins bearing N-terminal Cys (Figs 2 and 3) can be reconstituted in an *in vitro* system (Fig. 4).

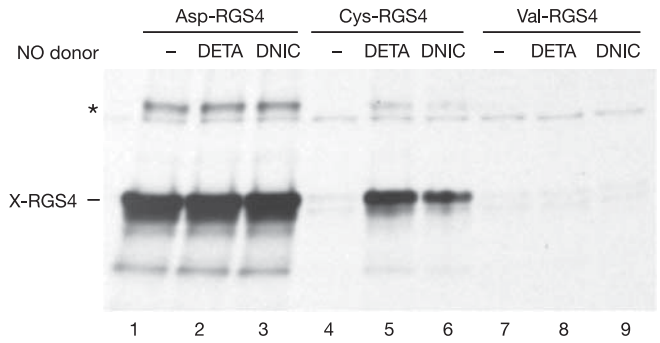

Figure 4 | *In vitro* reconstitution of nitric oxide-dependent arginylation of RGS4. Purified Asp-RGS4 (lanes 1–3), Cys-RGS4 (lanes 4–6) and Val-RGS4 (lanes 7–9) were incubated with [^{3}H]arginine under conditions of the arginylation assay described in the text, followed by SDS–PAGE and fluorography. Lanes 1, 4 and 7, no pretreatment of X-RGS4 proteins. Lanes 2, 5 and 8, pretreatment of X-RGS4 proteins with DETA-NO. Lanes 3, 6 and 9, pretreatment of X-RGS4 proteins with DNIC-[GSH]$_2$. The asterisk denotes a minor ^{3}H-labelled species whose apparent molecular mass and relative levels indicate that it might be a dimer of X-RGS4.

Sequence motif of NO-dependent N-degron

The advances described above (Figs 1–4) accounted for the following, previously unexplained, observation: the conversion of the position 3 residue of RGS4 (position 2 after Met removal) from basic Lys to uncharged Ser stabilized the resulting RGS4$_{K3S}$ against degradation in a reticulocyte extract[38]. In contrast, RGS4$_{K3R}$, in which Lys 2 was replaced by Arg, remained short-lived[38]. Moreover, in contrast to wild-type RGS4, which was arginylated and bore $CysO_3H$ at position 2 (Fig. 3d and ref. 12), RGS4$_{K3S}$ that was transiently expressed in

mouse cells was found to have a blocked N terminus (I. Davydov and A.V., unpublished observations). These results can now be explained, because *S*-nitrosylation of internal (non-N-terminal) Cys by NO in polypeptides depends, in particular, on the presence of a Cys-proximal basic residue, which facilitates the abstraction of H^+ from the cysteine's thiol group[21,22]. In agreement with this explanation, the second residues of MetAP-processed RGS4, RGS5 and RGS16 are Lys, Lys and Arg, respectively. In a different examination of the consensus motif (N-terminal Cys-(basic residue)), we used an antibody against serine racemase[20], a protein with a Cys-Ala N-terminal sequence, to examine whether the levels of racemase were increased in *ATE1*$^{-/-}$ embryos or UBR1/2^{dnR2} cells. In contrast to results with RGS4, RGS5 and RGS16, no significant changes in racemase levels were observed (Fig. 2h, lanes 4–8), as would be expected from the requirement for a basic residue at position 2 for efficacious NO-mediated oxidation of N-terminal Cys.

Discussion

We have shown here that the oxidation of N-terminal Cys in a polypeptide is essential for the arginylation of Cys by *ATE1*-encoded R-transferases (Fig. 1c–j). We also discovered that the arginylation branch of the N-end rule pathway (Fig. 1a) is a sensor of nitric oxide (NO) that functions through its ability to destroy specific regulatory proteins bearing N-terminal Cys, at rates controlled by NO and apparently by oxygen as well (Figs 2–4). The first examples of these regulators, RGS4, RGS5 and RGS16, are also the first physiological substrates of mammalian N-end rule pathway. These proteins downregulate specific α subunits of G proteins by increasing their GTPase activity[30–32,35–37]. Through the conditional destruction of RGS4, RGS5 and RGS16, the N-end rule pathway is therefore involved in the regulation of signalling by G-protein-coupled receptors.

The above 'unification' of a ubiquitin-dependent proteolytic pathway and NO signalling opens up new vistas for understanding both. Most of the previously known regulation by NO was based on changes in the functional (for example enzymatic) activity of NO-modified proteins[19–23,27–29]. In contrast, a Cys-containing N-degron of a protein makes possible the NO-mediated control of circuits that contain this protein, through its NO-dependent degradation by the N-end rule pathway. The observed stabilization of RGS4 in cells grown at low oxygen concentration (Fig. 2g) suggests a possible involvement of oxygen or its derivatives in this NO-dependent regulation. That would also be expected from the NO results alone (Figs 3 and 4), given multiple links between the chemistries of NO and oxygen *in vivo*[19–23]. Our assay *in vitro* for NO-dependent arginylation of N-terminal Cys (Fig. 4) should eventually yield a detailed understanding of chemical transformations that result in oxidation of this uniquely positioned Cys residue.

The pathway of control by NO (Fig. 1a) targets proteins that bear N-terminal Cys followed by a basic residue. This motif is present in about 30 proteins encoded by the mouse (and human) genome, including RGS4, RGS5 and RGS16. More than half of non-RGS proteins in this set have entirely unknown functions, and the rest are barely characterized. The N-terminal Cys residues of RGS4, RGS5 and RGS16 can also be modified through palmitoylation[36]. The two modifications are expected to be mutually exclusive and also act in opposite ways, in that palmitoylation increases the activity of RGS proteins as downregulators of G proteins[36], whereas NO-dependent arginylation and destruction of RGSs reduce their activity by decreasing their levels in a cell.

RGS4 is a physiological inhibitor of angiogenesis and other tubulogenesis pathways[32]. Upregulation of RGS4 perturbs cardiovascular homeostasis in mice[31] and is a molecular correlate of human heart failure[30]. Mouse *ATE1*$^{-/-}$ embryos, which lack R-transferases and therefore lack arginylation, die before E17 with cardiovascular defects[12]. Our findings that the levels of RGS4, RGS5 and RGS16 are greatly increased in the hearts and other organs of *ATE1*$^{-/-}$ embryos (Fig. 2), and that NO is required for the proteolytic downregulation of these RGSs (Figs 3 and 4), are likely to account in part for the known role of NO, at physiologically optimal levels, in suppressing pathological changes in the heart[25,26,41]. The functions of NO in cardiovascular homeostasis include the stimulation of cyclic GMP formation by guanylyl cyclase and the regulation of cardiac contractility through *S*-nitrosylation of the calcium release channel[21,23,41]. Our results revealed an entirely different, mutually nonexclusive mechanism of NO signalling in the heart and other organs: the control of regulatory proteins bearing N-terminal Cys through their NO-dependent, arginylation-mediated degradation by the N-end rule pathway. Thus, pharmacological manipulation of activities or expression of R-transferases may provide an alternative, more selective route to clinically beneficial effects that are currently achieved through drugs that alter the levels of NO.

Mammalian R-transferases are strong sequelogues[34] of yeast (fungal) ATE1 R-transferases. However, whereas the inactivation of mouse *ATE1* results in embryonic lethality[12], a deletion of *S. cerevisiae ATE1* renders cells unable to degrade reporters with N-terminal Asp or Glu but has not been found to cause any other abnormal phenotype[2,33]. Our findings indicate that one function of arginylation in this and other organisms might be to serve as a sensor of nitrosative/oxidative stress (Supplementary Information). It remains to be determined whether the discovered signalling by NO proceeds exclusively through the oxidation of Cys-containing N-degrons (Fig. 1a) or whether NO can also function at other steps of the N-end rule pathway, for example through *S*-nitrosylation of its ubiquitin ligases or R-transferases.

A preponderance of circuits relevant to the findings of this work involve arginine: first, Arg is a direct precursor of NO (Fig. 1a); second, the levels of Arg are tightly controlled in a cell and are often downregulated by invading pathogens; third, Arg is a part of Arg-tRNA (a co-substrate of R-transferase), indicating a possible connection between the N-end rule pathway and regulation of translation; fourth, Arg is a primary destabilizing residue and is also conjugated to N-end rule substrates bearing N-terminal Asp, Glu or oxidized Cys (Fig. 1a); and last, some Arg residues in proteins undergo methylation or deimination, the latter a conversion of positively charged Arg to uncharged citrulline[42,43]. It remains to be determined whether methylation or deimination of Arg *in vivo* involves N-terminal Arg, and whether a set of circuits that has now been shown to connect the N-end rule pathway and the signalling by NO holds yet another Arg-linked surprise.

METHODS

Full technical details are provided in Supplementary Information.

Mouse embryos, immunoblotting and northern hybridization. *ATE1*$^{-/-}$ and wild-type embryos were produced as described[12]. E12.5 and E14.5 whole embryos or their organs were collected, followed by immunoblotting of embryo extracts with antibodies against RGS4 (a gift from S. Mumby), RGS16 (a gift from C. K. Chen and M. Simon), RGS5 (a gift from M. Greenwood), serine racemase (BD Biosciences), and mouse ATE1-1 R-transferase. The latter antibody was raised in rabbits against purified ATE1-1.

3T3^{toff}RGS4$_{fh}$, other cell lines, and treatments. Treatments of cells with cPTIO, with LMMA or with N3411 (L-N^{ω}-nitroarginine-2,4-L-diaminobutyric amide) were performed as described in Supplementary Information, followed by the preparation of extracts, SDS–PAGE and immunoblotting. The mouse 3T3^{toff}RGS4$_{fh}$ cell line, which expressed RGS4-Flag-His$_6$ (RGS4$_{fh}$) from a doxycycline-repressible promoter, was constructed with MEF/3T3 'Tet-off' cells (BD Biosciences). Other mouse cell lines were UBR1/2^{dnR2} (see the text; J.S., Z. Xia, Y. T. Kwon and A.V., unpublished observations), *ATE1*$^{-/-}$ EF cells and NIH-3T3 cells. Pulse–chase experiments were performed with 3T3^{toff}RGS4$_{fh}$ cells, essentially as described[12].

Reporter peptides and recombinant proteins. The eight-residue Asp-peptide (DHGSGAWL) and Cys-peptide (CHGSGAWL) were synthesized by standard methods. $CysO_3H$-peptide was synthesized as described in Supplementary Information. *S. cerevisiae* Arg-tRNA synthetase (RRS1) was expressed in *E. coli* and purified by chromatography. *S. cerevisiae* ATE1 R-transferase[33] was carboxy-terminally tagged with His$_6$, expressed in *E. coli* and purified

with Ni^{2+}-nitrilotriacetate (NTA)-agarose. Mouse ATE1-1 R-transferase[11,12] was C-terminally epitope-tagged, expressed with a baculovirus and purified with Ni^{2+}-NTA-agarose. X-RGS4 proteins were produced and purified as described in Supplementary Information.

Arginylation assays, NO donors, capillary electrophoresis, and other assays. A sample for performing the arginylation of a reporter peptide contained, in addition to reaction buffer, one of three eight-residue synthetic peptides, a mixture of *S. cerevisiae* tRNAs, purified *S. cerevisiae* Arg-tRNA synthetase, and either purified mouse ATE1-1 R-transferase or purified *S. cerevisiae* ATE1 R-transferase. Reactions were stopped by the addition of trifluoroacetic acid, followed by analyses of peptides by either capillary electrophoresis or MALDI–TOF MS. Details of the arginylation assay with [^{3}H]arginine, purified X-RGS4 proteins and the NO donors DNIC-$[GSH]_2$ and DETA-NO are described in Supplementary Information.

For N-terminal sequencing by Edman degradation, $RGS4_{fh}$ was isolated from $3T3^{toff}RGS4_{fh}$ cells, alkylated with iodoacetamide, fractionated by 12% SDS–PAGE and processed for sequencing[11].

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