Cell, Vol. 117, 941–952, June 25, 2004, Copyright ©2004 by Cell Press

Siah2 Regulates Stability of Prolyl-Hydroxylases, Controls HIF1α Abundance, and Modulates Physiological Responses to Hypoxia

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Summary

Hypoxia-inducible factor-1α (HIF1α) is a central regulator of the cellular response to hypoxia. Prolyl-hydroxylation of HIF1α by PHD enzymes is prerequisite for HIF1α degradation. Here, we demonstrate that the abundance of PHD1 and PHD3 are regulated via their targeting for proteasome-dependent degradation by the E3 ubiquitin ligases Siah1a/2, under hypoxia conditions. *Siah2* null fibroblasts exhibit prolonged PHD3 half-life, resulting in lower levels of HIF1α expression during hypoxia. Significantly, hypoxia-induced HIF1α expression was completely inhibited in *Siah1a/2* null cells, yet could be rescued upon inhibition of PHD3 by RNAi. Siah2 targeting of PHD3 for degradation increases upon exposure to even mild hypoxic conditions, which coincides with increased *Siah2* transcription. *Siah2* null mice subjected to hypoxia displayed an impaired hyperpneic respiratory response and reduced levels of hemoglobin. Thus, the control of PHD1/3 by Siah1a/2 constitutes another level of complexity in the regulation of HIF1α during hypoxia.

Introduction

Physiologic systems that regulate oxygen homeostasis are among the most extensively developed, reflecting the body's constant and absolute requirement for oxygen. Changes in oxygen tension are tightly monitored and addressed through a complex and rapidly implemented series of posttranslational modifications that affect the activity and expression of genes involved in control of cellular metabolism, growth, and death. HIF1, hypoxia-inducible factor, is a master regulator of oxygen homeostasis (reviewed in Safran and Kaelin, 2003). HIF1 target genes play key roles in development as well as physiological processes such as angiogenesis, vascular remodeling, erythropoiesis, glucose transport, glycolysis, iron transport, cell proliferation, and cell survival (reviewed in Semenza 2002, Pugh and Ratcliffe, 2003). HIF1 is a heterodimer composed of a hypoxia-inducible α subunit and a constitutively expressed β subunit (Wang et al., 1995).

Cellular O_2 concentration is a central determinant of HIF1α expression. In nonhypoxic conditions, HIF1α is constantly degraded through ubiquitination and proteasomal destruction, whereas these processes are inhibited under hypoxic conditions, resulting in rapid increase in HIF1α levels (Huang et al., 1998; Kallio et al., 1999; Salceda and Caro, 1997; Jiang et al., 1996). The constitutive degradation of HIF1α in normoxia requires O_2-dependent hydroxylation of proline residues 402 and 564 in HIF1α by prolyl 4-hydroxylases (PHD 1, 2, and 3 for *p*rolyl *h*ydroxylase *d*omain containing; Bruick and McKnight, 2001; Yu et al., 2001; Epstein et al., 2001). Hydroxylation of HIF1α is prerequisite for binding of the von Hippel-Lindau protein (pVHL), which forms an E3 ubiquitin ligase complex with elongin B, C, and Cullin 2, resulting in targeting of HIF1α for proteasomal degradation (Kamura et al., 1999; Bruick and McKnight, 2001; Ivan et al., 2001; Jaakkola et al., 2001; Maxwell et al., 1999). Loss of pVHL function in renal carcinoma cells or ES cells results in constitutive expression of HIF1α and induction of downstream target genes such as *VEGF* (Maxwell et al., 1997; Mazure et al., 1997; Richard et al., 1999). SM20, the rat homolog of PHD3, was originally identified in smooth muscle cells as a growth inhibitor-inducible gene (Wax et al., 1994). PHD3 exhibits high affinity and kinetics for hydroxylation of HIF1α (Bruick and McKnight, 2001). Further, inhibition of PHD enzymatic activities during hypoxia is thought to be important for stabilization of HIF1α (Ivan et al., 2001; Jaakkola et al., 2001; Epstein et al., 2001). Yet, mechanisms underlying the functional differences among the three PHDs are not well understood.

RING finger proteins consist of a characteristic cysteine-rich zinc binding domain defined by a pattern of conserved cysteine and histidine residues that can catalyze polyubiquitination, thereby functioning as E3 ubiquitin ligases (Borden 2000). Siah proteins are highly conserved mammalian homologs of *Drosophila* Seven in Absentia, which possesses potent RING finger E3 ubiquitin ligase activities implicated in the degradation of diverse proteins including DCC, β-catenin, N-CoR, c-myb, Numb, and TRAF2 (Hu et al., 1997; Tiedt et al., 2001; Matsuzawa and Reed, 2001; Matsuzawa et al., 1998; Zhang et al., 1998; Susini et al., 2001; Habelhah et al., 2002). In searching for novel targets of Siah2, we performed mass spectrometry analysis of Siah2-associated proteins and identified several enzymes that mediate oxidation and hydroxylation. Here, we identify PHD1 and 3 as new substrates of Siah2 and demonstrate that Siah2 limits PHD1 and PHD3 availability during hypoxia, thereby revealing a novel mechanism underlying the increase in HIF1α expression during hypoxic conditions.

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Results

Siah1a/2 Target PHD1/3 for a Proteasome-Mediated Degradation

To test the possibility that Siah2 may affect the stability of prolyl hydroxylases, we first examined the association of Siah2 with each of the 3 PHDs. PHD1, 2, and 3 were cotransfected with RING mutant Siah2 ($Siah2^{Rm}$), which lacks E3 ligase activity and is more highly expressed than wild-type Siah2 due to the absence of self-ubiquitination (Hu et al., 1997; Habelhah et al., 2002). Immunoprecipitation of each PHD revealed that PHD3 exhibited the highest degree of binding to Siah2 and that PHD1 also exhibited strong association. A substantially weaker association was found for PHD2 (Figure 1A). In light of these findings, further analysis was primarily carried out using PHD3.

Associations of wild-type Siah2 and PHD3 could be detected only in cells treated with a proteasome inhibitor (Figure 1B). The potent self-ubiquitination of Siah2 renders very low expression levels of endogenous Siah2 preventing detection of the association between endogenous proteins. Expression of Siah2 also resulted in decreased PHD3 steady-state level, which could be attenuated upon treatment with proteasome inhibitor (Figure 1B). These observations suggest that Siah2 and PHD3 associate in vivo and that this association subjects PHD3 to active degradation.

To further characterize the association between Siah2 and PHD3, we divided PHD3 into three fragments whose binding to $Siah2^{Rm}$ in vivo was assessed. This analysis revealed that Siah2 binds to the carboxy-terminal domain of PHD3 (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/117/7/941/DC1). In vitro binding assays using bacterially expressed and purified GST-Siah2 or GST-$Siah2^{Rm}$ and in vitro-translated ^{35}S-labeled PHD3 revealed that both wild-type and RING mutant Siah2 associate with PHD3 (Figure 1C). Incubation of immunopurified ^{35}S-labeled PHD3 with GST-Siah2 also confirmed their association (Supplemental Figure S2 available on *Cell* website).

Comparing Siah1a and Siah2's ability to affect the steady-state level of the PHDs revealed that both decreased abundance of PHD1 and PHD3, but not PHD2, and that Siah2 exhibited a stronger effect on both PHDs than Siah1a (Figure 1D). These findings show that both Siah1a and Siah2 (Siah1/2) reduce the steady-state levels of PHD1/3. Since Siah2's activity was more potent than Siah1a's, we focused on Siah2 in subsequent studies.

To identify the degradation pathway by which Siah2 decreases PHD3 stability, we tested the ability of different protease inhibitors to block degradation. Whereas the proteasome inhibitors MG101, MG132, lactacystin, and epoxomicin inhibited the degradation of PHD3 by Siah2, the lysosomal protease inhibitors E-64 or chloroquine failed to do so (Figure 1E).

We next assessed Siah2 effect on ubiquitination of PHD3. In vitro ubiquitination using bacterially produced and purified GST-Siah2 and ^{35}S-labeled PHD3 revealed Siah2-dependent ubiquitination of PHD3. This reaction required the presence of reticulocyte lysates, suggesting that further modification (i.e., posttranslational) or the presence of an adaptor protein is required for Siah2-mediated ubiquitination of PHD3 in vitro (Supplemental Figure S3 available on *Cell* website). In vivo analysis revealed a low basal ubiquitination of PHD3 (Figure 1F, lane 2), which was also seen in $Siah2^{-/-}$ cells and is likely to be noncanonical ubiquitination that does not affect PHD3 stability (data not shown). Exogenous expression of Siah2 resulted in a dose-dependent increase in PHD3 ubiquitination (Figure 1F, lanes 3 and 4). Higher amounts of Siah2 did not increase further the degree of PHD3 ubiquitination as it also reduced stability of PHD3 (Figure 1F, lanes 4 and 5). This ubiquitination can be inhibited by reducing PHD3 expression upon transfection of PHD3 RNAi, supporting the specificity of this ubiquitination. These results suggest that Siah2 targets the ubiquitination and degradation of PHD3 in a proteasome-dependent manner, as reported for other Siah2 substrates (Hu et al., 1997; Habelhah et al., 2002).

PHD3 Is More Stable in $Siah2^{-/-}$ Cells

We next elucidated changes in PHD3 stability in immortalized wild-type ($Siah2^{+/+}$) and *Siah2* null ($Siah2^{-/-}$) mouse embryonic fibroblasts (Habelhah et al., 2002). Initial analysis of steady-state levels of exogenously expressed PHD3 revealed almost undetectable expression in $Siah2^{+/+}$ cells, whereas it was clearly detected in $Siah2^{-/-}$ cells (Figure 2C, compare lanes 1 and 7). Pulse chase analysis of PHD3 revealed a half-life of about 1 hr in $Siah2^{+/+}$cells compared with over 2 hr in $Siah2^{-/-}$ cells (Figure 2A). Parallel analysis revealed that the half-life of PHD3 was shortened in $Siah2^{+/+}$ cells maintained under hypoxia compared to normoxia conditions (Figure 2B). A similar decrease in PHD3 half-life was also observed in $Siah2^{-/-}$ cells under hypoxia, compared with $Siah2^{+/+}$ cells, suggesting that other E3 ligases (e.g., Siah1) may also contribute to degradation of PHD3 (Figure 2B). To further confirm that PHD3 stabilization could be attributed to loss of Siah2 in $Siah2^{-/-}$ cells, we reintroduced Siah2 into these cultures. Reexpression of Siah2, but not $Siah2^{Rm}$, in $Siah2^{-/-}$ cells markedly decreased PHD3 stability and this effect was blocked by addition of proteasome inhibitor (Figure 2C, compare lanes 7, 8, 9, and 11).

To monitor the expression level of endogenous PHD3, we generated an affinity-purified rabbit polyclonal antibody against PHD3, which did not crossreact with ectopically expressed PHD1/2 (Supplemental Figure S4 available on *Cell* website). As expected, endogenous expression of PHD3 was higher in $Siah2^{-/-}$ than in $Siah2^{+/+}$ cells grown under normoxic conditions (Figure 2C, lower image). Higher level of PHD3 in $Siah2^{-/-}$ cells could be reduced upon exogenous expression of Siah2 (Figure 2C, lower image). Moreover, hypoxia treatment for 5 hr decreased steady-state levels of endogenous PHD3 (Figure 2D). This finding implicates Siah2's role in the degradation of endogenous PHD3 and also implies that Siah2 activity toward PHD3 may be enhanced during hypoxia (see below). The residual decrease in PHD3 levels following hypoxia in $Siah2^{-/-}$ cells is likely to be mediated by Siah1.

To test this possibility, given that Siah1 also affects PHD3 stability, we monitored changes in PHD3 half-life in MEFs obtained from *Siah1a/2* double-null mice. Indeed, half-life of PHD3 in *Siah1a/2* DKO MEFs was longer compared with wild-type MEF under normoxia conditions (>4 hr compared with 1 hr; Figure 2E). The

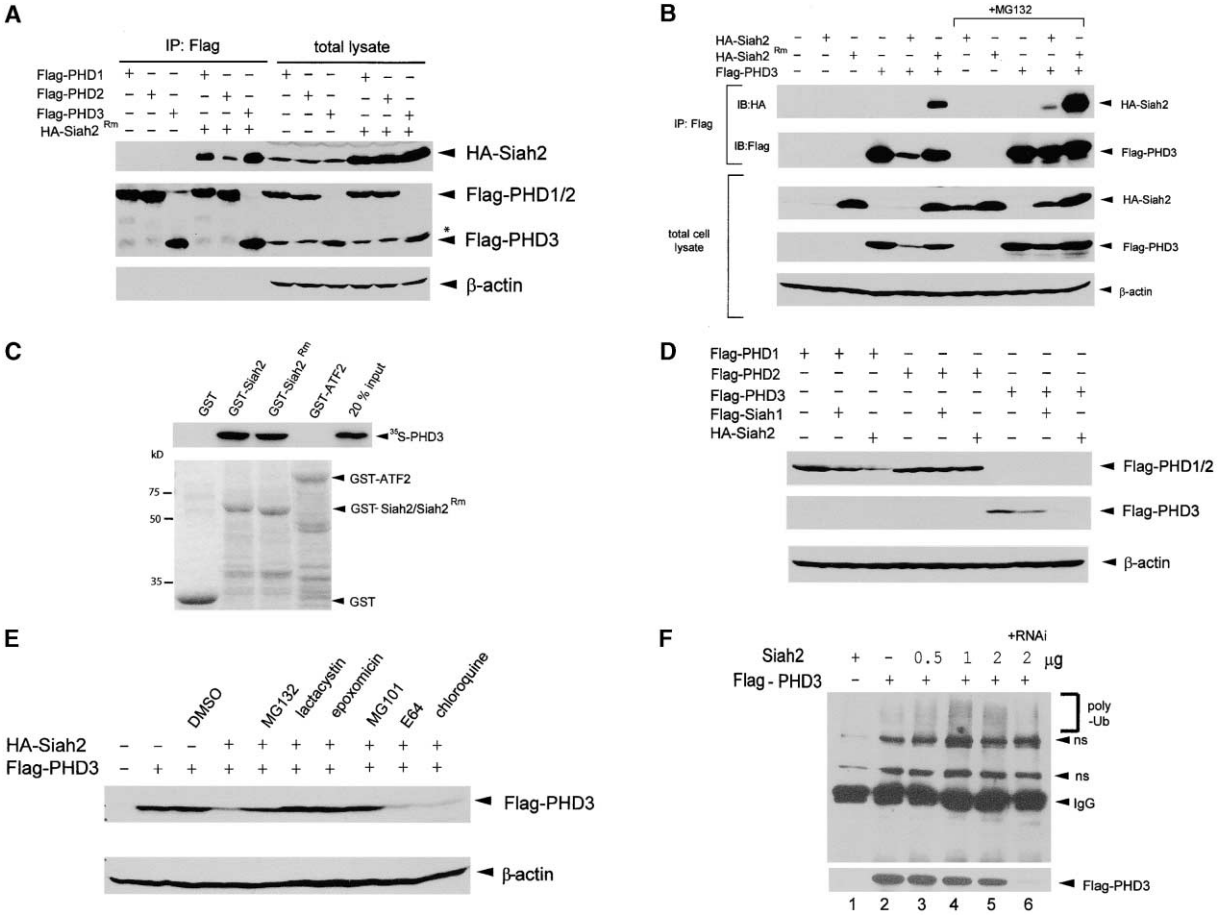

Figure 1. Interaction of PHDs with Siah2 and Their Degradation

(A) The HA-tagged RING mutant form of Siah2 (Siah2^{Rm}) and Flag-tagged PHD1, 2, and 3 were transfected into 293T cells to detect the association between Siah2 and PHDs. Cell lysates were immunoprecipitated with anti-Flag antibody and subjected to SDS-PAGE. Three PHDs coprecipitated HA-Siah2^{Rm} (top image). Expression of PHDs (middle image) and loading control β-actin (lower image) are shown. Asterisk indicates nonspecific band detected by Flag polyclonal antibody.

(B) HA-Siah2 or Siah2^{Rm} and Flag-PHD3 were transfected into 293T cells. Transfected cells, treated with or without MG132 (20 μM), were lysed and 1 mg of lysate was used for immunoprecipitation with anti-Flag antibody. Coprecipitation of Siah2^{Rm} as well as Siah2 in the MG132-treated condition was detected using anti-HA antibody (top image). Expressions of PHD3, Siah2, and β-actin are shown (three lower images).

(C) In vitro-translated ^{35}S-labeled PHD3 was incubated with bacterially purified GST-Siah2 or GST-Siah2^{Rm} fusion protein attached to glutathione beads in phosphate-buffered saline (PBS). GST and unrelated GST-ATF2 were used as negative controls. After incubation, the beads were extensively washed and subjected to SDS-PAGE. Association of ^{35}S-PHD3 and Siah2 in vitro was detected by autoradiography. Input of GST fusion proteins is shown as Coomassie blue-stained gel (lower image).

(D) Flag-Siah1 or HA-Siah2 with Flag-PHD1, 2, and 3 were transfected into 293T cells. The total cell lysate was subjected to Western blotting using anti-Flag tag antibody (top two images). β-actin was probed for the loading control (bottom image).

(E) Effect of various inhibitors was tested in 293T cells transfected with PHD3 and Siah2. Cells were treated with MG132 (20 μM), lactacystin (10 μg/ml), epoxomicin (2 μM), MG101 (10 μM), E64 (100 μg/ml), and chloroquine (1 μM) for 5 hr and harvested. Cell lysates were subjected to Western blotting and the membrane was probed with anti-Flag tag antibody (upper image). β-actin was probed for the loading control (lower image).

(F) Siah2-dependent ubiquitination of PHD3. Flag-PHD3 (4 μg), Siah2 expression vectors and pSup-PHD3 were cotransfected into 293T cells. After 48 hr, cells were harvested and lysed in 1% SDS/TBS followed by immediate boiling. Cell lysate was subjected to immunoprecipitation using anti-Flag antibody followed by separation on SDS-PAGE and immunoblot analysis with antiubiquitin- (upper image) or anti-Flag-antibody (lower image). Nonspecific (ns) and the IgG bands are indicated.

half-life of PHD3 under hypoxic conditions was also longer in *Siah1a/2* DKO cells, compared with wild-type cells (100 min compared with 50 min; Figure 2F), although some degradation of PHD3 was still noted and could be attributed to other E3 ligase. These findings reveal that *Siah1a/2* DKO cells further extended the already prolonged half-life of PHD3 seen in the *Siah2*$^{-/-}$ cells (compare images E and F with A and B in Figure 2). These results provide strong genetic evidence to support the role of Siah proteins in regulation of PHD3 stability and suggest that the decrease in PHD3 stability seen in the *Siah2*$^{-/-}$ cells can be attributed to Siah1a. Expression of Siah1a is not altered in *Siah2*$^{-/-}$ cells (Supplemental Figure S5 available on *Cell* website).

Siah2$^{-/-}$ Cells Exhibit Impaired Induction of HIF1α under Hypoxia

In light of the role identified for Siah2 in regulating PHD3 stability, we investigated the physiological implications of such regulation by monitoring levels of HIF1α in

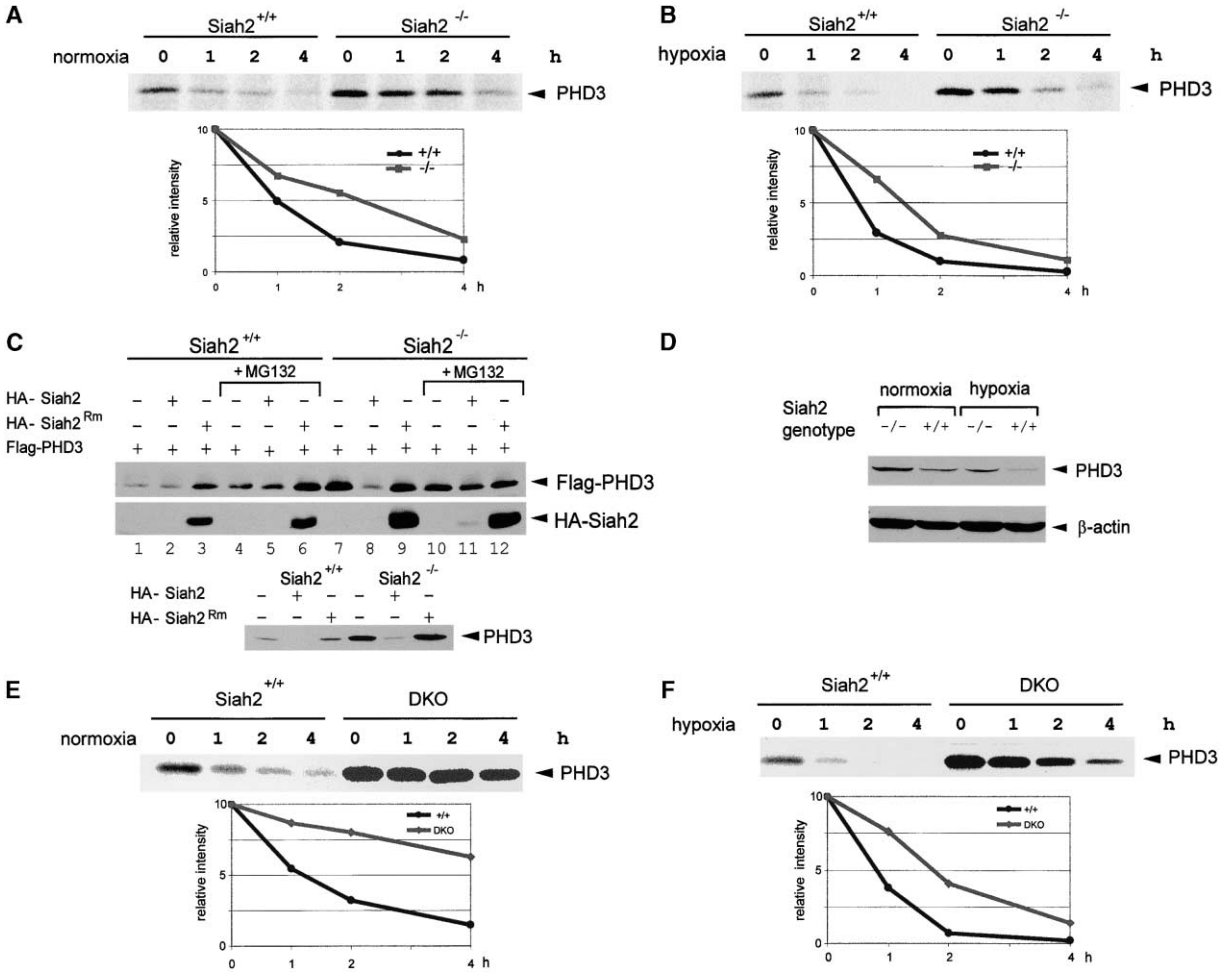

Figure 2. PHD3 Is Stabilized in *Siah2*$^{-/-}$ MEFs

(A and B) The half-life of PHD3 protein was monitored by a pulse-chase experiment. Five micrograms of Flag-PHD3 expression vector were transfected into *Siah2*$^{+/+}$ and *Siah2*$^{-/-}$ MEFs. 48 hr after transfection, cells were metabolically labeled with ^{35}S-Met for 2 hr followed by chase carried out during normoxia (A) or hypoxia (B) for the indicated time points. Flag-PHD3 was immunoprecipitated from the cell lysate with anti-Flag antibody and signals were detected by autoradiography.

(C) The effect of Siah2 on PHD3 was tested by introducing Siah2 into *Siah2*$^{-/-}$ cells. HA-Siah2 or Siah2^{Rm} and Flag-PHD3 were transfected into both *Siah2*$^{+/+}$ and *Siah2*$^{-/-}$ cells. Forty-eight hours after transfection, cells were mock treated or treated with MG132 (20μM) for 5 hr and harvested. Immunoprecipitates with anti-Flag antibody were subjected to SDS-PAGE followed by Western blotting using anti-Flag antibody. Expressions of HA-Siah2 (middle image) and endogenous PHD3 (bottom image) were also detected.

(D) Expression level of PHD3 in normoxia and hypoxia was detected in both *Siah2*$^{+/+}$ and *Siah2*$^{-/-}$ cells. Cells were treated by exposure to hypoxia (1% O_2) or normoxia for 5 hr. Total cell lysates were subjected to SDS-PAGE and detected with PHD3 antibody. β-actin was probed for the internal control.

(E and F) Half-life measurement of PHD3 in *Siah1a/2* double-null MEFs was carried out under normoxia (E) or hypoxia (F) conditions as detailed in (A and B) above.

Siah2$^{+/+}$ and *Siah2*$^{-/-}$ cells grown under normoxic and hypoxic conditions. Under normoxia, HIF1α expression was comparable between *Siah2*$^{+/+}$ cells and *Siah2*$^{-/-}$ cells. In contrast, after hypoxia treatment, the amount of HIF1α in *Siah2*$^{+/+}$ cells markedly increased compared with *Siah2*$^{-/-}$ cells (Figure 3A). These data are consistent with the notion that *Siah2*$^{-/-}$ cells are impaired in their ability to efficiently degrade PHD3, resulting in a concomitant decrease in the level of HIF1α expression under hypoxia.

We next examined changes in expression levels of HIF1α in *Siah1a/2* DKO cells. The lower levels of HIF1α expression seen in *Siah2*$^{-/-}$ cells was even more pronounced in DKO cells, where HIF1α expression was barely detectable following hypoxia treatment (Figure 3B). These data point to the role of Siah1a in complementing Siah2-targeted PHD3 degradation, which concomitantly affects HIF1α expression.

Lower HIF1α expression under hypoxia in *Siah2*$^{-/-}$ and *Siah1a/2* DKO cells is expected to cause a corresponding change in the expression of HIF1α target genes. Thus, we investigated the expression of *VEGF* mRNA, a representative HIF1α-inducible gene, by semiquantitative RT-PCR. Consistent with the HIF1α expression levels, the relative increase of *VEGF* mRNA in hypoxia-treated *Siah2*$^{+/+}$ cells was greater than that in

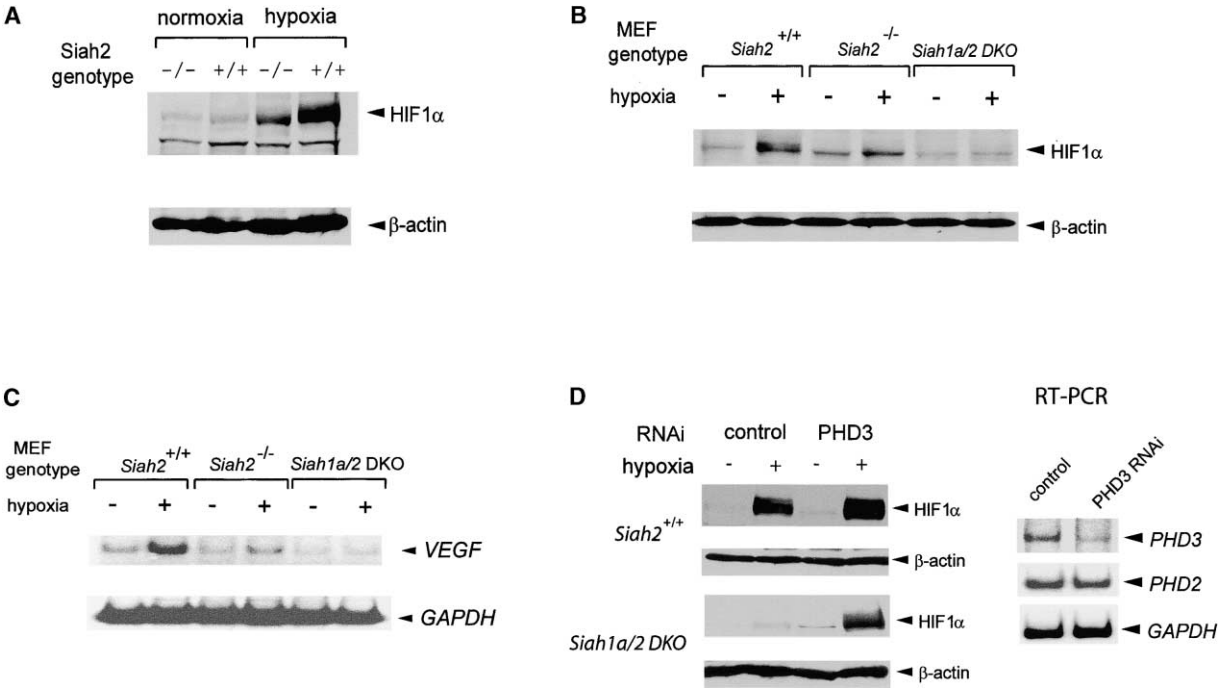

Figure 3. Siah2$^{-/-}$ Exhibit Impaired Induction of HIF1α in Response to Hypoxia

(A) Expression level of HIF1α in normoxia and hypoxia was detected in both *Siah2*$^{+/+}$ and *Siah2*$^{-/-}$ cells. Cells were treated by exposure to hypoxia (1% O_2) or normoxia for 5 hr. Total cell lysates were subjected to SDS-PAGE and detected with HIF1α antibody. β-actin was probed for the internal control.

(B) Expression level of HIF1α under hypoxia was compared among *Siah2*$^{+/+}$, *Siah2*$^{-/-}$, and *Siah1a/2* DKO MEFs. After hypoxia treatment (1% O_2), cells were harvested and subjected to SDS-PAGE. Blot was probed with HIF1α antibody (upper image) or β-actin antibody (lower image).

(C) Expression of *VEGF* mRNA in *Siah2*$^{+/+}$, *Siah2*$^{-/-}$, and *Siah1a/2* DKO MEFs was detected in normoxia and hypoxia. cDNA synthesized from the cells was subjected to a semiquantitative RT-PCR reaction using specific primer sets for *VEGF* and *GAPDH* genes. After PCR labeling with (α-^{32}P)dCTP, samples were separated on acrylamide gel and amplified bands were detected by autoradiography.

(D) Inhibition of PHD3 by RNAi increases HIF1α expression under hypoxia. pSuper control (empty vector) or pSuper carrying oligonucleotide designed for specific inhibition of PHD3 were transfected (6 μg each) into *Siah2*$^{+/+}$ and *Siah1a/2* DKO MEFs. 72 hr after transfection cells were subjected to normoxia or hypoxia for 5 hr. Proteins were analyzed for HIF1α expression by Western blotting. RNA extracted from the same cultures (*Siah2*$^{+/+}$ cells are shown) was analyzed for the expression of *PHD2*, *PHD3*, and *GAPDH* by RT-PCR followed by autoradiography.

Siah2$^{-/-}$ cells and barely detectable in *Siah1a/2* DKO cells (Figure 3C).

Inhibition of PHD3 Recovers HIF1α Expression in *Siah*-Deficient Mouse Fibroblasts

Given the effect of Siah1a and Siah2 on PHD3, but not on PHD2, we have assessed the changes in HIF1α expression in *Siah2*$^{+/+}$ and *Siah1a/2* DKO cells that were maintained in either normoxia or hypoxia, in which PHD3 expression was inhibited by a specific RNAi. Significantly, lack of HIF1α expression in *Siah1a/2* DKO cells subjected to hypoxia could be rescued upon inhibition of PHD3 expression via RNAi (Figure 3D). This finding provides direct support for the role of Siah1a/2 in the regulation of PHD3 expression under hypoxic conditions. Unlike the changes seen under hypoxia, there was no significant increase in the level of HIF1α under normoxia in cells in which PHD3 expression was inhibited (Figure 3D), as was observed upon inhibition of PHD2 expression (Berra et al., 2003). These findings suggest that whereas PHD2 primarily limits HIF1α under normoxia, PHD3, which is regulated by Siah2, determines HIF1α availability under hypoxic conditions.

Alteration of HIF1α Stability in *Siah2*$^{-/-}$ Cells Is Dependent on PHD3 Abundance

Since we have shown that PHD3 protein levels are higher in *Siah2*$^{-/-}$ cells (Figure 2C) and given the changes in HIF1α levels upon inhibition of PHD3 expression in cells that lack Siah, we tested whether altering the levels of PHD3 during hypoxia is sufficient to modulate HIF1α levels. Transfection of PHD3 expression vector into HeLa cells decreased, in a dose-dependent manner, basal as well as hypoxia-induced expression of HIF1α (Figure 4A). Similarly, exogenous expression of PHD3 decreased the HIF1α level in *Siah2*$^{+/+}$ cells in both normoxia and hypoxia to levels seen in *Siah2*$^{-/-}$ cells (Figure 4B). Thus, expression of PHD3 mimics the changes of HIF1α expression seen in *Siah2*$^{-/-}$ cells.

If the lower abundance of HIF1α in *Siah2*$^{-/-}$ cells under hypoxia results entirely from improper degradation of PHD3, then inhibition of PHD3-dependent (VHL-mediated) degradation should result in comparable levels of expression of HIF1α in mutant and wild-type cells. Treatment with proteasome inhibitor increased HIF1α abundance to similar levels in both genotypes of MEF (Figure 4C). Treatment with DFO, an iron chelator known to inhibit the activity of PHDs, increased HIF1α abun-

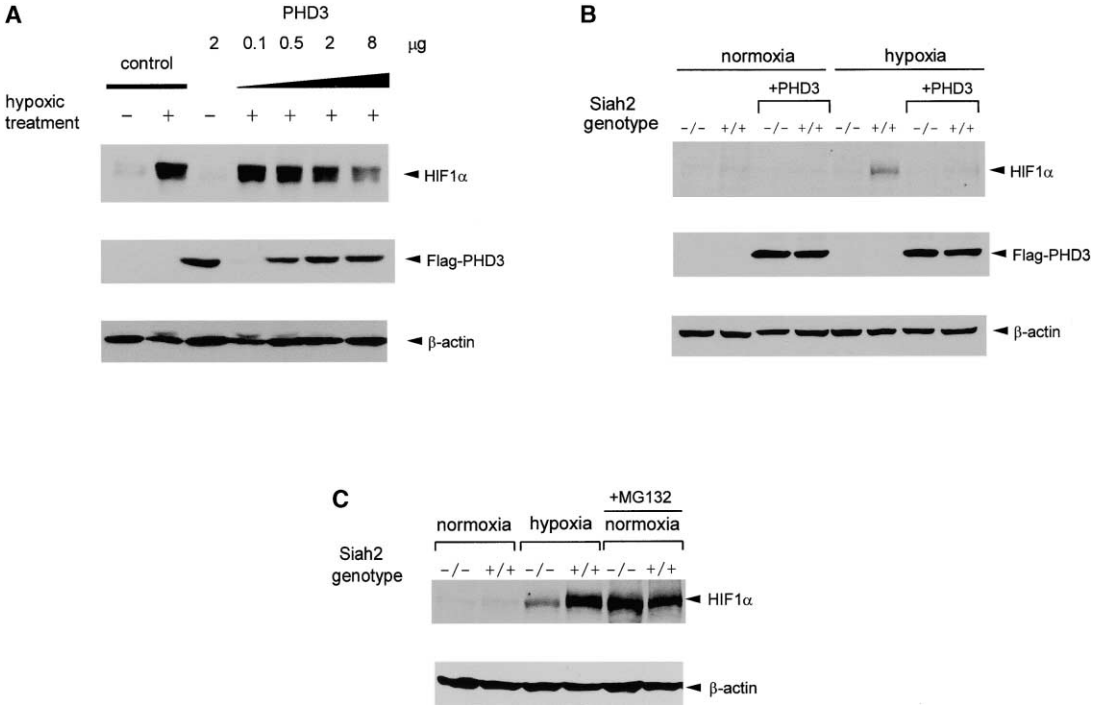

Figure 4. Expression of HIF1α Depends on PHD3 Abundance

(A) Different amounts of PHD3 expression plasmid were transfected into HeLa cells as indicated in the image. Thirty-six hours after transfection, cells were maintained under hypoxia (1% O_2) or normoxia, and harvested 5 hr after treatment. The total cell lysate was probed for HIF1α (upper image), Flag-PHD3 (middle image), and β-actin (lower image). PHD3 expression inhibited the induction of HIF1α in hypoxia in a dose-dependent manner.

(B) The effect of exogenously expressed PHD3 in *Siah2*$^{+/+}$ and *Siah2*$^{-/-}$ cells was tested. Six micrograms of PHD3 expression plasmid were transfected into cells of both types, which were either maintained under normoxia or hypoxia (1% O_2) for 5 hr. The total cell lysate was probed for HIF1α (upper image), Flag-PHD3 (middle image), and β-actin (lower image).

(C) The effect of MG132 treatment on HIF1α expression was examined in *Siah2*$^{+/+}$ and *Siah2*$^{-/-}$ cells. Cells were mock treated or treated with MG132 (20μM) for 5 hr and harvested. The total cell lysate was subjected to Western blotting with anti-HIF1α antibody.

dance to similar levels in both *Siah2*$^{+/+}$ cells and *Siah2*$^{-/-}$ cells under normoxia (Supplemental Figure S6 available on *Cell* website). Collectively, these results suggest that the relative abundance of PHD3 is one of the key determinants that regulate HIF1α expression under hypoxic conditions

Degradation of PHD3 Is Enhanced under Hypoxia

As the differences in HIF1α abundance between *Siah2*$^{+/+}$ cells and *Siah2*$^{-/-}$ cells are more striking under hypoxia than normoxia (Figure 3A), we hypothesized that Siah2 targeting of PHD3 may be increased by hypoxia. Consistent with this idea, we showed that hypoxia enhanced the ability of Siah2 to induce degradation of exogenously and endogenously expressed PHD3 (Figure 5A). Increase in Siah2 activity under hypoxia conditions was seen for both PHD3 and PHD1 (Figure 5B). Moreover, this effect was observed even under mild hypoxic conditions (5% and 10% oxygen concentration; Figure 5C compare lanes 3, 6, 9, and 12 and lanes 1, 4, 7, and 10), demonstrating that the regulation of PHD3 stability is sensitive to oxygen tension. Monitoring GFP driven from the same promoter as PHD3 revealed that neither Siah nor hypoxia affect transcription or translation of GFP (Supplemental Figure S7 available on *Cell* website). However, although hypoxia alone decreased PHD3 abundance and enhanced the ability of exogenous Siah2 to degrade PHD3, the degree of association between exogenously expressed PHD3 and Siah2^{Rm} was not altered by hypoxia under conditions where PHD3 was not the limiting factor (Figure 5A compare lanes 6 and 12). Thus, enhancement of interaction between PHD3 and Siah2 does not appear to account for increased degradation of PHD3 during hypoxia.

Siah2 Transcription Is Induced under Hypoxia

Since our biochemical and genetic experiments suggested that Siah2 expression levels are an important determinant of PHD3 abundance, we reasoned that hypoxia might increase Siah2 expression and hence potentiate PHD3 degradation. To test this possibility, semi-quantitative RT-PCR was used to monitor levels of *Siah2* mRNA following exposure to hypoxia. This analysis revealed a marked increase in the amount of *Siah2* transcripts as early as 2 hr after exposure to hypoxia. Further increase in the level of *Siah2* mRNA was observed within 5 hr after exposure to hypoxia, followed by a decrease after 14 hr to levels somewhat higher than that seen in normoxia (Figure 6A). Moreover, consistent with our findings of decreased PHD3 stability under even mild hypoxia (Figure 5C), we found that *Siah2* expression is induced to a similar extent by 1%, 5% or 10% oxygen

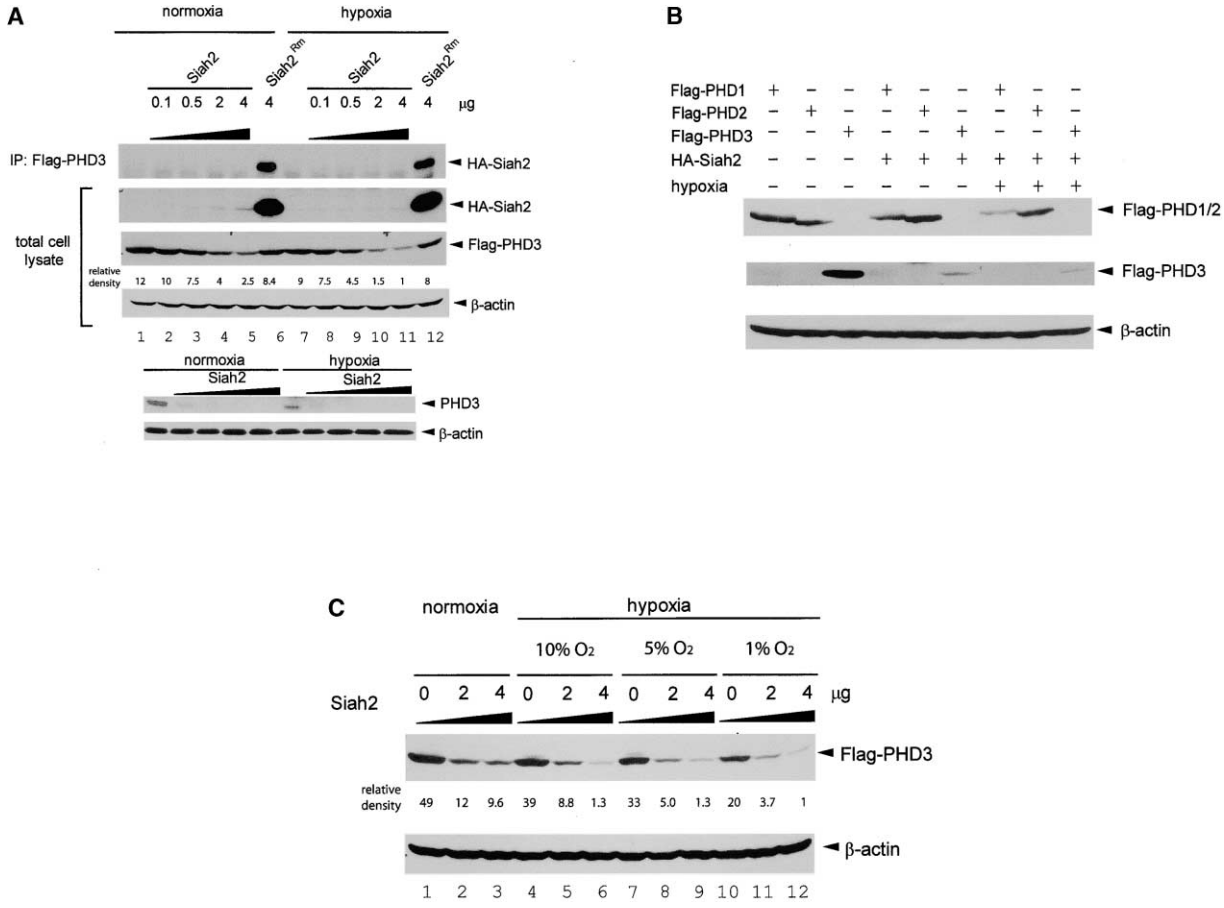

Figure 5. Siah2 Decreases Abundance of PHD1/3 also Under Mild Hypoxic Conditions

(A) Flag-PHD3 and HA-Siah2 or Siah2^{Rm} were transfected into 293T cells. Forty-eight hours later, cells were maintained under normoxia or hypoxia (1% O_2) for 5 hr and harvested. Cell lysates were immunoprecipitated with anti-Flag antibody. Total cell lysate or immunoprecipitates were subjected to Western blotting and probed for HA-Siah2 (two upper images), Flag-PHD3 (middle image), and β-actin (lower image). Expression of endogenous PHD3 was also detected with the increase amount of Siah2 transfection (bottom two images).

(B) Degradation of PHD1 and PHD3 by Siah2 increases under hypoxia. Expression vectors of Flag-PHD1, 2, 3, and HA-Siah2 were transfected into 293T cells. Forty-eight hours after the transfection, cell were maintained under normoxia or hypoxia (1% O_2) for 5 hr and harvested. Cell lysate was subjected to SDS-PAGE followed by probing with anti-Flag antibody (upper two images) or anti-β-actin antibody (lower image).

(C) Activity of Siah2 to target PHD3 was tested in 10%, 5%, and 1% O_2 concentrations. Cells transfected with different amount of Siah2 and PHD3 were treated under different hypoxic conditions for 5 hr. Cell lysates were subjected to Western blotting probed with anti-Flag tag antibody (upper image). The membrane was also probed with β-actin for internal control (lower image).

concentration (Figure 6B). Under the same conditions HIF1α expression is elevated as early as 2 hr following hypoxia and in mild hypoxic conditions (10% or 5%; Figures 6C and 6D). Since the increase in *Siah2* transcripts precedes the increase in HIF1α expression (at 10% hypoxia *Siah2* transcripts increase substantially, whereas HIF1α levels are minimally elevated), it is likely that factors other than HIF1α induce *Siah2* expression during hypoxia. Collectively, these findings lead us to propose a model in which transcriptional upregulation of Siah2 during hypoxia induces degradation of PHD3, providing a positive feed-forward mechanism to enhance HIF1α stability.

Defective Physiological Responses to Hypoxia in *Siah2* Null Mice

The role of Siah2 in the regulation of PHD3 stability and HIF1α abundance under hypoxic conditions suggested that *Siah2* null animals may exhibit altered physiological responses to hypoxia. Exposure of mice to chronic hypoxia induces an increase in red blood cell production (polycythemia), attributable to increased expression of the HIF1α target gene erythropoietin (Wenger, 2000). HIF1α heterozygous mice exhibit a delayed polycythemic response following chronic hypoxia (Yu et al., 1999), demonstrating the importance of HIF1α expression levels for this response. Consistent with our biochemical data showing that Siah2 is a positive regulator of HIF1α protein abundance, we found that *Siah2*$^{-/-}$ mice also display an impaired increase in red blood cell production, as measured by hemoglobin levels, following continuous treatment with hypoxia (10% oxygen) for 1–2 weeks (Figure 7A). This finding provides important physiological evidence demonstrating that Siah2 modulates HIF1α function in vivo.

Since mammals regulate ventilation and metabolism in

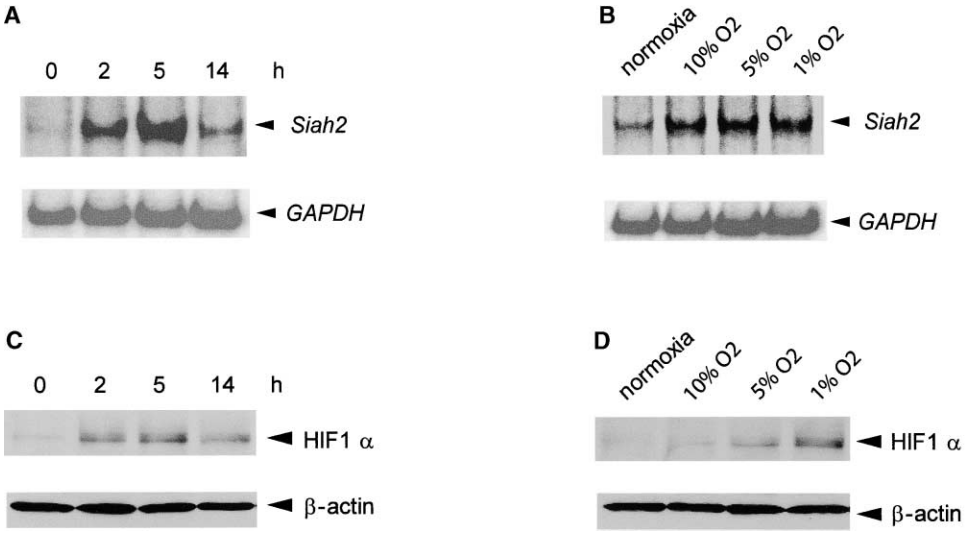

Figure 6. *Siah2* Transcripts Are Induced under Hypoxic Conditions

(A and C) Expression of *Siah2* mRNA and HIF1α in *Siah2*$^{+/+}$ cells was detected at different time points during hypoxia (1% O_2). cDNA synthesized from the cells was subjected to a semiquantitative RT-PCR reaction using specific primer sets for *Siah2* and *GAPDH* genes. PCR product labeled with (α-^{32}P)dCTP was separated on acrylamide gel and detected by autoradiography (A). Cell lysates prepared from those cells were subjected to SDS-PAGE and probed with anti-HIF1α antibody (C).

(B and D) Expression level of *Siah2* mRNA and HIF1α was detected in *Siah2*$^{+/+}$ cells treated with different hypoxic conditions for 5 hr. cDNA synthesized from the cells was subjected to a semiquantitative RT-PCR reaction using specific primer sets for *Siah2* and *GAPDH* genes. Amplified bands labeled with (α-^{32}P)dCTP were separated on acrylamide gel and detected by autoradiography (B). Cell lysates prepared from those cells were subjected to SDS-PAGE and probed with anti-HIF1α antibody (D).

response to available oxygen, we investigated whether Siah2 also regulates physiological oxygen-sensitive systems. Analysis of *Siah2*$^{+/+}$ and *Siah2*$^{-/-}$ mice under normoxia (Figure 7B; position 1) revealed identical rates of ventilation ($\dot{V}$ E) and metabolism ($\dot{V}$ O_2, rate of oxygen consumption) between genotypes. The average convective requirement determined for both genotypes ($\dot{V}$ E/$\dot{V}$ O_2) was close to 41, a value similar to that previously reported for mammals in general (Frappell et al., 1992). On exposure to acute hypoxia (Figure 7B, position 2), wild-type mice hyperventilate (shift to a new $\dot{V}$ E/$\dot{V}$ O_2 ratio of 82), achieved both by an increase in ventilation (hyperpnea) and a decrease in rate of oxygen consumption (hypometabolism). In contrast, *Siah2*$^{-/-}$ mice display the same hyperventilatory response ($\dot{V}$ E/$\dot{V}$ O_2 ratio of 82) but achieve it solely through a dramatic hypometabolic response and completely lack a hyperpneic response. Thus, although the absence of Siah2 does not prevent hyperventilation appropriate to the level of hypoxia, Siah2 is necessary for ventilatory changes (hyperpnea) in response to acute hypoxia. However, *Siah2*$^{-/-}$ mice display similar ventilatory and metabolic responses to those of wild-type mice during and following chronic hypoxia (data not shown).

Discussion

The present study identifies a novel layer in the regulation of HIF1α hydroxylation by PHD1/3. We demonstrate that PHD1 and PHD3 are regulated by members of the E3 ubiquitin ligase family, Siah2 and Siah1a, and that such regulation determines the level of HIF1α expression under hypoxic conditions. The biological significance of our findings is best illustrated by the finding that *Siah2*$^{-/-}$ cells exhibit lower HIF1α abundance than wild-type cells during hypoxia, and that *Siah1a/2* DKO cells fail to exhibit hypoxia-induced increase in HIF1α abundance. Consequently, there are correspondingly lower levels of expression of the HIF1α target gene *VEGF*, in *Siah2* null cells and *Siah1a/2* DKO cells during hypoxia.

Importantly, our findings demonstrate the effect of Siah1a/2 on PHD1/3, but not on PHD2, thereby pointing to a functional difference among the three PHD enzymes in the regulation of HIF1α. RNAi for PHD2 increases HIF1α expression under normoxia (Berra et al., 2003). In contrast, elevated expression of PHD3 suffices to decrease expression of HIF1α under hypoxia conditions in wild-type cells and RNAi-mediated inhibition of PHD3 rescues hypoxia-induced HIF1α expression in *Siah1a/2* DKO cells. We therefore conclude that whereas PHD2 primarily limits HIF1α expression under normoxia, PHD3 regulates HIF1α availability under moderate hypoxic conditions, under which sufficient oxygen molecules are available for its activity.

Since severe hypoxia or anoxia deprive PHDs of the oxygen molecules that are required for their enzymatic activities (Bruick and McKnight, 2001; Jaakkola et al., 2001; Epstein et al., 2001), it is necessary to highlight the physiological significance of Siah2-mediated degradation of PHD3 under hypoxia. The enzymatic activity of PHDs is inhibited in a graded manner with respect to decreasing oxygen concentration, with only partial inhibition in vitro at 10% oxygen (Epstein et al., 2001). Consistent with functional PHD under mild hypoxia conditions, both that level (mRNA) and activity (toward deg-

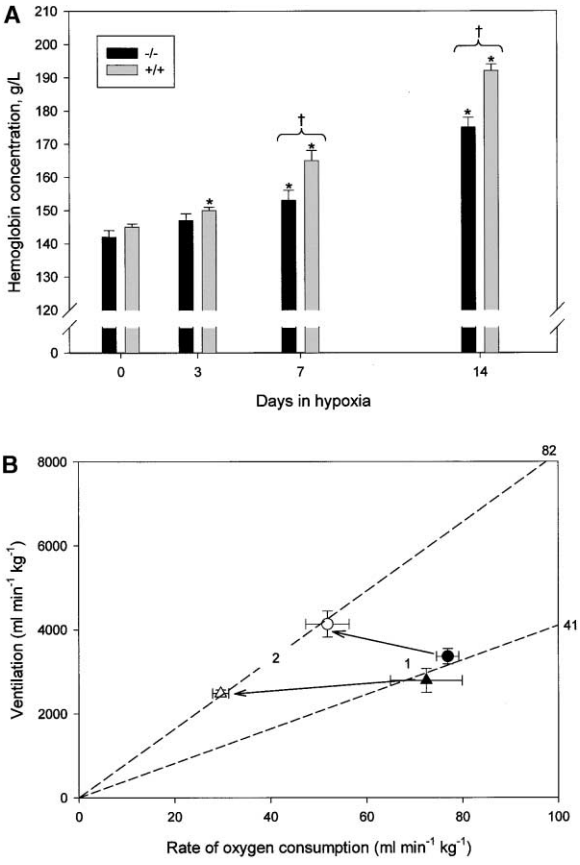

Figure 7. Defective Response to Hypoxia in Siah2 Null Mice

(A) The effect of hypoxia (10% O_2) on hemoglobin concentration in wild-type (+/+) and *Siah2* null mice (−/−). Bars are mean ± 1 SE, N = 7. *, Significant difference for same strain from day 0 (normoxia); †, significant difference between strains on the same day.

(B) The relationship between ventilation and rate of oxygen consumption in normoxia (position 1) and on exposure to acute hypoxia (10% O_2, position 2) for wild-type mice (●) and *Siah2* null mice (▲). Closed symbols are normoxic values; open symbols are hypoxic values. Dashed lines represent $\dot{V}_E/\dot{V}o_2$ isopleths (values given). Symbols are mean values ± 1 SE, N = 7. A significant difference exists between cohorts in $\dot{V}$ E and $\dot{V}$ O_2 at 2, no significant difference exists in either variable in normoxia.

radation of PHD3) of Siah2 are elevated upon exposure to even mild hypoxia (5% and 10% oxygen; Figures 5 and 6). We therefore propose that Siah2-regulation of PHD3 stability is of physiological importance within the range of moderate hypoxia where it is likely that there remains sufficient oxygen for PHD3 activity.

Increased expression of *PHD3* mRNA, but not *PHD1/2*, was observed in cardiac myocytes, smooth muscle cells, and endothelial cells subjected to hypoxia (Cioffi et al., 2003), while induced expression of both *PHD3* and *PHD2* mRNA under hypoxia conditions has been reported for certain cell lines, pointing to a tissue-specific regulation (Epstein et al., 2001; Metzen et al., 2003). As *Siah2* mRNA induction and enhancement of Siah2-mediated PHD3 degradation were as efficient at 10% and 1% oxygen, it is possible that Siah2-mediated PHD3 degradation may represent an oxygen-sensing mechanism that responds to even mild hypoxic conditions, thereby constituting a primary response to hypoxia.

Variations in oxygen concentration (or oxygen partial pressure) in different tissues have both physiological and pathophysiological implications. For example, oxygen tension is an important determinant of proliferation and differentiation of hematopoietic cells; whereas the formation of mature erythrocytes and megakaryocytes is more extensive under 20% O_2 (Laluppa et al., 1998), greater production of differentiated granulocytes is observed under 5% O_2 (Hevehan et al., 2000). Further, regulation of human placental development, lung branching morphogenesis, and angiogenesis are affected by altering oxygen tension (Genbacev et al., 1997; Adelman et al., 2000; Gebb and Jones 2003).

Our analyses of physiological responses to hypoxia in *Siah2* null mice provide strong evidence supporting our proposal that Siah2 is a component of oxygen-sensing systems. First, we showed that *Siah2* null mice exhibit a defect similar to that of HIF1α heterozygous mutant mice in induction of polycythemia following exposure to chronic hypoxia. Second, we observed that *Siah2* null mice lack a hyperpneic response to acute hypoxia and instead achieve hyperventilation solely through a dramatic decrease in metabolic rate. Interestingly, isolated carotid bodies of HIF1α heterozygous mutant mice displayed impaired neural activity on exposure to hypoxia (Kline et al., 2002). The normal ventilatory response to acute hypoxia in these mice was attributed to increased utilization of chemoreceptors other than the carotid body (Kline et al., 2002). In this context, it is particularly interesting that *Siah2* null mice lack a hyperpneic response to acute hypoxia, suggesting that Siah2 is important for the ventilatory response. Presumably the absence of Siah2 changes the expression of various genes encoding receptors or neurotransmitting enzymes in the chemoreceptors involved in transduction of the hypoxic signal to the central nervous system.

In summary, we propose that induction of Siah2 by hypoxia serves to enhance the degradation of PHD1/3 and consequently increase the abundance of HIF1α. Accordingly, Siah2 is part of a feed-forward mechanism that regulates PHD1/3 availability in hypoxia.

Experimental Procedures

Animals Genotype/Strains

Siah2$^{-/-}$ mice used for hypoxia studies are *c57/Bl6* strain. Mouse embryonic fibroblasts (MEF) used in the present study were prepared from *Siah2*$^{+/+}$, *Siah2*$^{-/-}$, and *Siah1a/2* DKO embryos of a 129 strain (Frew et al., 2003).

Cell Culture and Media

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum and antibiotics. HeLa cells were cultured in DMEM with 10% fetal bovine serum (FBS) and antibiotics. Immortalized *Siah2*$^{+/+}$ and *Siah2*$^{-/-}$, and *Siah1a/2 DKO* MEF were maintained in 10% FBS with 0.1 mM nonessential amino acids (Invitrogen), 0.2 mM 2-mercaptoethanol (Sigma), and antibiotics.

Antibodies and Chemical Reagents

Anti-Flag tag (monoclonal and polyclonal), Anti-β-actin antibodies were purchased from Sigma. Antiubiquitin antibodies were purchased from Covance. Anti-HIF1α antibody was obtained from NOVUS Biological. Anti-HA tag antibody was purified from 12CA5 clone. Anti-PHD3 antibody was raised in rabbit by injecting bacterial-purified PHD3

protein (Pocono Rabbit Farm & Laboratory Inc.). These antibodies were purified on a PHD3-coupled-HiTrap NHS-activated column (Amersham Biosciences) and tested for purity in immunoblotting experiments (Supplemental Figure S4 available on *Cell* website and data not shown). Mouse monoclonal antibodies against Siah1a were generated by D. Bowtell. Lactacystin, MG101, MG132, E64, chloroquine, and desferrioxiamine (DFO) were purchased from Sigma. Epoxomicin was purchased from Calbiochem.

Protein Identification by Mass Spectrometry

Proteins prepared from 293T cells that express exogenous Flag-Siah2^{Rm} were immunoprecipitated with Flag antibodies and bound material was eluted with the aid of Flag peptide. Flag-tagged empty expression vector was used as a control. Eluted proteins were separated on 2D gels and specific spots (compared with empty construct Flag IP) were identified via silver staining and subjected to mass spec analysis using matrix-assisted laser-desorption/ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS) (Reflex III; BRUKER Daltonics, Bremen, Germany), as described (Erdjument-Bromage et al., 1998), and also using an electrospray ionization (ESI) triple quadrupole MS/MS instrument (API300; ABI/MDS SCIEX, Thornhill, Canada) modified with an ultra-fine ionization source. Selected precursor or fragment ion masses were used to search a protein nonredundant database, as described (Winkler et al., 2002).

In Vitro Association Experiment

GST-Siah2 and GST-Siah2^{Rm} were purified from bacteria using glutathione-4B Sepharose (Amersham Bioscience). ^{35}S-labeled PHD3 was translated in vitro using a TNT-coupled reticulocyte lysate system (Promega). After preincubation in 0.1% BSA/PBS for 1 hr, GST-Siah2 or Siah2^{Rm} attached to glutathione-4B Sepharose and ^{35}S-labeled PHD3 were incubated at 4°C for 2 hr. The beads were extensively washed with PBS containing 0.5% NP40 and 150 mM NaCl three times before subjection to SDS-PAGE followed by autoradiography.

Immunoprecipitation and Western Blotting

293T cells were transfected using the calcium-phosphate method. HeLa cells and MEF were transfected using lipofectamine (Invitrogen). Transfected cells were lysed in lysis buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 1% Triton, 0.1 μg/ml PMSF, and 2 μg/ml leupeptin). Anti-Flag antibody (1 μg) was added to the lysate, followed by the addition of protein-G agarose (Invitrogen). The immunoprecipitates were subjected to SDS-PAGE and transferred onto nitrocellulose membrane (Osmonics Inc.). The membrane was probed with primary antibodies followed by a secondary antibody conjugated with horseradish peroxidase and detected by the ECL system (Amersham Bioscience).

RT-PCR Analyses

MEFs treated with or without hypoxia were harvested and total RNA was extracted using an RNA extraction kit (QIAGEN). Complementary DNA was synthesized from 2.5 μg total RNA using an oligo-dT (18) primer. Primers used for PCR were VEGF-forward: 5′ CCATGCA GATCATGCGGATCA 3′ and reverse: 5′ CCTTGGCTTGTCACATCT GCAA 3′; Siah2-forward: 5′ CTGTTTCCCTGTAAGTATGCTACC 3′ and reverse: 5′ CACTGACAGCATGTAGATATCGTG 3′; PHD2-forward 5′ ATGGCCAGTGACAGCGGC 3′ and reverse 5′ CAACGG CTTGGTCTGCCC 3′; and PHD3-forward 5′ ATGCCTCTGGGA CACATC 3′ and reverse 5′ TCAGTCTTTAGCAAGAGCA 3′; GAPDH-forward 5′ ACCACAGTCCATGCCATCAC 3′ and reverse: 5′ TCCAC CACCCTGTTGCTGTA 3′. The PCR reaction was carried out using (α-^{32}P)dCTP to amplify VEGF, Siah2, PHD2, PHD3, and GAPDH for 17, 25, 20, 25, and 15 cycles, respectively, and signals were detected by autoradiography.

Pulse-Chase Experiment

MEFs transfected with Flag-PHD3 were maintained in DMEM/10% FBS lacking Met and Cys for 1 hr. Following the starvation step, cells were incubated in DMEM/10% FBS containing a ^{35}S-Met/Cys mixture (50 μCi/ml) for 2 hr. Cells were then washed twice with PBS and labeling was stopped by addition of growth medium supplemented with an excessive amount of cold Met and Cys (3 mM). During the chase period, cells were subjected to normoxia or hypoxia treatments (see below), as indicated in Results.

Gene Silencing by RNAi

Of the 11 different RNAi sequences designed and tested for specific inhibition of *PHD3* mRNA expression, we found one that meets this criterion. Nucleotides corresponding to 357–375 nt of mouse *PHD3* coding sequence were synthesized and cloned into pSuper vector (pSup-PHD3). Empty pSuper vector was used as a control. RNAi expression vectors were introduced into MEFs by transfection (Lipofectamine +). Seventy-two hours later, cells were treated with or without hypoxia and harvested. Inhibition of PHD3 expression in both wt and *Siah1a/2* DKO cells (Figure 3D and data not shown) was confirmed via RT-PCR analysis using specific primer combination for mouse *PHD3* and *PHD2*. pSup-PHD3 was also transfected (Ca-phosphate) into 293T cells for in vivo ubiquitination experiment (Figure 1F).

Hypoxia Treatment

Cells were treated under hypoxia (10%, 5%, and 1% O_2) in a hypoxia/tissue-culture chamber (Billups-Rothenberg, Inc.). Next, cells were processed and in vitro reactions were carried out in a BioSpherix hypoxia working chamber monitored by a PROOX 110 sensor (BioSpherix NY).

Animal Studies

Experiments were performed on age- and sex-matched wild-type (+/+) and *Siah2* null (−/−) mice, n = 7 for both cohorts. To subject mice to chronic hypoxia, the nesting box was placed within a plexiglass chamber and the atmosphere within the chamber maintained at 10% O_2 by blending air and nitrogen with the flow adjusted so that CO_2 levels leaving the chamber (monitored daily) were maintained at less than 0.3%.

Hematology

Blood samples were obtained by retroorbital sinus puncture on day 0 (normoxia) and days 3, 7, and 14 in hypoxia. To determine hemoglobin concentration, [Hb] 10 μl of blood were drawn into a cuvette containing a modification of Vanzetti's reagents for azide methemoglobin (Vanzetti, 1966) and analyzed spectrophotometrically (Hb 201+, HemoCue AB, Sweden). Each sample was analyzed twice.

Respirometry

Unanesthetized animals were weighed and body temperature (Tb) measured before being placed in a 500 ml plexiglass chamber through which air flowed at ~500–600 ml min^{-1}. Measurements of metabolic rate and respiratory variables were made on day 0 (normoxia) and after acute exposure to hypoxia (10% O_2, 20 min), following three days in hypoxia and immediately on return to normoxia (after 20 min exposure). Metabolic rate and breathing pattern were determined as outlined in Frappell et al. (1992). Minute ventilation ($\dot{V}$ E = tidal volume x frequency) was determined using the barometric technique (Mortola and Frappell, 1998). All experiments were performed at room temperature (22°C), $\dot{V}$ O_2 and $\dot{V}$ CO_2 were expressed at STPD (273.15° K, 101.3 kPa, dry) and ventilatory volumes at BTPS (Tb, 101.3 kPa, saturated).

Statistics

Differences between genotypes and exposure to hypoxia were analyzed with repeated measures ANOVA and post hoc modified t-tests with Bonferroni correction. $P < 0.05$ was considered significant.

Acknowledgments

We thank Mark Taubman and Gregg Semenza for advice. We also thank A. Nazarian, Suparna Basu, and Gabriel Maulit for technical assistance; Z.Q. Pan and members of the Ronai Lab for extensive discussions. We also thank Reuven Agami for the pSuper plasmid and Jacques Pouyssegur for providing us with reagents. K.N. is supported by a JSPS postdoctoral fellowship. Support by NCI grants CA80058 and CA51995 (to Z.R.) and NCI Cancer Center Support Grant P30 CA08748 (to P.T.) is gratefully acknowledged.

Received: June 20, 2003
Revised: April 23, 2004
Accepted: April 27, 2004
Published: June 24, 2004

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