

# Vitamin C is dispensable for oxygen sensing in vivo

Katarzyna J. Nytko,<sup>1</sup> Nobuyo Maeda,<sup>2</sup> Philipp Schläfli,<sup>1</sup> Patrick Spielmann,<sup>1</sup> Roland H. Wenger,<sup>1</sup> and Daniel P. Stiehl<sup>1</sup>

<sup>1</sup>Institute of Physiology and Zürich Center for Integrative Human Physiology, University of Zürich, Zürich, Switzerland; and <sup>2</sup>Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC

**Prolyl-4-hydroxylation is necessary for proper structural assembly of collagens and oxygen-dependent protein stability of hypoxia-inducible transcription factors (HIFs). In vitro function of HIF prolyl-4-hydroxylase domain (PHD) enzymes requires oxygen and 2-oxoglutarate as co-substrates with iron(II) and vitamin C serving as cofactors. Although vitamin C deficiency is known to cause the collagen-disassembly disease scurvy, it is unclear whether cellular oxygen sensing is similarly affected. Here, we report that vitamin**

**C–deprived *Gulo*<sup>−/−</sup> knockout mice show normal HIF-dependent gene expression. The systemic response of *Gulo*<sup>−/−</sup> animals to inspiratory hypoxia, as measured by plasma erythropoietin levels, was similar to that of animals supplemented with vitamin C. Hypoxic HIF induction was also essentially normal under serum- and vitamin C–free cell-culture conditions, suggesting that vitamin C is not required for oxygen sensing in vivo. Glutathione was found to fully substitute for vitamin C requirement of all 3 PHD isoforms in vitro.**

**Consistently, glutathione also reduced HIF-1 $\alpha$  protein levels, transactivation activity, and endogenous target gene expression in cells exposed to CoCl<sub>2</sub>. A Cys201Ser mutation in PHD2 increased basal hydroxylation rates and conferred resistance to oxidative damage in vitro, suggesting that this surface-accessible PHD2 cysteine residue is a target of anti-oxidative protection by vitamin C and glutathione. (*Blood*. 2011;117(20): 5485-5493)**

## Introduction

Oxygen is essential for several physiologic processes, particularly for cellular respiration and energy metabolism. On the molecular level, response to hypoxia is mediated by hypoxia-inducible transcription factors (HIFs). Under continuous oxygen supply, 2 distinct prolyl residues within the oxygen-dependent degradation domain of HIF $\alpha$  subunits are hydroxylated by prolyl-4-hydroxylase domain-containing enzymes (PHDs). Hydroxy-HIF $\alpha$  is recognized by the von Hippel-Lindau tumor suppressor protein (pVHL) and subsequently targeted for proteasomal degradation.<sup>1,2</sup> When oxygen is limited, PHD activity ceases, and nonhydroxylated HIF $\alpha$  is stabilized and heterodimerizes with the HIF $\beta$  subunit to activate expression of numerous target genes.<sup>3</sup> Moreover, an asparaginyl hydroxylase termed factor-inhibiting HIF hydroxylates a C-terminal Asn residue of HIF $\alpha$  subunits in an oxygen-dependent manner, thereby regulating cofactor recruitment and HIFs' transcriptional activity.<sup>4</sup>

Three PHD isoforms have been characterized so far, termed PHD1, PHD2, and PHD3; they differ in size, subcellular localization, and tissue distribution.<sup>5</sup> PHD2 is the most ubiquitously expressed isoform, responsible for the normoxic control of HIF $\alpha$ .<sup>6</sup> Accordingly, genetic ablation of PHD2 but not PHD1 or PHD3 results in embryonic lethality in mice.<sup>7</sup> Suggesting a fundamental role in the hematopoietic and circulatory systems, somatic inactivation of PHD2 leads to increased erythropoiesis and angiogenesis as a result of HIF $\alpha$  stabilization followed by activation of its target genes, including erythropoietin (EPO) and vascular endothelial growth factor.<sup>8</sup> Knockout of either PHD1 or PHD3 had no effect on hematologic parameters. However, combined PHD1/PHD3 knockout animals showed a slight increase in hematocrit, hemoglobin,

and red blood cell counts.<sup>9</sup> Clinical data on patients with erythrocytosis revealed Pro317Arg or Arg371His mutations in the gene encoding for PHD2, altering the hydroxylation efficiency of the mutant protein.<sup>10,11</sup> A third point mutation in PHD2 (His374Arg) was found in a patient suffering from erythrocytosis and paraganglioma.<sup>12</sup> These case reports emphasize the critical role of PHD2 in regulating erythropoiesis and maintaining red blood cell homeostasis in humans.

PHDs belong to a larger superfamily of 2-oxoglutarate and Fe(II)-dependent dioxygenases. Similar to collagen prolyl-4-hydroxylase ([C-P4H]; EC 1.14.11.2), PHDs require molecular oxygen and 2-oxoglutarate as cosubstrates, as well as ferrous iron and probably L-ascorbic acid (vitamin C) as cofactors for enzymatic activity.<sup>13</sup>  $K_m$  values of PHDs for oxygen are strikingly higher than those of other prolyl-4-hydroxylases.<sup>13</sup> The relatively low oxygen affinity is essential for effective oxygen sensing, because even small changes in oxygen partial pressure can influence hydroxylation activity.<sup>13-15</sup>

In a previous study, we reported on the dose-dependent regulation of the in vitro activity of all 3 PHD isoforms by their essential cosubstrates and cofactors, including vitamin C.<sup>16</sup> Primates, including humans, lost the ability to synthesize vitamin C de novo and thus depend on dietary vitamin C intake. C-P4Hs hydroxylate proline residues to stabilize the collagen triple helix structure. Because ascorbate is an essential cofactor for C-P4Hs, persistent ascorbate deficiency results in disassembly of connective tissue structures, a common symptom of the nowadays rare disease scurvy.<sup>17</sup> With  $K_m$  values ranging from 140 to 180 $\mu$ M, the

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requirement of PHDs for vitamin C in vitro is only 2-fold lower than for C-P4H, suggesting that HIF hydroxylases also could well be affected by vitamin C malnutrition.<sup>13</sup> Mice lacking a functional *Gulo* gene have been described as a model to study vitamin C deficiency.<sup>18</sup> *Gulo* encodes for l-gulono-1,4-lactone-oxidase (EC 1.1.3.8), a key enzyme involved in the final step of vitamin C biosynthesis. Dietary vitamin C deprivation leads to body weight loss, anemia, aortic wall damage, and internal hemorrhages in these mice.<sup>18</sup>

Although the interaction between the target prolyl residue, molecular oxygen, 2-oxoglutarate, and iron during the reaction cycle in the active center of PHDs has been described in detail previously, the apparently inevitable presence of vitamin C for the in vitro function of the PHDs remains elusive.<sup>19,20</sup> Because of its antioxidative properties, vitamin C might maintain ferrous iron in the reduced state. Given the enzymatic relationship between HIF $\alpha$  and C-P4Hs, we set out to investigate the effect of dietary vitamin C on the regulation of the PHD-HIF oxygen-sensing pathway in *Gulo*<sup>-/-</sup> mice under normoxemic and hypoxemic conditions.

## Methods

### Cell culture

HeLa human cervix carcinoma cells were adapted to Ham nutrient mixture F-12 (Sigma-Aldrich), free of ascorbate and fetal calf serum (FCS), containing the following supplements: epidermal growth factor (50 ng/mL; Sigma-Aldrich), insulin (5  $\mu$ g/mL; Sigma-Aldrich), apo-transferrin (5  $\mu$ g/mL; Sigma-Aldrich), hydrocortisone (100 nM; Sigma-Aldrich) buffered with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid pH 7.4 as well as 100 IU/mL penicillin and 100  $\mu$ g/mL streptomycin. HepG2 human hepatoma cells stably transfected with a HIF-dependent firefly luciferase reporter gene termed HRG1 have been described previously.<sup>21</sup> If not indicated otherwise, all cells were maintained in DMEM with 10% FCS, 100 IU/mL penicillin, and 100  $\mu$ g/mL streptomycin. Cell number and viability were determined using a ViCell counter (Beckman Coulter).

### HIF transactivation activity

HeLa cells ( $5 \times 10^5$ ) were cotransfected with 500 ng of the HIF-dependent pH3SVL reporter vector containing 6 HIF binding sites in total derived from regulatory elements of the *transferrin* gene<sup>22</sup> and 40 ng of pRL-CMV *Renilla* luciferase expression plasmid (Promega) essentially as described previously.<sup>14</sup> Twenty-four hours after transfection, cells were split and exposed to graded oxygen concentrations (21%-0.2% oxygen) for 24 hours by using cross-calibrated oxygen-controlled CO<sub>2</sub> incubators (CB 150; Binder). Stably transfected HRG1 HIF reporter cells were adapted to 1% FCS overnight and treated with 50  $\mu$ M desferrioxamine mesylate (Dfx; Sigma-Aldrich) or 100  $\mu$ M CoCl<sub>2</sub> and 1 to 10 mM reduced l- $\gamma$ -glutamyl-l-cysteinyl-glycine ([GSH] 250 mM stock solution adjusted to pH 7.0) or 0.2 to 2 mM ascorbate for 24 hours. For hypoxic experiments, cells were grown under 2% O<sub>2</sub> for 24 hours and treated with GSH or ascorbate. HRG1 cells were transfected with pRL-SV40 *Renilla* luciferase to control for non-HIF-mediated effects of ascorbate and GSH on the heterologous simian virus 40 minimal promoter present in both constructs. Cells were lysed using passive lysis buffer, and luciferase activities were determined according to the manufacturer's instructions (Promega) by using a 96-well luminometer (Berthold Technologies). Data are expressed as relative luciferase activities per total cellular protein of experiments performed in triplicates by calculating the ratio of firefly/*Renilla* activities per well.

### Expression and purification of recombinant PHD enzymes

Recombinant PHD proteins were expressed and purified as glutathione transferase (GST)-fusion proteins from baculovirus-infected Sf9 insect cells as described previously.<sup>14</sup> Untagged enzyme preparations were

obtained by introducing a PreScission protease cleavage site between the GST-tag and the PHD open reading frame. A Cys201Ser point mutation was introduced into the human PHD2 expression plasmid by site-directed mutagenesis (Stratagene). Untagged PHD2 was expressed in Sf9 cells and purified by conventional ion-exchange chromatography (kind gift of Dr Felix Oehme, Bayer Healthcare, Wuppertal, Germany). Purity of the enzyme preparations was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie staining or immunoblotting.

### Prolyl-4-hydroxylation assay

Activity of recombinant PHD enzymes was measured by a microtiter plate-based peptide hydroxylation assay as described previously.<sup>23</sup> In brief, recombinant PHDs were used to hydroxylate a biotinylated peptide derived from HIF-1 $\alpha$  (amino acid residues 556-574) coupled to streptavidin-coated 96-well plates. Hydroxylation reaction was performed for 1 hour at room temperature in the presence of 10  $\mu$ M FeSO<sub>4</sub>, 0.5 mM 2-oxoglutarate, and 2 mM ascorbate in 20 mM Tris-HCl pH 7.5, 5 mM KCl, and 1.5 mM MgCl<sub>2</sub>. Hydroxylated peptides were detected by recombinant, thioredoxin-tagged von Hippel-Lindau/elongin B/elongin C (VBC) complex. Reactions were stopped by removing the reaction mix and adding 1 mM H<sub>2</sub>O<sub>2</sub>. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies and secondary horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (Sigma-Aldrich) by using the 3,3',5,5'-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding 2 M H<sub>2</sub>SO<sub>4</sub>, and absorbance was determined at 450 nm in a microplate reader. Background values as determined by using a mutant HIF-1 $\alpha$  (Pro564Ala) peptide were subtracted for each experiment.

### Ascorbate determination

Ascorbate content of in vitro hydroxylation assay samples was quantified by high-performance liquid chromatography (HPLC) as described previously.<sup>24</sup> In brief, a 10-fold dilution of the enzyme reaction mix containing 2 mM ascorbate was analyzed before and after 1 hour of hydroxylation reaction. After dilution in the mobile phase (60 mM phosphoric acid, pH 3.1), a 20- $\mu$ L sample was injected onto a Nucleosil C18 column (Macherey Nagel) and eluted applying an acetonitrile gradient (0%-60%). Ascorbate elution was monitored at 254 nm, corresponding to 96% absorbance of ascorbate and only 4% of dehydroascorbate.<sup>25</sup> Chromatograms and standard curve of pure ascorbate ranging from 25 to 200  $\mu$ M were used to calculate the content of ascorbate in study samples (supplemental Figure 2, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Ascorbate levels in plasma samples of mice were determined by HPLC (Swiss Vitamin Institute).

### Immunoblot analyses

Total soluble cellular proteins were extracted with a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was measured by the method of Bradford, and 60 to 70  $\mu$ g of cellular protein was subjected to immunoblot analyses. Membranes were probed using the following dilutions of mouse monoclonal (mAb) or rabbit polyclonal antibodies: mAb anti-HIF-1 $\alpha$  (1:1000; BD Transduction Laboratories), mAb anti-CA9 (M75; kindly provided by S. Pastorekova, Bratislava, Slovak Republic), mAb anti- $\beta$ -actin (1:5000; Sigma-Aldrich), rabbit anti-PHD1 (1:2000; Genway), rabbit anti-PHD2 (1:1000; Novus), or rabbit anti-PHD3 (1:1000; Novus), followed by secondary HRP-conjugated antibodies (1:2000; all Pierce).

### mRNA quantification

Total RNA purification and mRNA determination by real-time PCR has been described previously.<sup>14</sup> Transcript levels of the HIF-dependent and -independent genes were quantified by reverse transcription (RT) quantitative (q) PCR using SYBR Green qPCR reagent kit (Sigma-Aldrich) in combination with an MX3000P light cycler (Stratagene). Initial copy number of each sample was calculated by comparison with serial dilutions

of a calibrated standard. For mouse tissues, ribosomal protein S12 mRNA was used as a housekeeping gene, and ribosomal protein L28 mRNA served as control for samples from human cell lines. Primer sequences are given in supplemental Table 1.

### OxyBlot detection of protein oxidation

PHD2 carbonylation was determined with a protein oxidation detection kit (OxyBlot; Millipore). In brief, 50 ng/ $\mu$ L recombinant PHD2 was exposed to either 100  $\mu$ M CoCl<sub>2</sub>, 10  $\mu$ M FeSO<sub>4</sub>, 2mM ascorbate, 0.5mM 2-oxoglutarate, or 1mM H<sub>2</sub>O<sub>2</sub> in the presence of 400nM wild-type or Pro564Ala mutant HIF-1 $\alpha$  peptide in 20mM Tris-HCl, 5mM KCl, and 1.5mM MgCl<sub>2</sub> for 1 hour at room temperature. Then, 5  $\mu$ L of the reaction mix was mixed with 5  $\mu$ L of 12% sodium dodecyl sulfate, and the carbonyl groups were derivatized with 10mM 2,4-dinitrophenylhydrazine for 15 minutes. Dinitrophenyl groups were detected by immunoblotting using rabbit anti-DNP antibodies (1:150) followed by secondary goat anti-rabbit HRP-conjugated antibodies (1:300). For loading controls, PHD2 was detected using rabbit anti-PHD2 antibodies (Novus).

### Animal studies

*Gulo*<sup>-/-</sup> mice were maintained on vitamin C-supplemented water containing 0.33 g/L L-ascorbic acid and 0.01mM EDTA as described previously.<sup>18</sup> At 3 months of age, ascorbic acid supplement was withdrawn from 6 *Gulo*<sup>-/-</sup> males but continued for the control males. After 5 weeks, mice were killed with an overdose of 2,2,2-tribromoethanol, and tissues were collected and stored in RNAlater (Applied Biosystems) at -20°C until use. For hypoxia studies, 22 *Gulo*<sup>-/-</sup> males with an average age of 16 weeks were allocated to 4 groups so that no significant differences were observed in mean body weight and age of the animals at the beginning of the experiment. The body weight was determined every second day. After 36 days of ascorbate withdrawal, mice were exposed to 8% oxygen for 24 hours in a hypoxia tent (Coy Laboratory Products). Control animals were maintained at ambient oxygen concentration. Heparinized whole blood was collected from all mice by cardiac puncture after intraperitoneal injection anesthesia using 4 mg/mL xylazine and 20 mg/mL ketamine at a dosage of 0.1 mL/20 g body weight. Blood samples of hypoxic animals were collected inside the hypoxic tent. Animal experiments were conducted at 2 centers with the appropriate consent by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill for breeding and normoxic gene expression studies or by the Veterinary Office of the Canton Zürich (119/2010) for hypoxia studies.

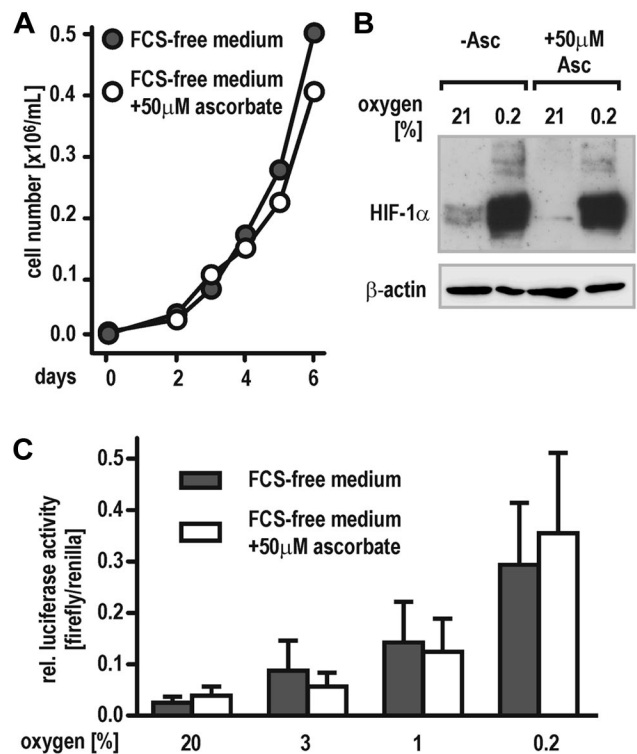
### Blood parameters and plasma EPO concentrations

Plasma EPO levels were measured by enzyme-linked immunosorbent assay following the procedures recommended by the manufacturer (Quantikine; R&D Systems). EPO concentrations were determined by comparison with a calibrated recombinant mouse EPO standard. Hematologic parameters of mouse whole blood were analyzed by the Division of Hematology (University Hospital, Zürich, Switzerland).

## Results

### Ascorbate is not required for HIF induction by hypoxia in HeLa cells

HeLa cells have been described previously to grow in serum-free medium supplemented with hormones and growth factors.<sup>26</sup> To achieve cell culture conditions avoiding ascorbate contamination derived from animal sera, HeLa cells were adapted to a chemically defined medium free of ascorbate for at least 2 weeks. Control cells were grown in the same medium supplemented with 50  $\mu$ M ascorbate. Both cell groups proliferated at the same rate with no differences noticed between ascorbate-free and supplemented cells (Figure 1A). Despite its need for PHD activity in vitro, hypoxic



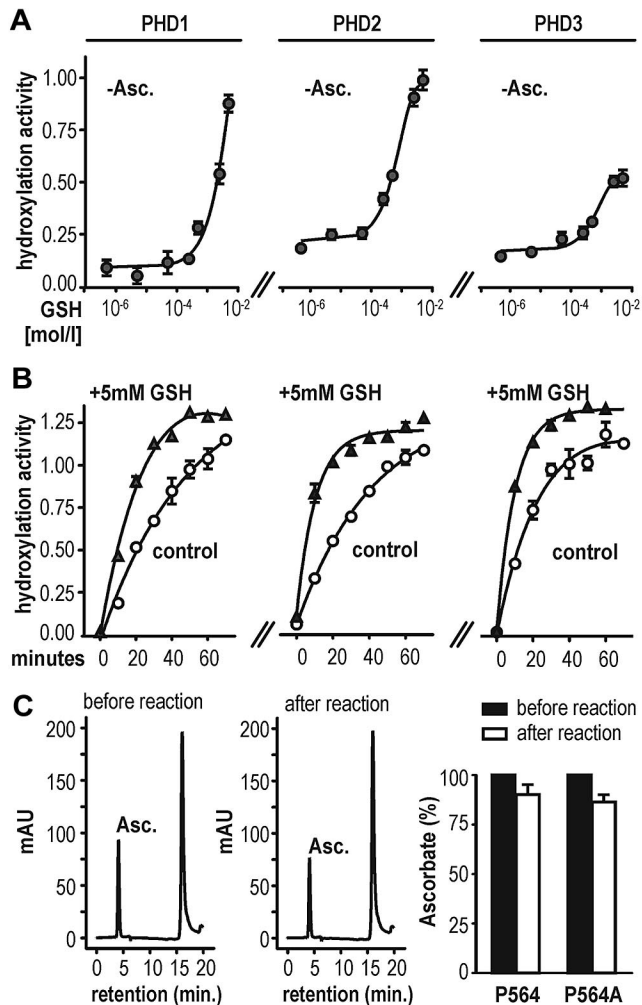
**Figure 1. Cellular oxygen sensing by the PHD-HIF pathway does not require vitamin C.** (A) Proliferation of HeLa cells growing in FCS-free, chemically defined medium containing either no or 50  $\mu$ M ascorbate. (B) Stabilization of HIF-1 $\alpha$  protein in HeLa cells maintained in FCS-free medium containing either no (-Asc.) or 50  $\mu$ M ascorbate. Cells were exposed to 21% and 0.2% oxygen for 6 hours, and then protein levels were analyzed by immunoblotting. (C) Induction of HIF-dependent luciferase activity (pH3SVL vector) in HeLa cells maintained in FCS-free medium containing either no or 50  $\mu$ M ascorbate and exposed to 0.2% to 21% oxygen for 24 hours.

HIF-1 $\alpha$  protein accumulation was similar in ascorbate-containing and -deficient cells. However, a faint normoxic induction of HIF-1 $\alpha$  could be observed in ascorbate-free cells only (Figure 1B). Accordingly, cells transfected with a HIF-responsive reporter gene (pH3SVL) and subsequently exposed to graded oxygen concentrations (0.2%, 1%, 3%, or 21% O<sub>2</sub>, respectively) revealed similar induction levels of luciferase activity under both culture conditions (Figure 1C).

### GSH can substitute for vitamin C in the hydroxylation reaction catalyzed by PHDs

Because HeLa cells grown in a medium containing no ascorbate maintained hypoxic HIF-1 $\alpha$  stabilization, we speculated that other antioxidants could compensate for vitamin C loss in these cells. Thus, several compounds with antioxidative properties were tested for their effects on PHD hydroxylation activity by using a previously described in vitro hydroxylation assay.<sup>23</sup> Surprisingly, some compounds such as *n*-propyl gallate<sup>16</sup> and the superoxide dismutase mimetic Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride were potent inhibitors of PHD enzymes (supplemental Figure 1A). However, GSH enhanced HIF $\alpha$  hydroxylation by all 3 PHDs. Because the recombinant enzyme preparations in the initial experiments were expressed and purified as GST-fusion proteins, we could not exclude interference of the tested antioxidants, particularly GSH, with the GST-tag. Therefore, a PreScission protease cleavage site was engineered in between the 2 fusion partners. Chimeric GST.PHD and tag-free PHD enzymes showed comparable hydroxylation activity (supplemental Figure 1B). GSH





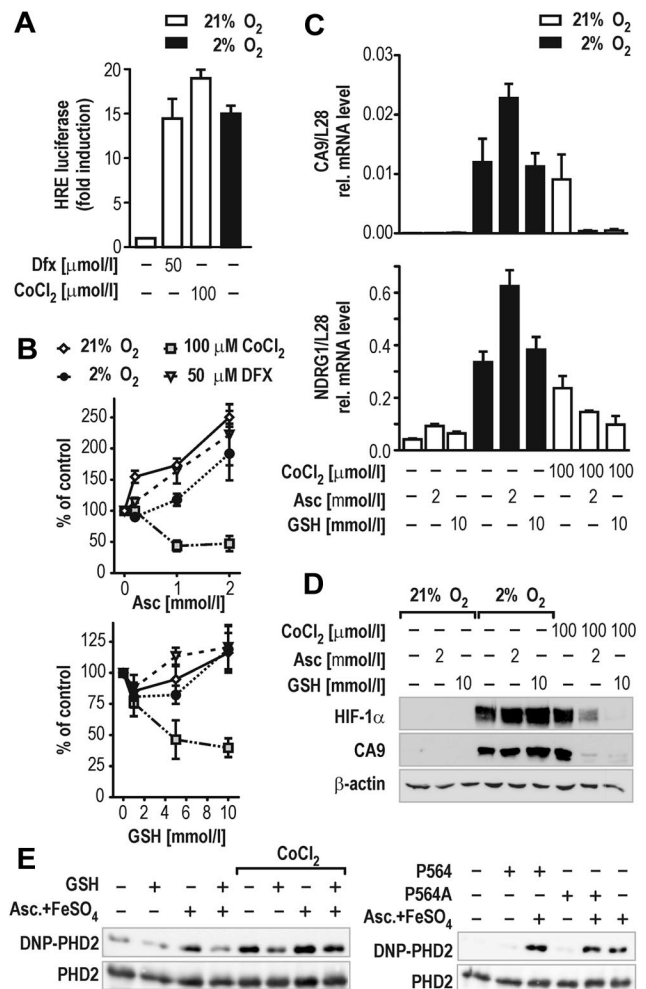
**Figure 2.** GSH substitutes for vitamin C as a cofactor in HIF-1 $\alpha$  hydroxylation in vitro. (A) GSH can enhance PHD hydroxylation activity in the absence of ascorbate (-Asc.) in a dose-dependent manner. Hydroxylation activity was determined using a multiwell VBC binding assay. (B) PHD-dependent hydroxylation reaction rate in the presence of 2mM ascorbate (control) or 2mM ascorbate combined with 5mM GSH (+5mM GSH). Shown are mean values  $\pm$  SEM of triplicates. (C) Ascorbate determination by HPLC before and after 1 hour of PHD2-dependent hydroxylation reaction (left panels). Ascorbate content is only slightly decreased after 1 hour of incubation and independent of target hydroxylation (right panel). Shown are mean values  $\pm$  SEM of 3 independent experiments normalized to values measured at time point zero.

increased the activity of all 3 untagged PHD isoforms, even in the absence of ascorbate (Figure 2A). Addition of GSH (+5mM in Figure 2B) to 2mM ascorbate increased the reaction rate compared with ascorbate alone (control in Figure 2B), suggesting 2 independent reaction modes of GSH, one mode replacing vitamin C and an additional mode enhancing the reaction rate. Of note, only minor changes in ascorbate oxidation were found before and after 1 hour of PHD2-mediated substrate hydroxylation (Figure 2C). The minor decrease in reduced ascorbate was probably because of air-dependent oxidation rather than enzymatic consumption because it was independent of the presence of the hydroxyl-acceptor substrate (Figure 2C right panel). In conclusion, as shown previously for C-P4H,<sup>27</sup> ascorbate is not consumed during coupled PHD-catalyzed hydroxylation reactions.

#### GSH decreases HIF activity in CoCl<sub>2</sub>-treated hepatoma cells

In cell culture models, Co(II) and Ni(II) have been shown to substantially decrease cellular ascorbate content by catalyzing

ascorbate oxidation to dehydroascorbate followed by irreversible hydrolysis to diketogulonate.<sup>28</sup> Interestingly, exogenous ascorbate administration completely blunted the Co(II)-induced hypoxic response in lung epithelial cells.<sup>29</sup> To test whether GSH could similarly compensate for reduced ascorbate levels after Co(II) stimulation, HIF transcriptional activity was further studied in HepG2 hepatoma cells stably transfected with an HIF-dependent luciferase reporter gene (HRG1 cells). We first determined the concentrations of CoCl<sub>2</sub> required to activate HIF-dependent reporter gene expression to a similar extent as exposure of the cells to 2% oxygen or the hypoxia mimicking iron chelator Dfx (Figure 3A). Subsequently, HRG1 cells were treated with 100 $\mu$ M CoCl<sub>2</sub> or 50 $\mu$ M Dfx under 21% or 2% oxygen. Control cells were kept at



**Figure 3.** GSH impairs HIF activation in cells. (A) Induction of HIF-dependent luciferase reporter gene activity in stably transfected HRG1 hepatoma cells by 2% O<sub>2</sub>, 50 $\mu$ M Dfx, or 100 $\mu$ M CoCl<sub>2</sub> for 24 hours. (B) Effects of ascorbate (Asc, 0.2-2mM; top panel) or GSH (1-10mM; bottom panel) in combination with hypoxia, Dfx, or CoCl<sub>2</sub> treatment on HIF-dependent luciferase activity relative to the protein concentration of the lysates. Shown are mean values  $\pm$  SEM of 3 independent experiments normalized to the reporter activity in the absence of either ascorbate or GSH (control). (C) CA9 and NDRG1 HIF target gene mRNA levels in HRG1 cells after treatment with 2mM ascorbate or 10mM GSH combined with 2% O<sub>2</sub> or 100 $\mu$ M CoCl<sub>2</sub> for 24 hours. Shown are mean values  $\pm$  SEM of 3 independent experiments relative to the mRNA content of ribosomal protein L28. (D) HIF-1 $\alpha$  and CA9 protein levels in HRG1 cells after treatment with 2mM ascorbate or 10mM GSH combined with 2% O<sub>2</sub> or 100 $\mu$ M CoCl<sub>2</sub>. (E) OxyBlot analyses of recombinant PHD2 protein carbonylation. GSH (5mM) reduced PHD2 carbonylation by either 2mM ascorbate/10 $\mu$ M FeSO<sub>4</sub> or 100 $\mu$ M CoCl<sub>2</sub> (left panel). PHD2 oxidation is independent of target hydroxylation as shown by using a wild-type or a Pro564Ala mutant HIF-1 $\alpha$  hydroxyl-proline acceptor peptide in in vitro hydroxylation reactions (right panel).

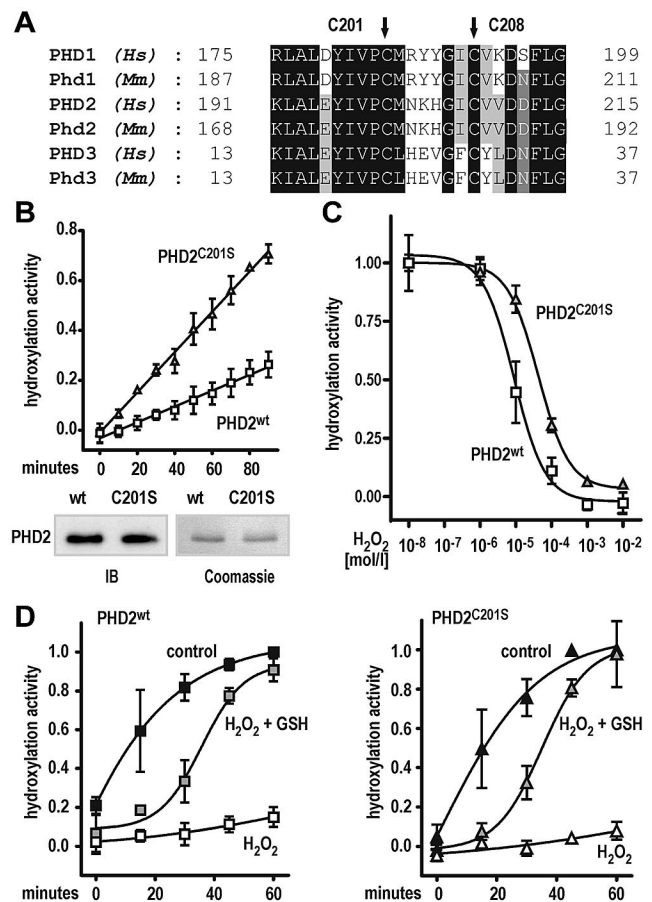
ambient oxygen concentrations. All cells were cotreated with 1 to 10mM GSH or 0.2 to 2mM ascorbate. Indeed, ascorbate and GSH reduced HIF activity exclusively in  $\text{CoCl}_2$ -treated HRG1 cells (Figure 3B). A substantial increase of HIF activation was noted particularly when cells were treated with 2mM ascorbate, which might be explained by the pro-oxidative function ascorbate exerts if applied at high concentration (Figure 3B top panel).<sup>30</sup> In line with these observations, both ascorbate and GSH reduced the expression levels of the endogenous HIF target genes *CA9* and *NDRG1*<sup>3</sup> only in cells treated with  $\text{CoCl}_2$ , whereas 2mM ascorbate enhanced hypoxic activation of both genes by almost 2-fold (Figure 3C). To evaluate whether these effects reflected differential activities of cellular PHD enzymes, HIF-1 $\alpha$  protein accumulation was analyzed by immunoblotting. As expected, only  $\text{CoCl}_2$ -stabilized HIF-1 $\alpha$  was down-regulated by cotreatment with ascorbate or GSH (Figure 3D). Interestingly, 10mM GSH was a more potent inhibitor of  $\text{CoCl}_2$ -induced HIF-1 $\alpha$  stabilization than 2mM ascorbate, a concentration that showed saturated inhibition of the HIF-reporter in the same cell line (Figure 3B top panel).

### GSH protects PHD2 from metal-catalyzed oxidation

Enzymatic activity of the PHDs is sensitive to reactive oxygen species and transition metal ions.<sup>31</sup> However, the mechanism(s) by which reactive oxygen species or metal ions inhibit hydroxylase activity remained speculative. Besides its general antioxidative properties as radical scavenger, vitamin C actively interferes with the oxidation state of metal ions by serving as electron donor in a redox reaction. As such, it largely differs from GSH that is a major cellular antioxidant protecting cysteinyl and methionyl residues in proteins from oxidative modifications. Both  $\text{CoCl}_2$  and  $\text{H}_2\text{O}_2$  inhibited all 3 PHD isoforms in vitro, with PHD2 being slightly more resistant to  $\text{CoCl}_2$  (supplemental Figure 3). To directly determine protein oxidation by these compounds, carbonyl group formation in PHD2 was estimated by OxyBlot technology. As shown in Figure 3E (left panel), 2mM ascorbate and 10 $\mu\text{M}$   $\text{FeSO}_4$  (as used in the standard reaction buffer for in vitro hydroxylation) substantially increased carbonylation of recombinant PHD2 during 1 hour of hydroxylation reaction. Surprisingly,  $\text{CoCl}_2$  increased carbonylation of PHD2 whether ascorbate/ $\text{FeSO}_4$  was present or not. After addition of 5mM GSH, oxidation of PHD2 by ascorbate/ $\text{FeSO}_4$ ,  $\text{CoCl}_2$ , and  $\text{H}_2\text{O}_2$  was markedly reduced. To examine whether PHD2 protein oxidation is coupled to its dioxygenase activity, the reaction was performed in the presence of a mutant Pro564Ala HIF-1 $\alpha$  peptide substrate. As shown in Figure 3E (right panel), PHD2 protein oxidation was independent of the presence of a hydroxylation acceptor proline, providing evidence that protein oxidation is not caused by the hydroxylation reaction cycle.

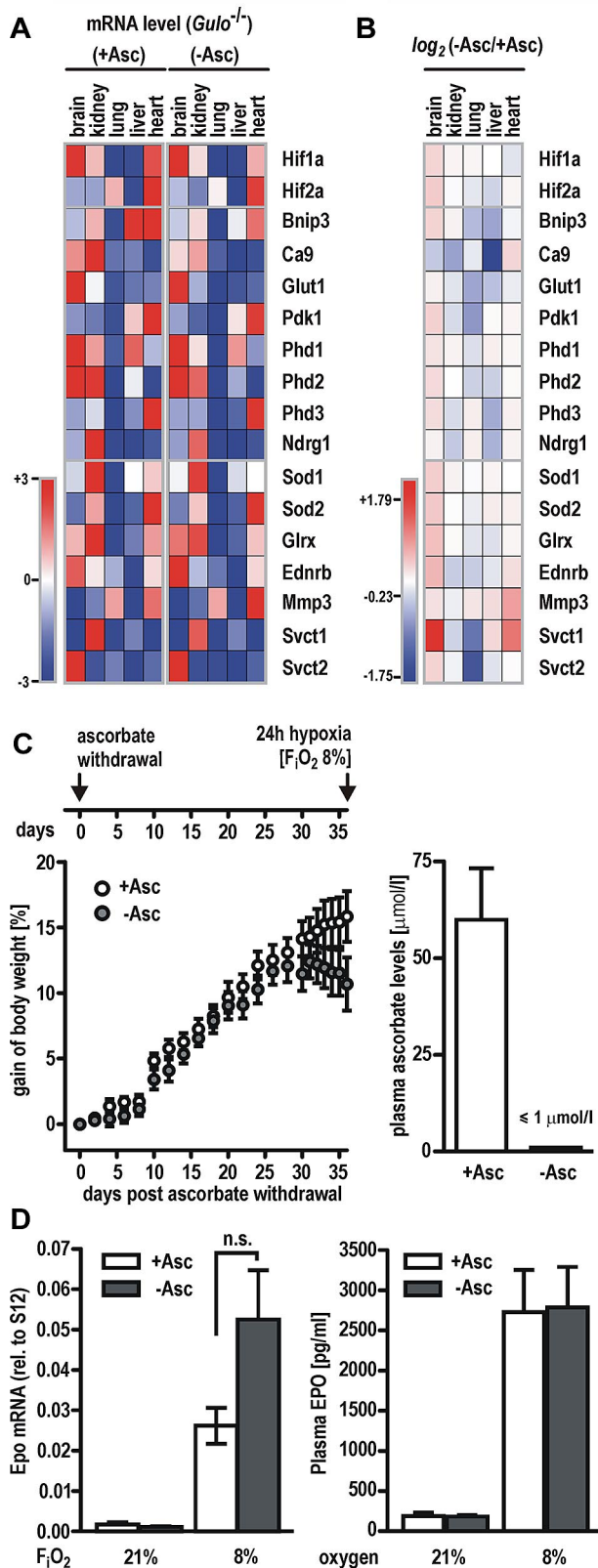
### Cysteine 201 affects PHD2 hydroxylation activity

Recently, cysteine residue 201 within the catalytic domain of PHD2 has been identified as a surface-accessible, highly nucleophilic residue predominantly interacting with thiol compounds.<sup>32</sup> Moreover, Cys201 and Cys208 were proposed to provide an additional metal binding site in PHD2.<sup>33</sup> Both, Cys201 and Cys208 are highly conserved among all 3 human and mouse PHD isoforms (Figure 4A). To investigate the functional relevance of Cys201, recombinant PHD2, wild type, or Cys201Ser mutant was purified from Sf9 cells. Surprisingly, the Cys201Ser mutation significantly ( $P < .0001$ ) increased the PHD2 reaction



**Figure 4. A Cys201Ser mutation enhances PHD2-dependent hydroxylation reaction rate and protects from protein oxidation.** (A) Conservation of cysteine residues (Cys201 and Cys208) in human (Hs) and mouse (Mm) PHD isoforms. (B) Increased reaction rate of Cys201Ser mutant PHD2 as measured by the hydroxylation-dependent VBC binding assay. Shown are mean values  $\pm$  SEM of 3 independent experiments. Linear regression analyses were performed, revealing highly different slopes ( $P < .0001$ ). (C) The Cys201Ser mutation confers resistance of PHD2 to  $\text{H}_2\text{O}_2$ -mediated inhibition of hydroxylation activity. Shown are mean values  $\pm$  SEM of a representative experiment performed in triplicates. (D) GSH can rescue PHD2 wild type and Cys201Ser hydroxylation activities after  $\text{H}_2\text{O}_2$ -mediated enzyme damage. In brief, enzyme preparations were preincubated with 1mM  $\text{H}_2\text{O}_2$  for 30 minutes ( $\text{H}_2\text{O}_2$ ) or left untreated for a similar period (control). For rescue experiments, enzymes after  $\text{H}_2\text{O}_2$  treatment were incubated with 5mM GSH ( $\text{H}_2\text{O}_2$  + GSH) for 15 minutes. Hydroxylation reactions were carried out at standard assay conditions for 60 minutes. Note that all reactions contained 2mM ascorbate freshly added when hydroxylation reactions were started. Data are given as mean values  $\pm$  SEM of 3 independent experiments normalized to hydroxylation activities of control reactions obtained after 60 minutes.

rate by 2.5-fold (Figure 4B top panel). Equal concentrations of wild-type and mutant PHD2 proteins were confirmed by Coomassie staining of the undiluted stock solutions and immunoblotting of the diluted assay solutions (Figure 4B bottom panel). To further test the hypothesis that the Cys201Ser mutation might protect the PHD2 enzyme from oxidative damage, the effect of  $\text{H}_2\text{O}_2$  on hydroxylation activity was measured. As shown in Figure 4C, the half-maximal inhibitory concentration for  $\text{H}_2\text{O}_2$  was roughly 5-fold higher for the Cys201Ser mutant compared with wild-type PHD2 (half-maximal inhibitory concentration of  $8.9 \times 10^{-6}$  and  $4.3 \times 10^{-5}$  M  $\text{H}_2\text{O}_2$  for wild-type and Cys201Ser mutant PHD2, respectively). We further tested whether the  $\text{H}_2\text{O}_2$ -mediated loss of PHD2 activity could be rescued by sequential addition of GSH. For both enzyme preparations,



**Figure 5. Hypoxic response is fully functional in vitamin C-depleted *Gulo*<sup>-/-</sup> mice.** *Gulo*<sup>-/-</sup> male mice received a diet with (+Asc) or without (-Asc) ascorbate for 5 weeks. (A) Transcript levels of HIF target genes (*Bnip3*, *Ca9*, *Glut1*, *Pdk1*, *Phd2*, *Phd3*, and *Ndr1*) as well as genes involved in antioxidative defense (*Sod1*, *Sod2*, and *Glrx*), the ascorbate transporters *Svct1* and *Svct2*, or oxygen-independent genes (*Ednrb*, *Mmp3*, and *Phd1*) were quantified by RT-qPCR in brain, kidney, lung, liver, and heart. Values are expressed relative to S12 mRNA levels and visualized in a heatmap (Genepattern; Broad Institute). Lowest and highest mRNA levels of

preincubation with 1mM H<sub>2</sub>O<sub>2</sub> for 30 minutes inhibited subsequent substrate hydroxylation reactions, despite 2mM ascorbate being freshly added to start the hydroxylation reaction (Figure 4D). Interestingly, further addition of 5mM GSH could similarly reactivate both enzyme preparations, although reaction kinetics was substantially slower for the reactivated enzymes (Figure 4D).

#### Oxygen sensing is fully functional in ascorbate-deficient *Gulo*<sup>-/-</sup> mice

*Gulo*<sup>-/-</sup> mice received a diet with or without vitamin C for 5 weeks. Transcript levels of known HIF target genes (*Bnip3*, *Ca9*, *Glut1*, *Pdk1*, *Phd2*, *Phd3*, and *Ndr1*) as well as genes involved in antioxidative defense (*Sod1*, *Sod2*, and *Glrx*), the ascorbate transporters *Svct1* and *Svct2*, or oxygen-independent genes (*Ednrb*, *Mmp3*, and *Phd1*) were determined by RT-qPCR in brain, lung, kidney, heart, and liver. Similar tissue-specific expression levels for most genes involved in different pathways were observed in both groups (Figure 5A). Conclusive with our finding from cell culture experiments, expression levels of most of the HIF target genes remained largely unaffected by vitamin C deficiency or even showed reduced levels (blank and blue squares, respectively, in the heatmap shown in Figure 5B). Expression of the ascorbate transporter *Svct1*, however, was moderately induced in animals fed without ascorbate, possibly reflecting compensatory mechanisms for ascorbate deprivation.

To further test whether the absence of ascorbate limits PHD function under hypoxic conditions, *Gulo*<sup>-/-</sup> males were deprived of vitamin C for 5 weeks, whereas control animals were supplemented with vitamin C. As described previously,<sup>18</sup> the animals developed a scorbutic phenotype marked by a substantial loss of body weight after 35 days on an ascorbate-free diet, indicating that systemic stores of vitamin C have been exhausted (Figure 5C left panel). In line with this observation, plasma ascorbate levels in *Gulo*<sup>-/-</sup> mice fed an ascorbate-free diet were below the detection limit ( $\leq 1\mu\text{M}$ ), whereas plasma of control animals contained 40.3 to 123.8 $\mu\text{M}$  vitamin C (Figure 5C right panel), corresponding to ascorbate plasma levels in healthy humans.<sup>34</sup>

After 5 weeks of vitamin C deprivation, mice were breathing 8% oxygen for an additional period of 24 hours, whereas control groups were kept under ambient oxygen concentration (see scheme in Figure 5C). Both ascorbate-deficient and supplemented animals responded to the hypoxic treatment with a robust induction of Epo mRNA in the kidney that was higher in *Gulo*<sup>-/-</sup> males fed without ascorbate, although differences did not reach the level of significance ( $P = .07$ , Student *t* test; Figure 5D left panel). Circulating EPO protein levels in mouse plasma were induced by hypoxic exposure to a similar extent in both groups (Figure 5C right panel).

**Figure 5 (continued).** each gene were arbitrarily defined as -3 (dark blue) and +3 (dark red), respectively. (B) Heatmap of gene expression changes following a vitamin C-deficient diet.  $\log_2(-Asc/+Asc)$  ratios revealed that the majority of HIF target genes remained either unchanged or showed a slightly reduced expression pattern. Data ranged from -1.75 (dark blue) and +1.79 (dark red), respectively. (C) Scheme depicting the experimental setup for hypoxic experiments with vitamin C-depleted *Gulo*<sup>-/-</sup> animals (top panel). Relative gain of body weight of *Gulo*<sup>-/-</sup> mice ( $n = 11$  animals per group) after ascorbate withdrawal (-Asc) or ascorbate supplementation (+Asc) for 36 days (left panel). Ascorbate levels in the plasma of *Gulo*<sup>-/-</sup> mice ( $n = 6$  animals per group) after 5 weeks of ascorbate withdrawal compared with mice kept on an ascorbate-supplemented diet (right panel). (D) Epo mRNA (left panel) and circulating EPO protein (right panel) levels in *Gulo*<sup>-/-</sup> mice maintained on a diet with (+Asc) or without (-Asc) for 5 weeks followed by exposure to 8% or 21% oxygen for 24 hours. Data represent mean values  $\pm$  SEM derived from at least 5 animals per group; n.s., indicates not significant.



Of note, no significant changes of the red cell lineage hematologic parameters were observed in *Gulo*<sup>-/-</sup> mice after 5 weeks of ascorbate depletion, indicating that the oxygen transport capacity was similar in both treatment groups. Hypoxic increases in hematocrit values are known to be delayed and reach the level of significance not before 72 hours of continuous exposure to hypoxia,<sup>35</sup> explaining the lack of an increase of either hematocrit values or red blood cell counts in our experimental setting with a hypoxic period of only 24 hours (supplemental Table 2).

## Discussion

Ascorbic acid and ferrous iron have been reported as essential cofactors for PHD-dependent HIF $\alpha$  hydroxylation *in vitro*.<sup>13</sup> Unexpectedly, we found a fully functional cellular oxygen-sensing pathway in HeLa cells maintained under strictly ascorbate-free culture conditions, indicating that ascorbate is dispensable for HIF $\alpha$  hydroxylation *in vivo*. In a search for the nature of antioxidative compounds substituting for ascorbate during prolyl-4-hydroxylation, we identified GSH as a potent activator of all 3 PHDs *in vitro*, increasing HIF $\alpha$  peptide hydroxylation in a dose-dependent manner. Notably, ascorbate and GSH are the most abundant reducing compounds within eukaryotic cells.<sup>36</sup> Given the distinct antioxidative properties of vitamin C and GSH, the 2 compounds might affect prolyl-4-hydroxylation by different ways. Ascorbate might be required to reduce occasionally oxidized ferric Fe(III) generated in the active center of PHDs by uncoupled reaction cycles as it has been described for C-P4H.<sup>27</sup> However, to the best of our knowledge, no experimental evidence has been reported for enzymatic activity of PHDs in the absence of a hydroxyl-acceptor substrate. Strikingly, the major iron form bound to purified PHD2 is ferrous Fe(II) even when purified under oxygenated conditions,<sup>37</sup> arguing against an essential role of ascorbate in reducing PHD iron. In support of this notion, iron and 2-oxoglutarate have been reported to copurify with 50% and 5%-10% of PHD2, respectively, whereas ascorbate did not copurify at all.<sup>37</sup> Recent work by Flashman et al<sup>38</sup> showed that ascorbate does not directly interact with the catalytic domain of PHD2; however, its intrinsic ene-diol-reducing moiety was found to be important to promote hydroxylation by PHD2.

GSH fully stimulated *in vitro* PHD hydroxylation activity only at rather high concentrations, which is in line with findings reported previously for N-terminally truncated PHD2.<sup>38</sup> The millimolar GSH concentrations used in our study reflect physiologically relevant levels of this compound in living cells.<sup>39</sup> Moreover, we found that ascorbic acid is not consumed by coupled substrate hydroxylation, suggesting that exogenously added GSH does not simply regenerate potentially copurified oxidized dehydroascorbate. Our data rather favor an alternative function of GSH by preventing oxidative damage to the enzyme itself. Physiologic concentrations of GSH were able to reduce transition metal- or peroxide-induced PHD enzyme carbonylation. Despite being generally referred to as antioxidant, ascorbate, together with oxygen and transition metals such as Fe(III) or Cu(II), also exerts pro-oxidative effects by generating hydroxyl radicals in a Fenton-like reaction.<sup>40</sup> Indeed, we found increased PHD2 carbonylation by ascorbate-iron *in vitro*, suggesting that GSH might protect PHDs from the adverse effects of ascorbate. In line with 2 distinct reaction modes, addition of GSH to hydroxylation reactions containing saturating ascorbate concentrations markedly increased the hydroxylation rate of PHDs *in vitro*. Of note, Co(II) induced

ascorbate depletion, as suggested for cultured cells,<sup>28,29</sup> cannot account for PHD inhibition in our cell-free assays, because we showed previously that Co(II) only inefficiently catalyzes ascorbate oxidation by air under these assay conditions.<sup>16</sup> Direct interference of Co(II) with the enzymes is supported by the observation that Co(II) strongly carbonylated purified PHD2 even in the absence of ascorbate. Moreover, ascorbate and GSH exclusively blunted Co(II)-induced HIF activation in our cellular models, demonstrating a complementary function of GSH and ascorbate in oxygen sensing by living cells. Although simple chelation of Co(II) by ascorbate and GSH cannot be fully excluded in cell culture experiments, it should be mentioned that metal chelators naturally occurring in serum (eg, histidine, glutamic acid, and albumin but also GSH) are essential to facilitate Co(II)-induced ascorbate oxidation, because “free” Co(II) is unable to directly oxidize ascorbate in simple aqueous solutions at neutral pH (for a review, see Salnikow and Kasprzak<sup>28</sup>). Thus, the actual redox potential of the ion in such ternary complexes—rather than the intracellular concentration of “free” metal—determines its efficacy to act as a “hypoxia mimetic.”

One of the specific GSH functions is to prevent or reduce inappropriate disulfide bond formation. Two recent reports identified PHD2 cysteinyl residues Cys201 and Cys208 to be highly nucleophilic and surface accessible.<sup>32,33</sup> Moreover, crystallographic analyses predicted that these 2 cysteines might form disulfide bonds.<sup>32,33</sup> Cys201Ser mutation protects recombinant PHD2 from oxidative damage *in vitro* and results in a 2.5-fold higher specific hydroxylation activity. One might speculate that a certain fraction of wild-type PHD2 enzyme constantly undergoes oxidative modification of Cys201, leading to reduced activity. As such, PHD enzymes could combine oxygen- and redox-sensing properties, providing a possible explanation for previous work on redox factors modulating PHD activity.<sup>41-43</sup> However, potentiation of PHD activity by GSH clearly involves mechanisms distinct from Cys201 oxidation, because both wild-type and Cys201Ser mutated PHD2 enzymes could be efficiently reactivated from H<sub>2</sub>O<sub>2</sub>-induced damage by GSH.

Translating our biochemical and cellular findings into a systemic context, we did not observe marked alterations in HIF target gene expression after dietary vitamin C deprivation in *Gulo*<sup>-/-</sup> mice, providing evidence that other antioxidants might substitute for vitamin C *in vivo*. Accordingly, the hypoxic response of *Gulo*<sup>-/-</sup> mice with low or undetectable ascorbate in the plasma was similar to that of mice receiving an ascorbate-supplemented diet. Consistent with antioxidant redundancy *in vivo*, a study using the same animals backcrossed to a BALB/c genetic background found vitamin C-independent *de novo* synthesis of collagen in allografted tumors and unchanged levels of hydroxyproline-collagen. Dermal hydroxyproline content of collagen was even increased.<sup>44</sup> Of note, treatment of vitamin C-deprived guinea pigs with a cell-permeable GSH monoethyl ester significantly attenuated the severity of scurvy-related symptoms, also suggesting a cooperative function of both antioxidants for P4H function *in vivo*.<sup>45</sup> Moreover, *Gulo*<sup>-/-</sup> mice have increased levels of total glutathione in brain and liver, possibly explained by a compensatory mechanism for antioxidative defense in these animals.<sup>46</sup>

Interestingly, tumor growth and angiogenesis were retarded in a syngenic tumor model using vitamin C-deprived *Gulo*<sup>-/-</sup> mice, but no changes were observed for HIF-1 $\alpha$  protein levels in the respective tumor tissue.<sup>47</sup> The possible value of vitamin C in cancer therapy recently experienced a renaissance, because it has been shown that pharmacologic doses of vitamin C decrease growth of

tumor xenografts in mice by increasing peroxide levels in neoplastic tissue.<sup>30,48</sup> Such tumoricidal effects of antioxidants might, at least partially, involve destabilization of HIF $\alpha$  after increased hydroxylase activity.<sup>49,50</sup> Our data support a model of cooperative function of GSH and vitamin C in regulating the efficiency of PHD oxygen sensors. Translated to chemotherapy of cancers, combined treatment with both clinically approved molecules might even boost their antitumorigenic function.

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## Authorship

Contribution: K.J.N. designed and performed experiments, analyzed data, and wrote the manuscript; K.J.N., N.M., P. Schläfli, and P. Spielmann performed in vivo experiments; R.H.W. designed experiments and wrote the manuscript; and D.P.S. designed experiments, analyzed data, and wrote the manuscript.

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Correspondence: Daniel P. Stiehl, Institute of Physiology, University of Zürich, Winterthurerstr 190, CH-8057 Zürich, Switzerland; e-mail: daniel.stiehl@access.uzh.ch.

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