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

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Organisms must respond to hypoxia to preserve oxygen homeostasis. We identify a thiol oxidase, previously assigned as cysteamine (2-aminoethanethiol) dioxygenase (ADO), as a low oxygen affinity (high-K_mO_2) amino-terminal cysteine dioxygenase that transduces the oxygen-regulated stability of proteins by the N-degron pathway in human cells. ADO catalyzes the conversion of amino-terminal cysteine to cysteine sulfinic acid and is related to the plant cysteine oxidases that mediate responses to hypoxia by an identical posttranslational modification. We show in human cells that ADO regulates RGS4/5 (regulator of G protein signaling) N-degron substrates, modulates G protein-coupled calcium ion signals and mitogen-activated protein kinase activity, and that its activity extends to other N-cysteine proteins including the angiogenic cytokine interleukin-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 human oxygen sensors (1). These enzymes are low oxygen affinity (high-K_mO_2), 2-oxoglutarate (2-OG)-dependent dioxygenases that catalyze trans-4-prolyl hydroxylation of the transcription factor HIF (2, 3) to target it for proteolysis. By this process, these hydroxylases regulate a broad range of transcriptional responses to hypoxia [reviewed in (4)]. Although the prolyl hydroxylation of HIF was unprecedented as a signaling mechanism, 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). After the action of methionine aminopeptidases, oxidation of N-terminal cysteines creates a substrate for arginyltransferases, which catalyze the addition of this N-terminaldestabilizingresidue,promoting degradation. No cysteine-modifying enzyme was defined, but N-terminal cysteine oxidation was shown to be affected by nitric oxide (9) and, on the basis of in vitro studies, has been considered likely to be nonenzymatic. Subsequently, it was shown in plants 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 cysteine 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 green fluorescent protein (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 nonenzymatic, metal-catalyzed oxidation. Exposure of the transfected cells to hypoxia and to the nonspecific iron chelator dipyridyl resulted in accumulation of the RAP_{1-50}:GFP:V5 reporter protein, but not that of a C2A mutant, without affecting reporter transcript levels (fig. S1). By contrast, neither reporter was activated by nonspecific 2-OG dioxygenase inhibitors [desferrioxamine (DFO) and dimethyloxalylglycine (DMOG)] or a HIF prolyl hydroxylase inhibitor, all of which robustly induced HIF (Fig. 1A). In cells exposed to hypoxia for 16 hours and then treated with cycloheximide before being reoxygenated 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 that of the HIF prolyl hydroxylases and that operates on amino-acid sequences from plant RAP2.12, in a manner dependent on cysteine at position 2.

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 hemagglutinin (HA)-tagged RGS4 (RGS4:HA) and an RGS4:GFP fusion ($RGS4_{1-20}$:GFP), each encoding wild-type or C2A mutant sequences, revealed accumulation of wild-type, but not mutant, proteins in cells exposed to hypoxia and dipyridyl, but not DMOG or DFO (Fig. 1C and fig. S3). Endogenous RGS4 and RGS5 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 an enzyme(s) similar to the PCOs.

The human genome contains two thiol dioxygenases with a predicted structure similar to that of 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 cotransfected with a gene encoding RGS4:HA 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

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to constitutive up-regulation of endogenous and transfected RGS4 and RGS5 proteins irrespective of oxygen levels (Fig. 2B and figs. S6 and 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 reexpression 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

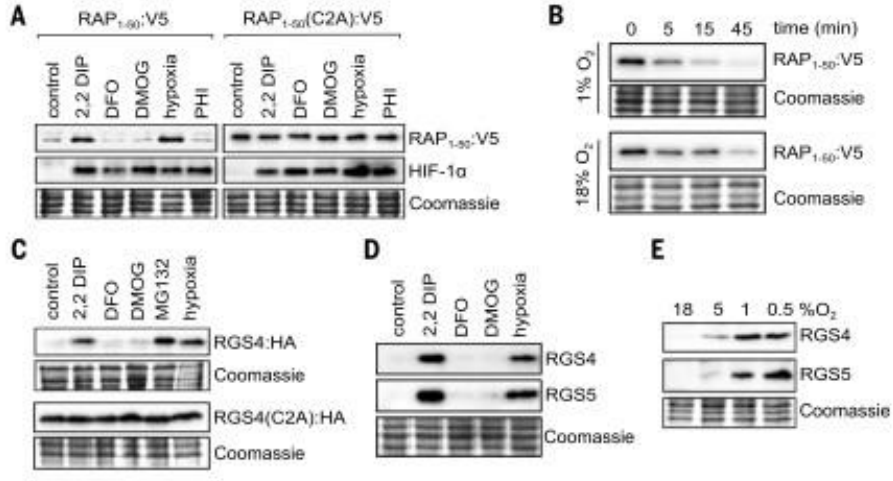

Fig. 1. Regulation of plant and animal N-degron substrates by oxygen in human cells. (A) Levels of fusion proteins linking the N-terminal 1-50 residues of plant RAP2.12 or a C2A mutant to a GFP:V5 cassette [RAP_{1-50}:V5; RAP_{1-50}(C2A):V5] in stably transfected U-2OS cells exposed to hypoxia or the indicated inhibitors. (B) RAP_{1-50}:V5 reporter protein half-life in cells incubated in hypoxia (16 hours, 1% O_2), treated with cycloheximide (100 mM, 10 min), and then maintained in hypoxia or reoxygenated for the indicated times. (C) C-terminal 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 mM); DFO, desferrioxamine (100 mM); DMOG, dimethyloxalylglycine (1 mM); PHI, prolyl hydroxylase inhibitor (125 mM); MG132, proteasomal inhibitor (25 mM). All exposures of cells to hypoxia or inhibitors were for 4 hours unless otherwise indicated. HIF-1a immunoblots are provided in (A) for comparison.

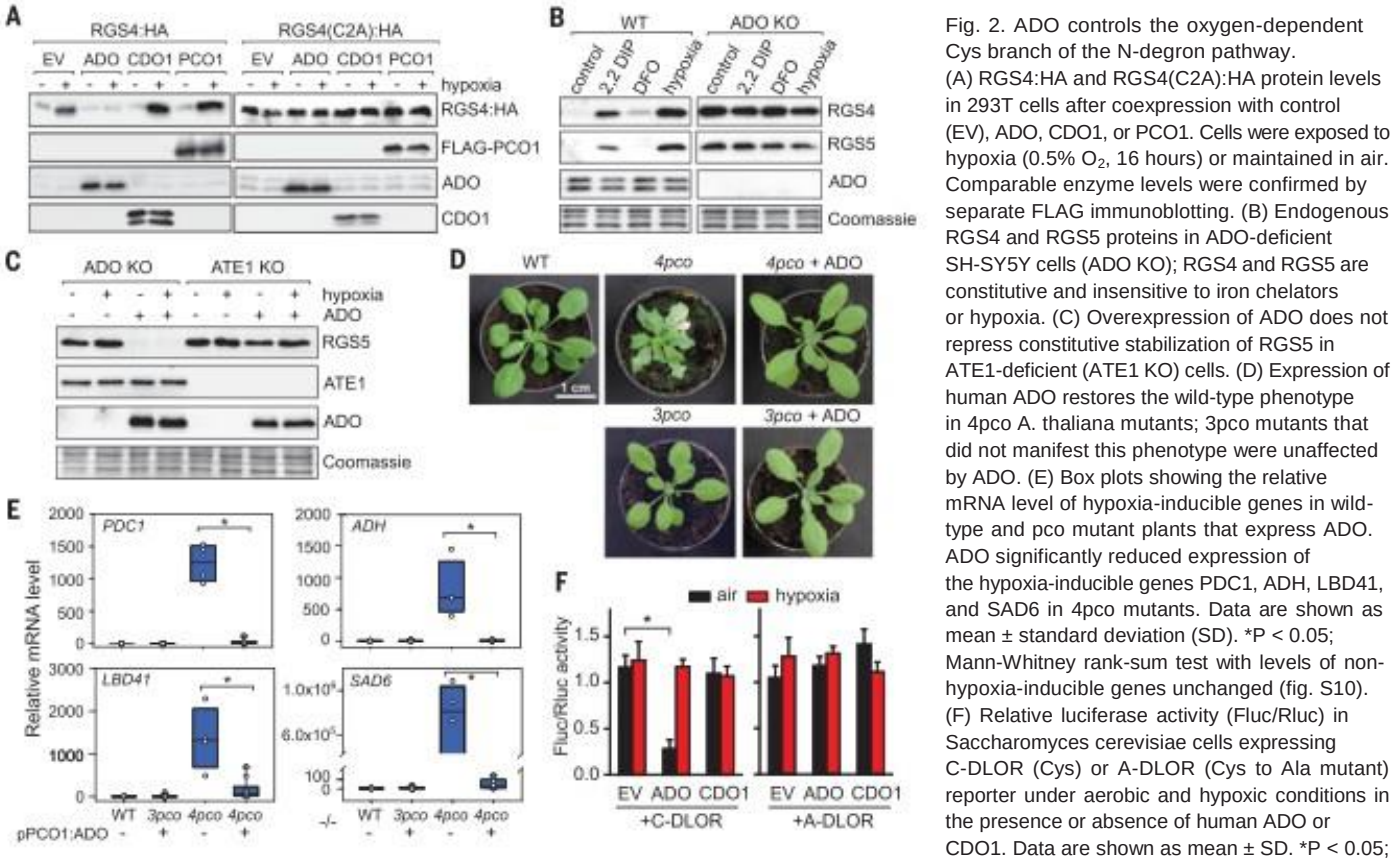

Fig. 2. ADO controls the oxygen-dependent Cys branch of the N-degron pathway. (A) RGS4:HA and RGS4(C2A):HA protein levels in 293T cells after coexpression with control (EV), ADO, CDO1, or PCO1. Cells were exposed to hypoxia (0.5% O_2, 16 hours) or maintained in air. Comparable enzyme levels were confirmed 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) Overexpression of ADO does not repress constitutive stabilization of RGS5 in ATE1-deficient (ATE1 KO) cells. (D) Expression of human ADO restores the wild-type phenotype in 4pco A. thaliana mutants; 3pco mutants that did not manifest this phenotype were unaffected by ADO. (E) Box plots showing the 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. Data are shown as mean ± standard deviation (SD). *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 Saccharomyces 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. Data are shown as mean ± SD. *P < 0.05; two-way analysis of variance (ANOVA) followed by Holm-Sidak post hoc test.

of the proposed cysteine oxidation in the N-degron pathway. Up-regulation of RGS5 in ATE1- 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 determine whether ADO can complement deficient PCO in plants, we generated a PCO-depleted A. thaliana mutant by crossing plants in which four of the five known PCO (1, 2, 4, and 5) genes were inactivated by transferred DNA insertional mutagenesis. Homozygous quadruple pco1/2/4/5 (4pco), but not triple pco1/2/4 (3pco), mutant plants manifested severe developmental defects (Fig. 2D) and up-regulation 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 (Fig. 2, D and E, and fig. S10). Consistent with complementation of defective PCO function, coexpression of ADO in A. thaliana protoplasts caused dose-dependent suppression of RAP2.12 luciferase fusion protein activity (fig. S10).

In budding yeast, cysteine is not a destabilizing N-terminal residue (18). To determine whether deficiency of an ADO-like enzyme might be responsible for the stability associated with N-cysteine in yeast, we introduced ADO or CDO1 into yeast cells together with a ratiometric 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 $RAP_{2\text{-}28}$-FLuc protein, but not a C2A mutant (Fig. 2F and fig. S11). Consistent with this, phylogenetic analyses revealed potential ADO and CDO orthologs 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 that 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 to 15 of human RGS4 and RGS5, and examined the products by mass spectrometry (MS). 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 $^{18}O_2$ or in the presence of ^{18}O-labeled water ($H_2{}^{18}O$). These experiments revealed a single +36 Da mass addition in $^{18}O_2$ and a single +32 Da mass addition in the presence of ^{18}O-labeled 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-cysteine residues in RGS4 and RGS5 to cysteine sulfinic acid. Kinetic measurements on human ADO (Fig. 3C and fig. S15) revealed high turnover (k_{cat}) values of 20.1 and 16.9 s^{-1} on RGS4 and RGS5 peptides under atmospheric conditions, respectively, but marked sensitivity to oxygen (apparent K_mO_2 > 500 mM). Thus, ADO resembles the HIF prolyl hydroxylase enzymes in manifesting a K_mO_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 a cysteamine dioxygenase, we also examined competition of N-cysteine peptide dioxygenation by free cysteamine and cysteine, but found inhibition only at high concentrations of these metabolites (IC_{50}, 37 and 13 mM, respectively, fig. S16).

RGS4 and RGS5 regulate heterotrimeric G protein signaling by enhancing Gα-coupled guanosine 5′-triphosphate hydrolysis and hence attenuating G protein signals. Because the catalytic activity of ADO lowers the levels of RGS4 and RGS5, ADO-deficient cells in which levels of these proteins are increased should manifest attenuation of G protein signaling on relevant pathways. Gα 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 arginyltransferase 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-knockout (KO), but not ATE1-KO, cells (Fig. 4B). To test the effects

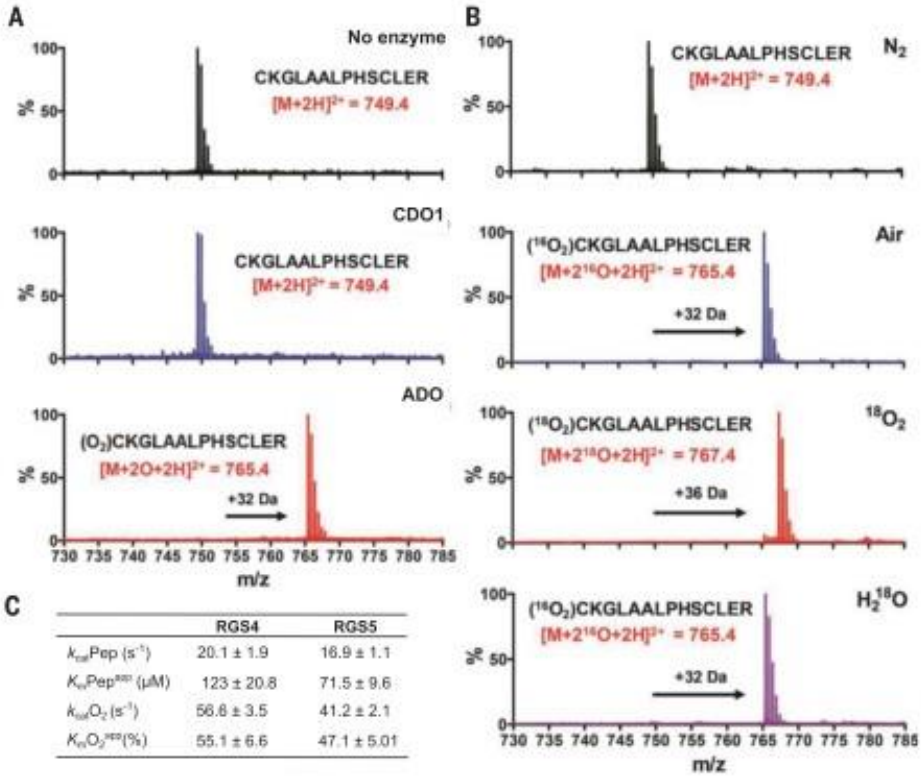

Fig. 3. ADO catalyzes the dioxygenation of the N-terminal Cys of RGS4/5 peptides. (A) MS results showing a mass shift of +32 Da when RGS5 N-terminal peptide was incubated with recombinant human ADO, but not with recombinant human CDO1. Similar results were obtained when an RGS4 N-terminal peptide was 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 labeling demonstrates incorporation of two 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_{cat}Pep and K_mPep^{app}) and O_2 levels using a fixed, nonlimiting concentration of peptide ($k_{cat}O_2$ and K_mO_{2app}) were examined to determine sensitivities to both substrates. m/z, mass-to-charge ratio. Source data are provided in fig. S15.

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 Ca^{2+}. Attenuation of Ca^{2+} mobilization in response to carbachol (Fig. 4C), but not to the receptor-independent ionophore ionomycin (Fig. 4D), was observed in ADO-deficient cells and was reversed by reexpression 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, this study establishes a role for ADO in the regulation of G protein signaling, consistent with its role as a cysteine-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 is 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-cysteine polypeptides and then measured dioxygenation (+32 Da mass shift) by MS. These experiments revealed substrate-dependent catalytic activity of ADO ranging from near zero to levels that were similar to those in experiments using RGS5 peptide (figs. S17 and S18). We then examined endogenous protein levels corresponding to peptide substrates that supported high [interleukin-32 (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 because IL-32 was not detected in SH-SY5Y cells. Immunoblotting revealed that the abundance of IL-32, but not ASNS or JunB, was

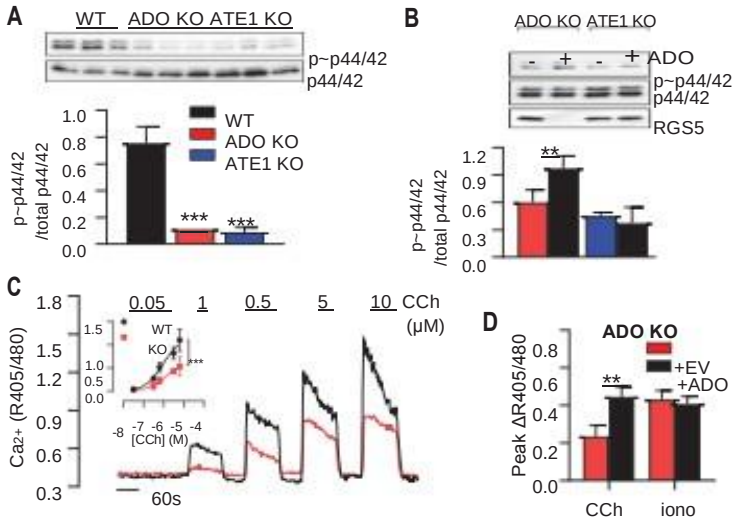

Fig. 4. ADO regulates G protein signaling. ADO regulates G protein signaling in SH-SY5Y cells. (A) MAPK (p44/42) phosphorylation in wild-type, ADO KO, and ATE1 KO cells. Immunoblot lanes represent separate biological replicates, with densitometric analysis provided below. Data are shown as mean ± SD. n = 3 independent clones. ***P < 0.001, one-way ANOVA with Holm-Sidak post hoc test. (B) Reexpression of ADO increases phosphorylated p44/42 in ADO KO, but not ATE1 KO cells. Data are shown as mean ± SD. n = 3 independent experiments. **P < 0.01, two-way ANOVA with Holm-Sidak post hoc test. (C) Carbachol (CCh)-stimulated rises in $[Ca^{2+}]_i$ 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 to 12 individual cells. ***P < 0.001, three-parameter nonlinear regression analysis. (D) Ionomycin (0.1 mM) is equipotent at stimulating Ca^{2+} release in ADO KO cells infected with either control (EV) or ADO-containing lentivirus, whereas responses to CCh are recovered by ADO reexpression. Data are shown as mean ± SD. n = 6 to 7 individual cells. *P < 0.05, two-way ANOVA with Holm-Sidak post hoc test.

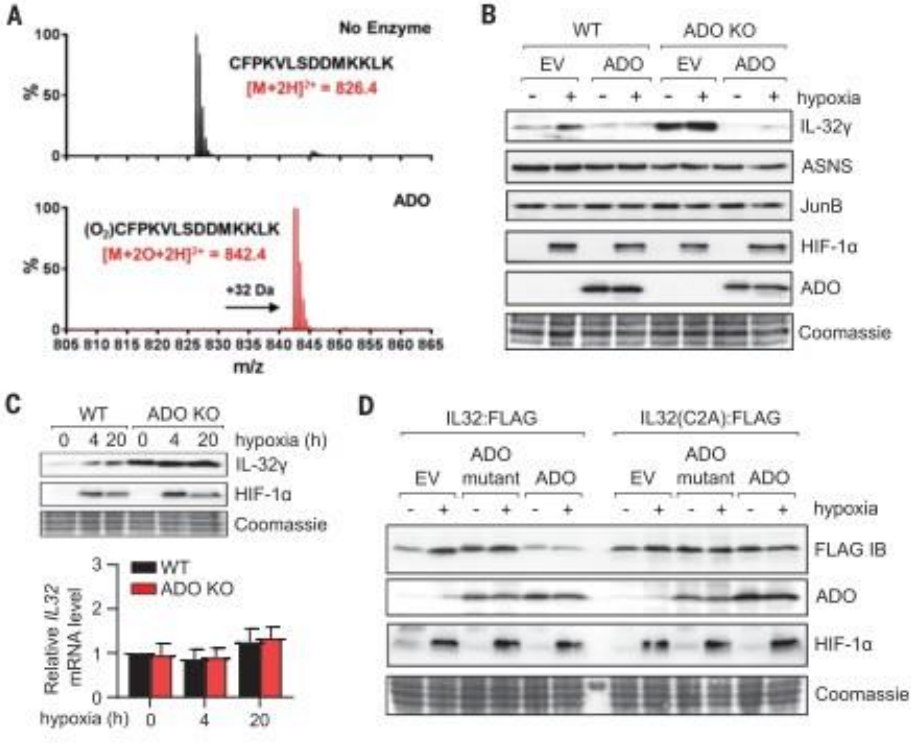

Fig. 5. IL-32 is a target of ADO-catalyzed N-terminal cysteine dioxygenation. (A) MS analyses of the indicated IL-32 N-terminal peptide incubated aerobically with or without recombinant ADO (1 hour at 37°C), showing a +32 Da shift when incubated with ADO, indicative of the addition of O_2. The small 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, is regulated by hypoxia (1% O_2, 4 hours) and ADO in RKO cells. (C) ADO-dependent regulation of IL-32 by hypoxia is observed at the protein but not the mRNA level. (D) 293T cells cotransfected 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% O_2) for 16 hours. 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, cotransfected cells, whereas C2A mutation abolished sensitivity to both hypoxia and ADO overexpression. Note that cotransfection with mutant ADO appears to increase basal levels of IL-32, consistent with possible competition with endogenous ADO for substrate binding.

increased in hypoxic cells, accumulated constitutively in ADO-deficient cells, and was reduced by reexpression of transfected ADO (Fig. 5B). Experiments in wild-type and ADO-deficient RKO cells confirmed IL-32 regulation at the protein but not the mRNA level (Fig. 5C). Further experiments confirmed dioxygenation of the N-terminal cysteine after reaction of the IL-32 peptide with recombinant ADO (fig. S18) and showed that this residue was necessary for ADO-mediated suppression of cotransfected 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-cysteine polypeptides that might be generated in cells and it is therefore likely that other ADO-regulated human targets exist. 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 Cys branch 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-catalyzed dioxygenation, 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. By contrast, direct operation of ADO on the protein stability of signaling molecules has the potential to transduce more rapid responses to hypoxia than those mediated by the transcriptional output of 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 proinflammatory cytokine networks and angiogenic growth factors (24, 25). Although our findings identify ADO as an essential regulator of the 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 cells, but not in other cells. These findings predict that in specific cellular settings in which 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.

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