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Conserved N-terminal cysteine dioxygenases transduce responses to hypoxia in animals and plants

Norma Masson^{1,*}, Thomas P. Keeley^{1,*}, Beatrice Giuntoli^{3,4,*}, Mark D. White^{2,*}, Mikel Lavilla Puerta⁴, Pierdomenico Perata⁴, Richard J. Hopkinson², Emily Flashman^{2,a}, Francesco Licausi^{3,4,a}, Peter J. Ratcliffe^{1,5,a,**}

¹Ludwig Institute for Cancer Research, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7FZ, UK

²Chemistry Research Laboratory, University of Oxford, Mansfield Road, Oxford OX1 3TA, UK

³Department of Biology, University of Pisa, Via Luca Ghini 13, Pisa 56126, Italy

⁴Plantlab, Institute of Life Sciences, Scuola Superiore Sant'Anna, Via Guidiccioni 8/10, Pisa 56124, Italy

⁵The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK

Abstract

Organisms must respond to hypoxia to preserve oxygen homeostasis. We identify a thiol oxidase, previously assigned as cysteamine (2-aminoethanethiol) dioxygenase (ADO), as a high K_m O₂ N-terminal cysteine dioxygenase that transduces the oxygen-regulated stability of proteins by the N-degron pathway in human cells. ADO catalyzes conversion of N-terminal cysteine to cysteine sulfinic acid and is related to the plant cysteine oxidases that mediate responses to hypoxia by an identical post-translational modification. In human cells we show that ADO regulates the RGS4/5 (regulator of G-protein signalling) N-degron substrates, modulates G-protein coupled Ca²⁺ signals and MAPK (mitogen-activated protein kinase) activity, and that its activity extends to other N-Cysteine proteins including the angiogenic cytokine IL-32. Identification of a conserved enzymatic oxygen sensor in multicellular eukaryotes opens routes to better understanding and therapeutic targeting of adaptive responses to hypoxia.

Oxygen homeostasis is critical for most forms of life, and is impaired in most human diseases. Previous work identified the hypoxia inducible factor (HIF) prolyl hydroxylases as

**Corresponding author. peter.ratcliffe@ndm.ox.ac.uk.

*Joint first authors

^aJoint senior authors

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Competing interests: PJR is a scientific co-founder holds equity in Reox Ltd, has served on the Research Advisory Board of GSK Ltd., and is co-inventor on patents disclosing methods and means of assaying for HIF hydroxylases inhibitors and their use in the treatment of hypoxic diseases (US8535899, EP1379630), which have been licensed to Reox Ltd. PJR, FL, EF, NM, TK, BG, MDW are inventors on a patent application related to this work (United Kingdom Patent Application No. 1908332.8). The other authors declare no competing interests.

Data and materials availability

All data is available in the manuscript or the supplementary materials.

human oxygen sensors(1). These regulatory enzymes are 2-oxoglutarate (2-OG) dependent dioxygenases, with high K_mO_2 values, which catalyze *trans*-4-prolyl hydroxylation of the transcription factor HIF(2, 3), to target it for proteolysis. Hence they regulate a broad range of transcriptional responses to hypoxia (reviewed in(4)). Although the prolyl hydroxylation of HIF was unprecedented as a signaling system, subsequent work has revealed different systems of enzymatic protein oxidation, which signal hypoxia in representatives of all four eukaryotic kingdoms (5–7). In each system the protein oxidation event is linked to protein degradation.

Of particular interest is the Cys-branch of the N-degron pathway(8). Following the action of methionine aminopeptidases, oxidation of N-terminal cysteines creates a substrate for arginyl-transferases, which catalyze addition of this N-terminal destabilizing residue, promoting degradation. No cysteine-modifying enzyme was defined, but N-terminal cysteine oxidation was shown to be affected by nitric oxide (9), and based on *in vitro* studies, cysteine oxidation has been considered likely to be non-enzymatic. Subsequently, in plants, it was shown that the Cys-branch of the N-degron pathway controls the stability of ethylene response transcription factors (ERF-VII) (10, 11). Further studies in *Arabidopsis thaliana* revealed that Cys-oxidation is catalyzed by a series of plant cysteine oxidases (PCOs), which act as oxygen sensors directing hypoxic adaptation (7, 12). These findings led us to further investigate the mechanism of N-terminal cysteine oxidation in animals.

First, we created human osteosarcoma U-2OS and colon cancer RKO cells that stably express a fusion protein comprising N-terminal sequences that are sufficient for oxygen-dependent degradation of the ERF-VII transcription factor RAP2.12 (Related to APETALA2) in plants, linked to a GFP:V5 reporter, and exposed these cells to hypoxia. To distinguish responses from those transduced by HIF, we also tested known inhibitors of the HIF prolyl hydroxylases that differ in their specificity both for other iron-dependent dioxygenases and non-enzymatic metal catalysed oxidation. Exposure of the transfected cells to hypoxia and to the non-specific iron chelator, dipyriddy, resulted in accumulation of the RAP₁₋₅₀:GFP:V5 reporter protein, but not that of a C2A mutant, without affecting reporter transcript levels (fig. S1). In contrast, neither reporter was activated by non-specific 2-OG dioxygenase inhibitors (DFO and DMOG), or a HIF prolyl hydroxylase inhibitor (PHI), all of which robustly induced HIF (Fig. 1A). For cells exposed to hypoxia for 16 h, then treated with cycloheximide, before being re-oxygenated or maintained in hypoxia, we found that hypoxia prolonged the reporter protein half-life from ~5 to 35 min (Fig. 1B and fig. S2). These findings demonstrated an iron and oxygen-dependent activity in human cells that is distinct from the HIF prolyl hydroxylases and that operates in a Cys2-dependent manner on amino-acid sequences from plant RAP2.12.

We next compared this response with that of members of the R4 group of RGS proteins, which are targets of the Cys-branch of the N-degron pathway in humans and mice (13, 14). Experiments on RKO cells stably expressing HA-tagged RGS4 (RGS4:HA) and an RGS4:GFP fusion (RGS4₁₋₂₀:GFP), each encoding wild-type or C2A mutant sequences, revealed accumulation of wild-type, but not mutant proteins in cells exposed to hypoxia and dipyriddy, but not DMOG or DFO (Fig. 1C and fig. S3). Endogenous RGS4 and RSG5 proteins in human neuroblastoma SH-SY5Y cells responded identically to the same set of

compounds (Fig. 1D). Responses of these RGS proteins to graded hypoxia were further examined in a series of human (SH-5Y5Y, RKO, human endothelial EA.hy926) and mouse embryonic sarcoma (C3H/10T1/2) cells, revealing progressive accumulation of RGS4 or RGS5 proteins in response to physiological hypoxia (Fig. 1E and fig. S4). These changes were observed at the level of proteins and not mRNAs, with the exception of RGS4 in SH-SY5Y. Thus, plant and human reporter proteins and endogenous RGS proteins appeared to be regulated similarly, suggesting that human cells might regulate their stability using enzyme(s) similar to the PCOs.

The human genome contains two thiol dioxygenases with similar predicted structure to the PCOs, cysteine dioxygenase (CDO1) and an enzyme previously assigned as cysteamine (2-aminoethanethiol) dioxygenase (ADO) (15, 16). Genes encoding these enzymes and PCO1 were co-transfected with the RGS4:HA gene into human embryonic kidney 293T cells. Overexpressed ADO, but not CDO1, suppressed hypoxic induction of RGS4:HA, in a manner dependent on cysteine at position 2 (Fig. 2A). At these levels of overexpression, RGS4:HA was not suppressed by PCO1. The ability of ADO to suppress RGS4:HA was inhibited by combined exposure to hypoxia and dipyridyl and ablated by H112A+H114A mutations (fig. S5) that prevent assembly of the catalytic iron center (15). These experiments indicated that the catalytic activity of overexpressed ADO was sufficient to suppress RGS4:HA.

We next inactivated ADO and CDO1 in SH-SY5Y and RKO cells, using CRISPR/Cas9-mediated gene editing. Inactivation of ADO but not CDO1 led to constitutive upregulation of endogenous and transfected RGS4 and RGS5 proteins irrespective of oxygen levels (Fig. 2B and figs. S6, S7). In view of reported actions of nitric oxide on RGS proteins (9, 17), we also tested responses to the nitric oxide donor DETA-NO in wild type and ADO-deficient cells. Suppression of hypoxic RGS4 levels by DETA-NO in wild-type SH-SY5Y and RKO cells was also abrogated in ADO-deficient cells (fig. S8). Stable re-expression of ADO, but not overexpression of CDO1, suppressed levels of RGS proteins (figs. S7 and S9). Under these conditions, expression of PCO1 also suppressed RGS proteins and restored regulation by oxygen, demonstrating oxygen-dependent activity of the plant enzyme on endogenous human proteins. In SH-SY5Y cells, we then inactivated the arginyl-transferase ATE-1 that operates downstream of the proposed Cys-oxidation in the N-degron pathway. Up-regulation of RGS5 in ATE1-deficient and ADO-deficient cells was similar, and was not suppressed by overexpression of ADO in ATE1-deficient cells (Fig. 2C). Thus ADO is required for oxygen-dependent degradation of RGS proteins. This activity was dependent on the integrity of ATE1, consistent with ADO acting upstream of ATE1 in the N-degron pathway.

To explore whether ADO can complement deficient PCO in plants, we generated a PCO-depleted *Arabidopsis thaliana* mutant by crossing plants in which four of the five known PCO (1, 2, 4 and 5) genes were inactivated by T-DNA insertional mutagenesis. Homozygous quadruple *pco1/2/4/5* mutant plants (*4pco*), but not triple *pco1/2/4* (*3pco*), manifested severe developmental defects (Fig. 2D) and upregulation of hypoxia-responsive genes under aerobic conditions (Fig. 2E). When human ADO, but not CDO1, was introduced into *4pco* plants under control of the PCO1 promoter, the constitutive upregulation of anaerobic genes in air was corrected and the plants developed normally (Figs. 2D and 2E, and fig. S10).

Consistent with complementation of defective PCO function, co-expression of ADO in *A. thaliana* protoplasts caused dose-dependent suppression of RAP2.12 luciferase fusion protein activity (fig. S10).

Interestingly, in budding yeast, Cys is not a destabilizing N-terminal residue (18). To determine if deficiency of an ADO-like enzyme might be responsible for the stability associated with N-Cys in yeast, we introduced ADO or CDO1 into yeast cells together with a ratio-metric reporter in which the activity of a RAP2.12-Firefly Luciferase fusion protein or a C2A mutant is normalized to Renilla Luciferase (fig. S11). Expression of human ADO but not CDO1 reduced the activity of, and conferred hypoxic regulation on, the RAP₂₋₂₈-FLuc protein but not a C2A mutant (Fig. 2F and fig. S11). Consistent with this, phylogenetic analyses revealed potential ADO/CDO orthologues across most plants, animals and at least some protist species, but not fungi (fig. S12). Together, these findings explain the lack of activity of cysteine as a destabilizing residue in yeast, but suggest the pathway might otherwise operate widely in eukaryotic species.

Cross-complementation suggested that ADO catalyzes a form of N-terminal cysteine dioxygenation similar to that catalyzed by the PCOs. To test this, we produced recombinant human ADO and CDO1 in *E. coli*, reacted these enzymes with synthetic peptides corresponding to residues 2-15 of human RGS4 and RGS5 and examined the products by mass spectrometry. We found that the peptides were modified by +32 Da mass addition by ADO but not CDO1 (Fig. 3A and fig. S13) and that this modification was suppressed in anaerobic conditions (Fig. 3B). To confirm dioxygenation, we conducted the reactions in an atmosphere of ¹⁸O₂ or in the presence of ¹⁸O-labelled water (H₂¹⁸O). These experiments revealed a single +36 Da mass addition in ¹⁸O₂, and a single +32 Da mass addition in the presence of ¹⁸O-labelled water, demonstrating that two oxygen atoms were incorporated directly into the peptide from molecular oxygen and confirming dioxygenation (Fig. 3B). MS2 assigned the modification to the N-terminal cysteine (fig. S14). Thus, human ADO catalyzes the dioxygenation of N-Cys residues in RGS4 and RGS5 to cysteine sulfinic acid. Kinetic measurements on human ADO (Fig. 3C and fig. S15) revealed high k_{cat}^{Pep} values of 20.1 and 16.9 s⁻¹ on RGS4 and RGS5 peptides under atmospheric conditions, but marked sensitivity to oxygen ($K_m^{O_2^{app}} > 500 \mu M$). Thus ADO resembles the HIF prolyl hydroxylase enzymes in manifesting a $K_m^{O_2}$ that is significantly above the physiological range, a property that may underpin a role in oxygen homeostasis. Given the original assignment of ADO as cysteamine dioxygenase we also examined for competition of N-Cys peptide dioxygenation by free cysteamine and cysteine, but found inhibition only at high concentrations of these metabolites (IC₅₀ 37 and 13mM, respectively, fig. S16).

RGS4 and RGS5 regulate heterotrimeric G-protein signaling by enhancing G α -coupled GTP hydrolysis and hence attenuating G-protein signals. Since the catalytic activity of ADO lowers levels of RGS4 and 5, ADO-deficient cells in which levels of these proteins are increased, should manifest attenuation of G-protein signaling on relevant pathways. G α proteins can regulate the activity of mitogen-activated protein kinase (MAPK) pathways (14, 19). Consistent with this, mouse cells and embryos with a defective N-degron pathway due to loss of the arginyl-transferase ATE1, have been shown to exhibit reduced activation of MAPK kinase (14). We therefore assayed phosphorylation of MAPK (p44/p42) in ADO-

deficient SH-SY5Y cells (Fig. 4A). These experiments revealed reduced levels of phosphorylated MAPK in ADO-deficient SH-SY5Y cells that were similar to levels of phosphorylated p44/p42 in ATE1-deficient SH-SY5Y cells. Reduction in phosphorylated p44/p42 was reversed by expression of ADO in ADO KO but not ATE1 KO cells (Fig. 4B). To test the effects of ADO on responses to a specific G-protein coupled agonist, we examined carbachol, a cholinergic agonist whose muscarinic receptor is coupled via Gαq to the regulation of intracellular Ca²⁺. Attenuation of Ca²⁺ mobilization in response to carbachol (Fig. 4C), but not the receptor-independent ionophore, ionomycin (Fig. 4D), was observed in ADO-deficient cells and was reversed by re-expression of ADO. Given the complexity of interactions among RGS proteins and G-protein signaling pathways, we cannot be certain that these effects are entirely caused by effects of ADO on RGS4 and RGS5 proteins. Nevertheless, the work establishes a role for ADO in the regulation G-protein signaling, consistent with its role as a Cys-modifying enzyme in the N-degron pathway regulating RGS proteins.

N-terminal sequence analyses of proteins encoded by plant and animal genomes have suggested the existence of many other potential substrates for the Cys-branch of the N-degron pathway (7, 9) and ADO is more widely expressed in human cells and tissues than RGS4/5(20). We therefore sought to determine whether ADO-mediated oxygen-dependent regulation of human proteins extended beyond the identified RGS proteins. To pursue this, we first reacted recombinant ADO with a diverse series of peptides derived from proteins predicted to be processed to generate N-Cys polypeptides, and monitored for dioxygenation (+32 Da mass shift) by MS. These experiments revealed substrate-dependent catalytic activity of ADO, ranging from close to zero, to levels that were similar to those using RGS5 peptide (figs. S17, S18). We then examined endogenous protein levels corresponding to peptide substrates that supported high (IL-32, Fig. 5A) or very low (asparagine synthetase, (ASNS) and JunB) ADO-catalyzed dioxygenation using available antibodies. These experiments were conducted in the previously engineered ADO-deficient RKO cells as IL-32 was not detected in SH-SY5Y cells. Immunoblotting revealed that the abundance of IL-32, but not ASNS or JunB, was increased in hypoxic cells, accumulated constitutively in ADO-deficient cells and was reduced by re-expression of transfected ADO (Fig. 5B). Experiments in wild type and ADO-deficient RKO cells confirmed IL-32 regulation at the protein but not mRNA level (Fig. 5C). Further experiments confirmed dioxygenation of the N-terminal cysteine following reaction of the IL-32 peptide with recombinant ADO (fig. S18), and that this residue was necessary for ADO-mediated suppression of co-transfected IL-32 in cells (Fig. 5D). These findings demonstrate that ADO target proteins do extend beyond RGS proteins and identify human IL-32 as one such protein. The tested peptides represent only a small fraction of N-Cys polypeptides that might be generated in cells and it is therefore likely that other ADO-regulated human targets exist. Interestingly, the transcriptional regulator LITTLE ZIPPER 2 and a component of Polycomb Repressor 2 Complex, VERNALIZATION 2, have recently been identified as new oxygen-regulated targets of the N-degron pathway in plants(21, 22). Given the emerging complexity of N-degron regulation(8), in which other processes may compete with ADO-catalysed deoxygenation, it cannot be assumed that high levels of ADO-catalysis on isolated peptides will necessarily predict physiological regulation by ADO, or indeed that all protein

regulation by ADO operates through the same downstream pathways. Identification of human ADO as an enzymatic human oxygen sensor should open the way to understanding responses to hypoxia that are transduced by these pathways.

Conservation of ADO and the PCOs as human and plant oxygen sensors contrasts with the absence of conservation of their known substrates, and with different challenges to oxygen homeostasis that are encountered by animals and plants. In plants, the ERF-VII pathway directs transcriptional responses to hypoxia that require time for the transcriptional output to engage adaptive responses. In animal cells the principal process regulating transcriptional responses to hypoxia is the prolyl hydroxylation of HIF. In contrast, direct operation of ADO on the protein stability of signaling molecules has the potential to transduce more rapid responses to hypoxia than HIF. RGS4 and RGS5 have been implicated in oxygen homeostasis in mammals through effects on the cardiovascular system and angiogenesis(13, 19, 23). IL-32 is an atypical cytokine that regulates pro-inflammatory cytokine networks and angiogenic growth factors(24, 25). Although our findings identify ADO as an essential regulator of responses of these proteins to hypoxia, it is of interest that RGS4, RGS5 and IL-32 have all been reported to be transcriptional targets of HIF(26–28). Consistent with the reported cell-type specific regulation of RGS4 by HIF(26), we did observe induction of RGS4 mRNA by hypoxia in SH-SY5Y, but not other cells. These findings predict that in specific cellular settings where both systems are operative the ADO and HIF prolyl hydroxylase systems will interact to generate physiological responses to hypoxia. In conclusion, our work defines an enzymatic human oxygen sensor, most likely operating physiologically on a shorter time scale than the transcriptional responses transduced by the HIF prolyl hydroxylases and opens a new route to the investigation of adaptive responses to hypoxia, potentially including their therapeutic augmentation by catalytic inhibitors of ADO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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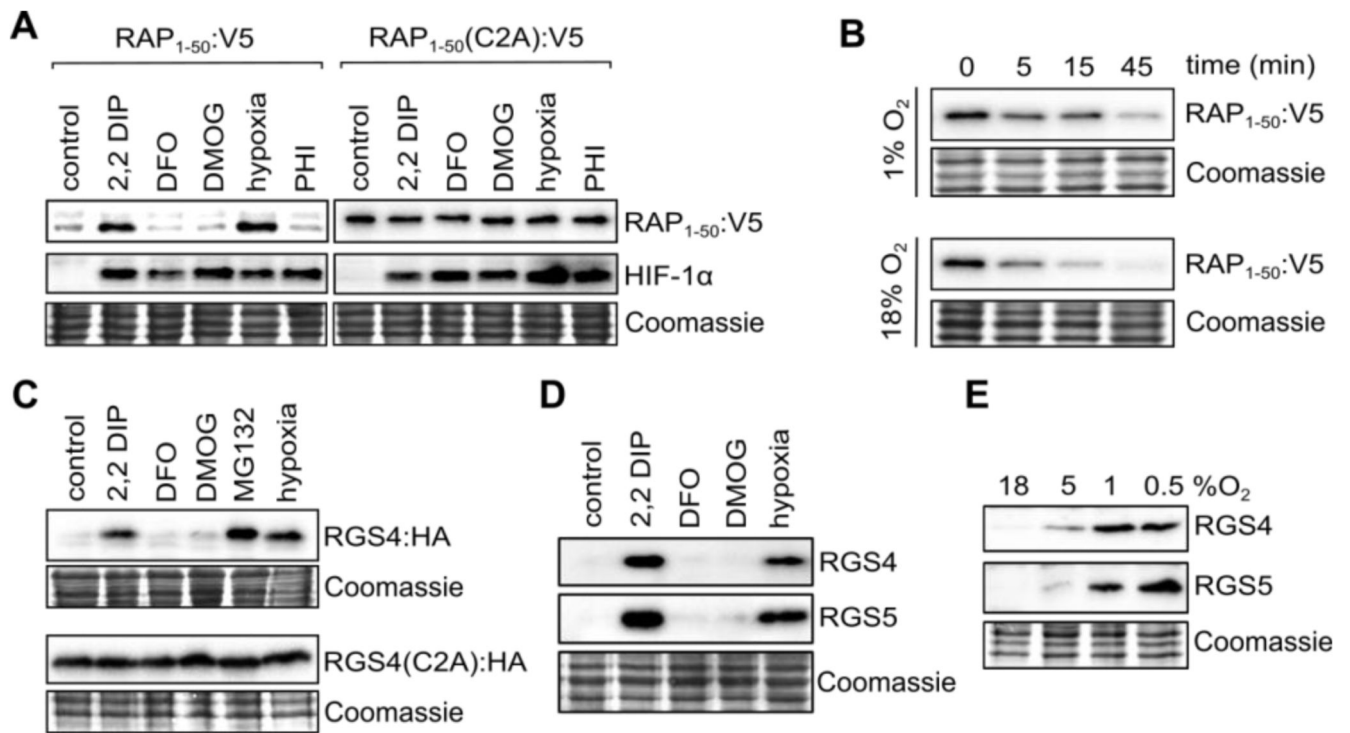


Fig. 1. Regulation of plant and animal N-degron substrates by oxygen in human cells.

(A) Levels of fusion proteins linking the N-terminal 1-50 residues of plant RAP2.12 or a C2A mutant to a GFP:V5 cassette (RAP₁₋₅₀:V5; RAP₁₋₅₀(C2A):V5) in stably transfected U-2OS cells exposed to hypoxia or the indicated inhibitors. (B) RAP₁₋₅₀:V5 reporter protein half-life in cells incubated in hypoxia (16 h, 1% O₂) then treated with cycloheximide (100 μM, 10min), then maintained in hypoxia or re-oxygenated for the indicated times. (C) C-terminal hemagglutinin (HA) tagged human RGS4, (RGS4:HA) or a C2A mutant in stably transfected RKO cells exposed to hypoxia or inhibitors. (D and E) Endogenous RGS4 and RGS5 proteins in SH-SY5Y cells exposed to inhibitors (D) or graded hypoxia (E). Similar patterns of response were observed for the plant fusion-protein reporter, transfected RGS4:HA and endogenous RGS4/5 proteins; responses of exogenous proteins were abolished by C2A mutation. 2,2 DIP, 2,2-dipyridyl (100 μM); DFO, desferrioxamine (100 μM); DMOG, dimethylxalylglycine (1 mM); PHI, prolyl hydroxylase inhibitor (125 μM); MG132, proteasomal inhibitor (25 μM). All exposures of cells to hypoxia or inhibitors were for 4 h unless otherwise indicated. In panel A HIF-1α immunoblots are provided for comparison.

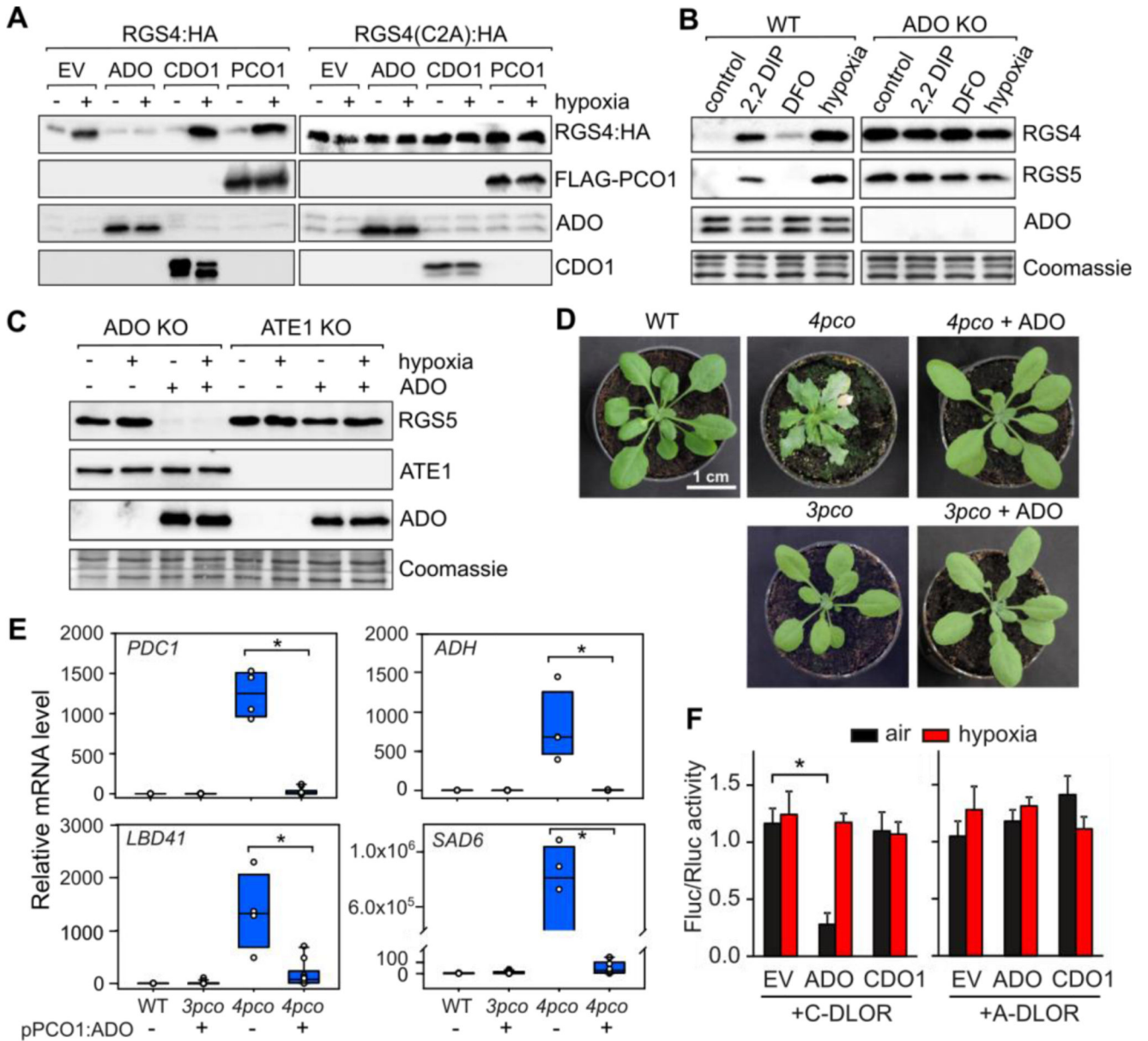


Fig. 2. ADO controls the oxygen dependent Cys-branch of the N-degron pathway. (A) RGS4:HA and RGS4(C2A):HA protein levels in 293T cells after co-expression with either control (EV), ADO, CDO1 or PCO1. Cells were exposed to hypoxia (0.5% O₂, 16 h) or maintained in air. Comparable enzyme levels were confirmed by separate FLAG-immunoblotting. (B) Endogenous RGS4 and RGS5 proteins in ADO-deficient SH-SY5Y cells (ADO KO); RGS4 and RGS5 are constitutive and insensitive to iron chelators or hypoxia. (C) Over-expression of ADO does not repress constitutive stabilization of RGS5 in ATE1-deficient (ATE1 KO) cells. (D) Expression of human ADO restores wild type phenotype in *4pco* *A. thaliana* mutants; *3pco* mutants which did not manifest this phenotype were unaffected by ADO. (E) Box plots showing relative mRNA level of hypoxia-inducible genes in wild type and *pco* mutant plants that express ADO. ADO significantly reduced

expression of the hypoxia-inducible genes *PDC1*, *ADH*, *LBD41* and *SAD6* in *4pco* mutants, Mean \pm S.D. *P<0.05; Mann-Whitney Rank Sum Test) with levels of non-hypoxia-inducible genes unchanged (fig. S10). (F) Relative luciferase activity (Fluc/Rluc) in *S. cerevisiae* cells expressing C-DLOR (Cys) or A-DLOR (Cys to Ala mutant) reporter under aerobic and hypoxic conditions in the presence or absence of human ADO or CDO1, mean \pm S.D. *P<0.05; 2-way ANOVA followed by Holm-Sidak post hoc test.

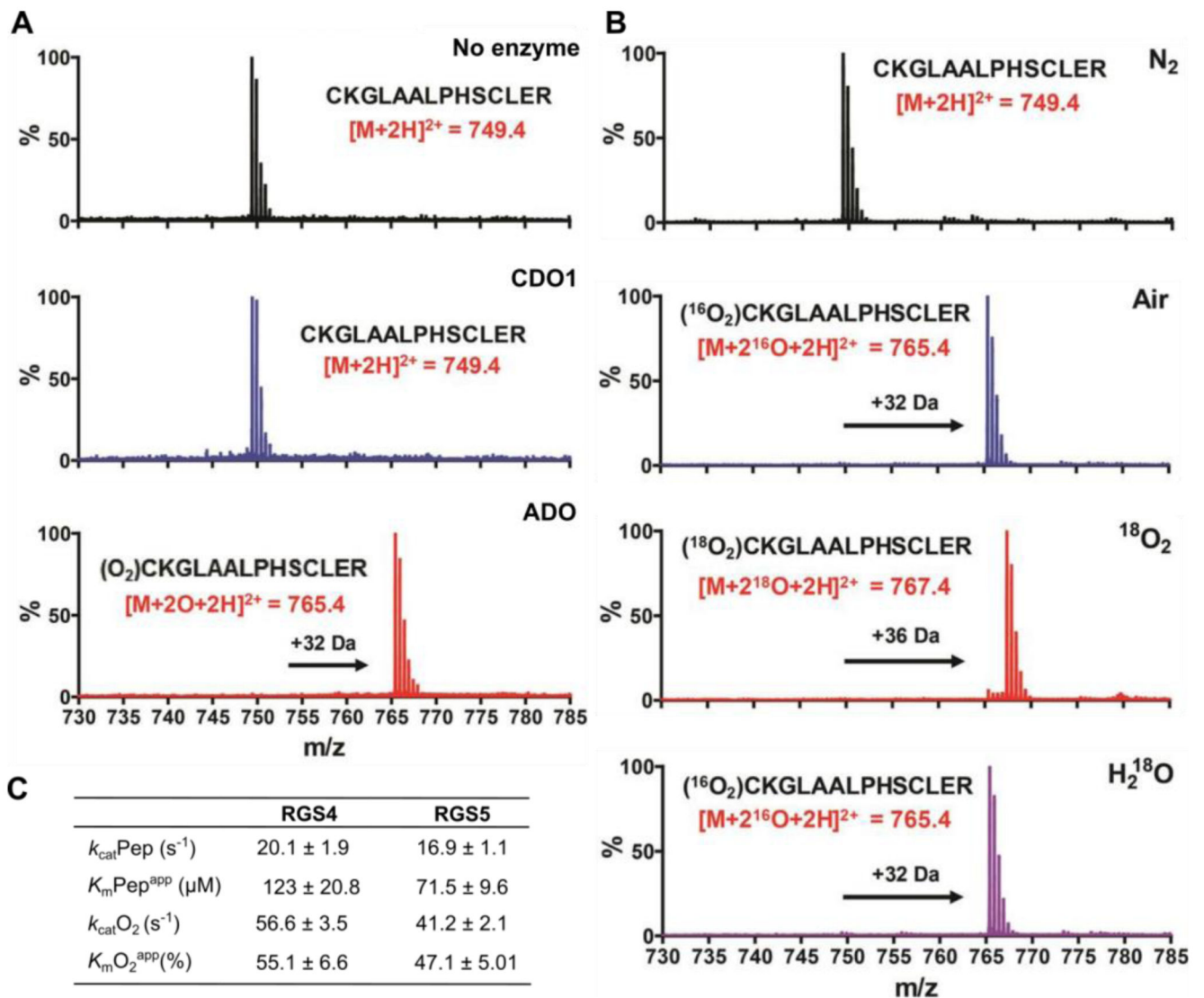


Fig. 3. ADO catalyzes the dioxygenation of the N-terminal Cys of RGS4/5 peptides.

(A) MS spectra show a mass shift of +32 Da when RGS5 N-terminal peptide was incubated with recombinant human ADO, but not with recombinant human CDO1. Similar results were obtained when an RGS4 N-terminal peptide was used (fig. S13). (B) The ADO-catalyzed +32 Da mass addition is absent when reactions were conducted under anaerobic (100% N_2) conditions; ^{18}O labelling demonstrates incorporation of 2 oxygen atoms derived directly from molecular O_2 and not H_2O . (C) Summary table of reaction kinetics for ADO-catalyzed dioxygenation of N-terminal RGS4/5 peptides. The influence of varying peptide concentration under atmospheric conditions ($k_{\text{cat}}^{\text{Pep}}$ and $K_{\text{m}}^{\text{Pep}^{\text{app}}}$) and O_2 levels using a fixed, non-limiting concentration of peptide ($k_{\text{cat}}^{\text{O}_2}$ and $K_{\text{m}}^{\text{O}_2^{\text{app}}}$) were examined to determine sensitivities to both substrates. Source data are in fig. S15.

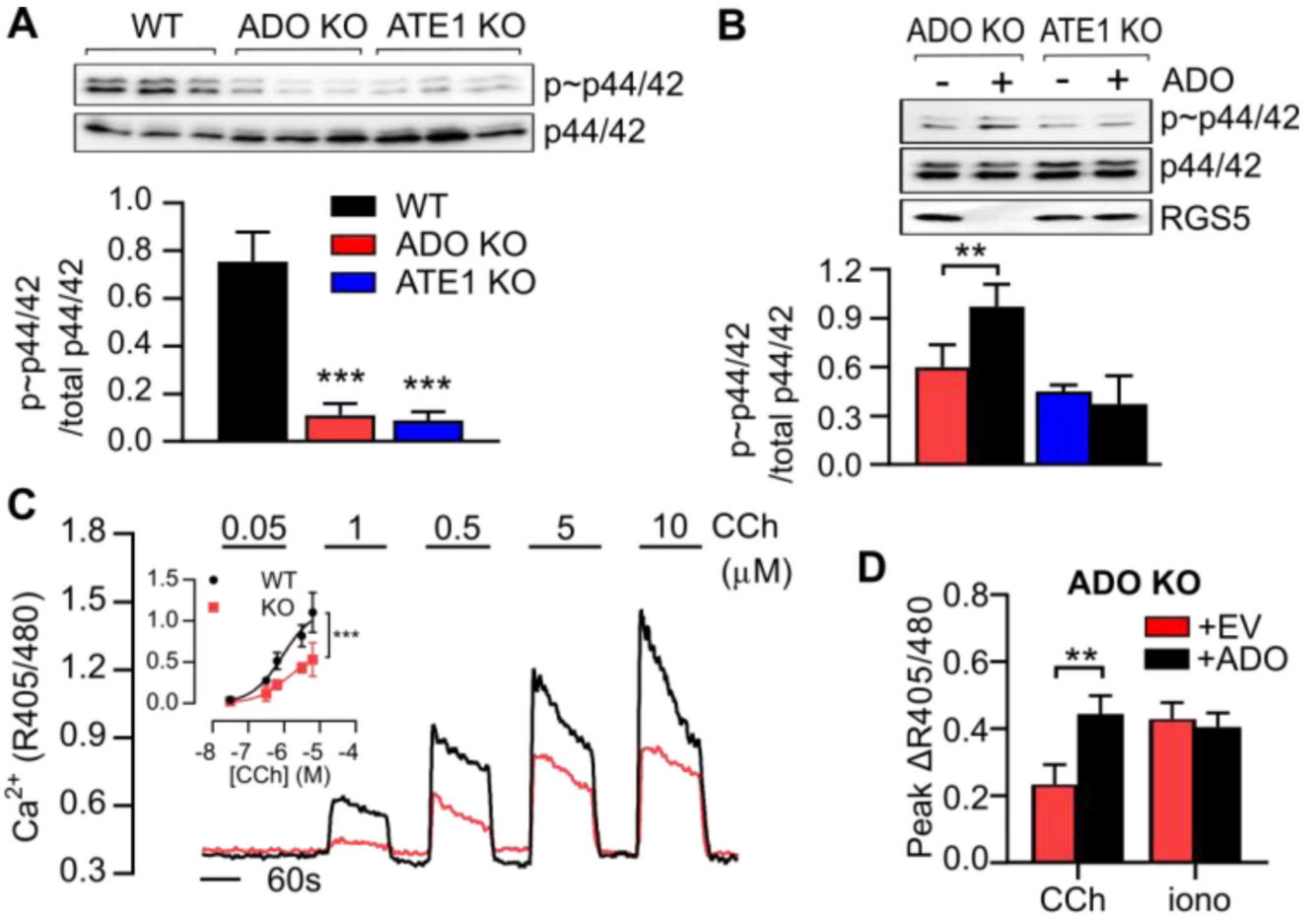


Fig. 4. ADO regulates G-protein signalling.

(A-D) ADO regulates G-protein signalling in SH-SY5Y cells. (A) MAPK (p44/42) phosphorylation in WT, ADO KO and ATE1 KO cells. Immunoblot lanes represent separate biological replicates, with densitometric analysis provided below. Mean ± S.D. n=3, ***P<0.001 one-way ANOVA with Holm-Sidak post hoc test. (B) Re-expression of ADO increases phosphorylated p44/42 in ADO KO, but not ATE1 KO cells, mean ± S.D. n=3, **P<0.01 two-way ANOVA with Holm-Sidak post hoc test. (C) Carbachol (CCh) stimulated rises in [Ca²⁺]_i are attenuated in ADO-deficient (KO) compared with wild-type (WT) cells. A representative trace is provided and mean peak change in R405/495 intensity at each CCh concentration is shown (inset). n=8-12, ***P<0.001, 3-parameter non-linear regression analysis. (D) Ionomycin (0.1μM) is equipotent at stimulating Ca²⁺ release in ADO KO cells infected with either control (EV) or ADO-containing lentivirus, whereas responses to CCh are recovered by ADO re-expression. Mean ± S.D. n=6-7, *P<0.05, two-way ANOVA with Holm-Sidak post hoc test.

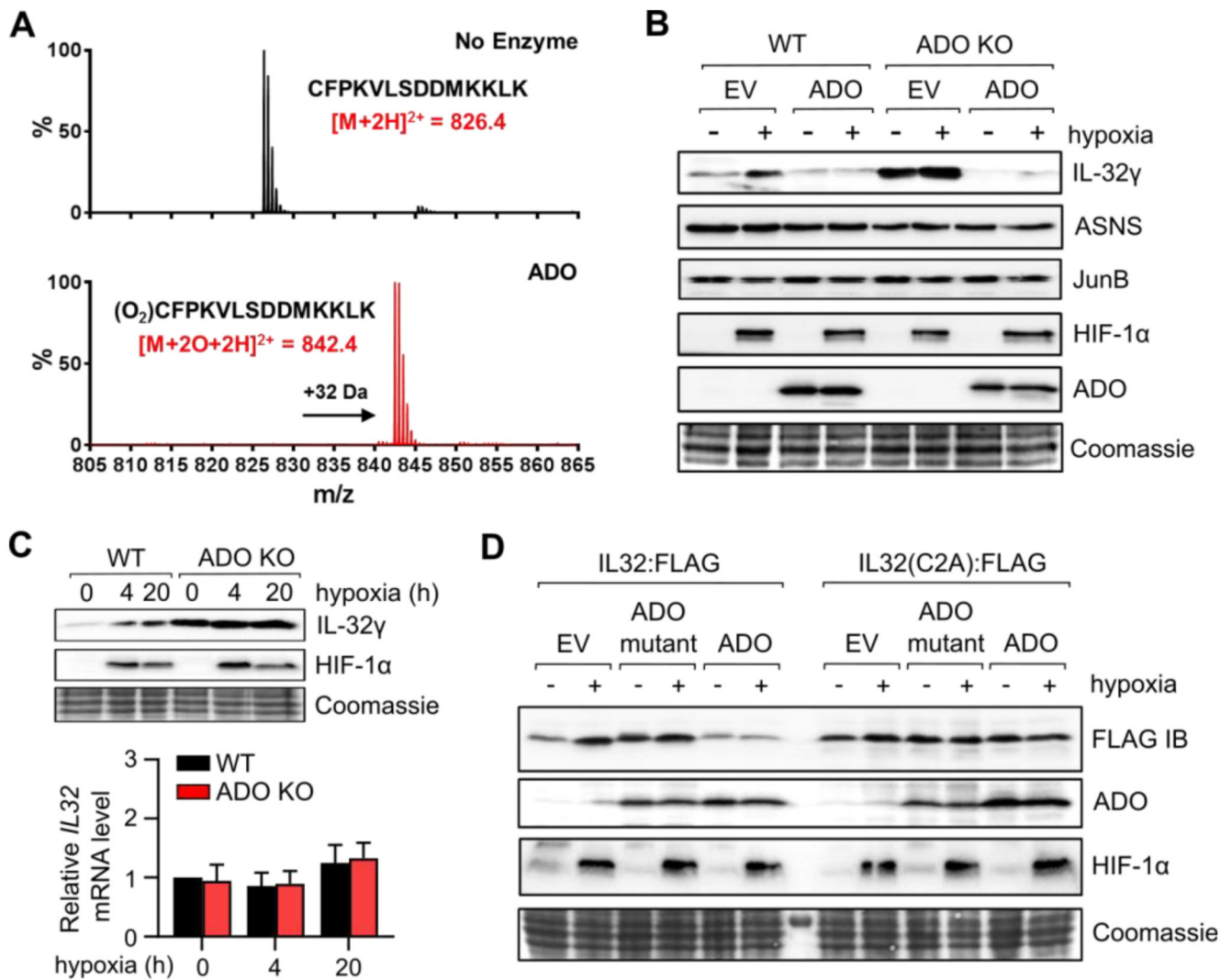


Fig. 5. IL-32 is a target of ADO-catalyzed N-terminal cysteine dioxygenation.

(A) Mass spectrometry analyses of the indicated IL-32 N-terminal peptide incubated aerobically with or without recombinant ADO (1h; 37°C), showing a +32 Da shift when incubated with ADO, indicative of the addition of O₂. The small peak at ~845 m/z in the absence of ADO (top panel) was confirmed to correspond to a potassium adduct of the unoxidized peptide. (B) IL-32, but not asparagine synthetase (ASNS) or JunB, are regulated by hypoxia (1% O₂, 4 h) and ADO in RKO cells. (C) ADO-dependent regulation of IL-32 by hypoxia is observed at the protein but not mRNA level. (D) 293T cells co-transfected with plasmids encoding C-terminally FLAG-tagged IL-32 or an IL-32(C2A) mutant, and either empty pRRL vector (EV), ADO or a catalytically inactive ADO mutant (H112A+H114A), and exposed to hypoxia (1% O₂) for 16 h. IL-32 levels were assessed using an anti-FLAG antibody. Hypoxic accumulation of IL-32 was evident in EV and mutant ADO, but not ADO, co-transfected cells, whilst C2A mutation abolished sensitivity to both hypoxia and ADO overexpression. Note that co-transfection with mutant ADO appears to increase basal

levels of IL-32, consistent with possible competition with endogenous ADO for substrate binding.