Prolyl hydroxylase 3 stabilizes the p53 tumor suppressor by inhibiting the p53–MDM2 interaction in a hydroxylase-independent manner

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ABSTRACT
Prolyl hydroxylase 3 (PHD3) has initially been reported to hydroxylase hypoxia-inducible factor α (HIFα) and mediate HIFα degradation. More recent studies have shown that, in addition to HIFα, PHD3 has also other substrates. Moreover, PHD3 is believed to act as a tumor suppressor, but the underlying mechanism remains to be elucidated. Here, we demonstrate that PHD3 stabilizes p53 in a hydroxylase-independent manner. We found that PHD3 overexpression increases and PHD3 knockdown decreases p53 levels. Mechanistically, PHD3 bound MDM2 proto-oncogene (MDM2) and prevented MDM2 from interacting with p53, thereby inhibiting MDM2-mediated p53 degradation. Interestingly, we found that PHD3 overexpression could enhance p53 in the presence of the prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG). And the prolyl hydroxylase activity–deficient variant PHD3-H196A also inhibited the p53–MDM2 interaction and stabilized p53. Genetic ablation of PHD3 decreased p53 protein levels in mice intestinal epithelial cells, but a genetic knock-in of PHD3-H196A did not affect p53 protein levels in vivo. These results suggest that the prolyl hydroxylase activity of PHD3 is dispensable for its ability to stabilize p53. We found that both PHD3 and PHD3-H196A suppress the expression of the stem cell–associated gene NANOG and inhibited the properties of colon cancer stem cells through p53. Our results reveal an additional critical mechanism underlying the regulation of p53 expression and highlight that PHD3 plays a role in the suppression of colon cancer cell stemness in a hydroxylase-independent manner.

INTRODUCTION
Prolyl hydroxylases (PHD1, 2 and 3) are dioxygenases that use oxygen and
2-oxoglutarate (2-OG) as co-substrates. PHDs are involved in the cellular response to oxygen by hydroxylating conserved prolyl residues of hypoxia inducible factor α (HIFα) (1-3). The hydroxylated HIFα is recognized by von Hippel-Lindau protein and then subjected to ubiquitination and proteasomal degradation. In hypoxia, the prolyl hydroxylase activity is suppressed, resulting in accumulation of HIFα. HIFα is a master transcription factor. It dimerizes with HIF-1β and translocates into the nucleus, where it initiates the transcription of many genes. The hydroxylase activity of PHD is strictly controlled by oxygen availability over the entire physiologic range (2). These properties make PHDs well suited to act as oxygen sensors.

Recent studies have shown that, besides HIFα, PHDs have other substrates. For example, PHD3 was found to mediate oxygen-dependent stability of the activating transcriptional factor 4 (ATF4) (4) and the β-2-Adrenergic receptor (5), and to prevent degradation of myogenin (6). PHD3 regulates DNA damage response through hydroxylating the human homolog of the *Caenorhabditis elegans* biological clock protein CLK-2 (7). A recent study demonstrates that PHD3 hydroxylates and stabilizes MAPK6 (8). We found that PHD3 repressed IKK/NF-κB signaling (9).

A few studies have demonstrated that PHD3 acts as a tumor suppressor. Downregulation of PHD3 was found in a few cancers (9-11). PHD3 upregulation was linked to cell apoptosis (12) and its activation suppressed xenograft growth of melanoma cells (13). PHD3 caused apoptosis of cervical cancer HeLa cells (14) and inhibited proliferation of gastric cancer cells (15) and renal carcinoma cells (16). Epidemiology studies showed that expression of PHD3 was correlated with good prognostic factors in breast cancers (17) and it was a favorable prognosticator for gastric cancer (18). PHD3 was shown to inhibit tumor growth via EGFR signaling (19).

Though studies have indicated that PHD3 functions as a tumor suppressor, the underlying mechanism remains unclear. In this manuscript we demonstrate that PHD3 blocks the interaction of p53 and MDM2, thereby inhibiting the MDM2-mediated p53 destruction, in a hydroxylase-independent mechanism. The PHD3-induced p53 stabilization inhibits NANOG expression, leading to inhibition of colon cancer stem cells. Our findings reveal a new mechanism underlying the regulation of p53 stability through PHD3 and highlight the role of PHD3 in suppression of cancer cell stemness independent of its hydroxylase activity.

**RESULTS**

**PHD3 stabilizes p53**

This study was kindled by an accidental discovery that PHD3 influenced the expression of p53. We found that overexpression of PHD3 enhanced the protein levels of p53 in colon cancer RKO and normal colon epithelial CCD841 cells (Fig. 1A). Overexpression of PHD3 did not affect the transcript level of *TP53* (Fig. 1B). In agreement with the above results, knockdown of PHD3 reduced p53 protein (Fig. 1C) with little effect on *TP53* transcript levels (Fig. 1D). The data imply that PHD3 regulates the expression of p53 in a post-transcriptional manner. We found that overexpression of PHD3 retarded degradation of p53 and increased half-life of p53 (Fig. 1E). We determined the effect of PHD3-knockdown on half-life of p53 in and the results show that knockdown of PHD3
increased the rate of p53 degradation (Fig. 1F). These results suggest that PHD3 stabilizes p53. We next did experiments to verify that PHD3 could modulate the expression of p53 in vivo. We employed Phd3^IEC-KO mice that Phd3 was deleted in intestinal epithelial cells. Generation of Phd3^IEC-KO mice is as described in Experimental Procedures. The results show that genetic ablation of Phd3 led to a dramatic decrease of p53 in both small intestine and colon epithelial cells in mice (Fig. 1G). We examined other cells and found that overexpression of PHD3 increased p53 in cervical cancer Hela, breast cancer MCF-7, and renal cancer ACHN and Caki-1 cells, but not in colon cancer HCT116 cells (Fig. 1H). We also examined human embryonic kidney 293T cells and overexpression of PHD3 had no effect on expression of p53 in the cells (Fig. 1H). This is probably that 293T cells express adenoviral oncoproteins e1a/e1b55k which prevents p53 degradation (20, 21). All these cells we examined have wild-type p53.

PHD3 stabilizes p53 through MDM2
As MDM2 is the primary E3 ubiquitin ligase of p53 (22), we presumed that PHD3 might stabilize p53 through MDM2. To this end, we firstly determined whether PHD3 associated with MDM2. We found that both exogenous (Fig. 2A) and endogenous (Fig. 2B) PHD3 interacted with MDM2. The bacterial produced His-PHD3 and GST-DM2 bound each other, suggesting that PHD3 binds MDM2 directly (Fig. 2C). Thus, PHD3 might bind MDM2 to interfere with p53-MDM2 interaction. As expected, overexpression of PHD3 blocked the interaction of p53 and MDM2 (Fig. 2D). Consistent with the results, knockdown of PHD3 enhanced the interaction of p53 and MDM2 (Fig. 2E). Furthermore, we found that PHD3 inhibited p53-MDM2 interaction in a dose-dependent manner (Fig. 2F).

We determined the effect of PHD3 on ubiquitination of p53. The results show that overexpression of PHD3 decreased (Fig. 2G) and knockdown of PHD3 increased p53 ubiquitination (Fig. 2H). We did in vitro p53 ubiquitination assay and the results show that PHD3 decreased the MDM2-mediated ubiquitination of p53 (Fig. 2I).

It is known that the central acid domain of MDM2 binds p53, which is essential for MDM2 to mediate p53 ubiquitination (23-25). We asked whether PHD3 bound this central domain of MDM2 to impede p53-MDM2 interaction. To know this, we constructed a vector encoding GST-DM2(Δ217-367) that did not have the central domain. Our results show that PHD3 didn’t bind GST-DM2(Δ217-367) (Fig. 2J). We also constructed a vector encoding GST-DM2(217-367) and found that PHD3 bound GST-DM2(217-367) (Fig. 2J). These results suggest that PHD3 binds the central domain of MDM2, which prevents MDM2 from mediating p53 destruction.

PHD3 stabilizes p53 independent of its hydroxylase activity
PHD3 has prolyl hydroxylase-independent functions (9, 26). We wanted to known whether the prolyl hydroxylase activity is required for PHD3 to stabilize p53. To this end, we employed the prolyl hydroxylase inhibitor DMOG in our work. Treatment of the cells with DMOG increased HIF1α dramatically (Fig. 3A), indicating that the prolyl hydroxylase activity is suppressed. Interestingly, overexpression of PHD3 still enhanced p53 protein in the presence of DMOG (Fig. 3A). We next employed a vector coding prolyl hydroxylase-deficient PHD3(H196A) in our work (27). We found
that overexpression of PHD3(H196A) also enhanced p53 protein (Fig. 3B). Moreover, we found that PHD3(H196A) increased the half-life of p53 as PHD3 did (Fig. 3C, D). These results suggest that PHD3 stabilizes p53 in a hydroxylase-independent manner. We found that PHD3(H196A) associated with MDM2 (Fig. 3E) and overexpression of PHD3(H196A) suppressed the interaction between p53 and MDM2 (Fig. 3F). Thus, PHD3(H196A) may enhance p53 in a same way as PHD3 does.

In hypoxia, the expression of PHD3 is transcriptionally activated by HIFα (28). A few studies have shown that hypoxia also induces expression of p53 (29-32). So, we asked whether PHD3 was involved in hypoxic induction of p53. To know this, we determined the effect of knockdown of PHD3 on expression of p53 in hypoxia. We found that hypoxia enhanced p53 and knockdown of PHD3 attenuated the hypoxia-induced p53 in CCD841 and RKO cells (Fig. 3G). These results suggest that PHD3 is required for hypoxic induction of p53. Further, we found that knockdown of PHD3 had little effect on HIF-1 and -2α protein levels, suggesting that PHD3 does not regulate hypoxic induction of p53 via HIFα.

To confirm that PHD3 regulates p53 expression in a hydroxylase-independent manner, we employed intestinal epithelial PHD3(H196A)-knockin (Phd3(H196A)IEC-KI) mice in our work. PHD3 of mouse is quite similar to that of human (97% identities). Both human PHD3 and mouse PHD3 consist of 239 amino acids and they both have His196 residue. We constructed mouse PHD3 and PHD3(H196A) vectors and found that overexpression of mouse PHD3, but not PHD3(H196A), decreased HIF-1α and HIF-2α proteins (Fig. 4A), implying that the mouse PHD3(H196A) is deficient of prolyl hydroxylase activity. We found that overexpression of mouse PHD3(H196A) enhanced p53 protein as wild-type PHD3 did (Fig. 4A).

Generation of Phd3(H196A)IEC-KI mice is as described in Experimental Procedures. The structures of wild-type and mutant allele of Phd3 of mice were shown (Fig. 4B). PCR results show that the homozygous Phd3(H196A) had a band of 1184 bp and the wild-type Phd3 had a band of 656 bp (Fig. 4C). Genotyping of Phd3(H196A)FF and Phd3(H196A)IEC-KI are shown in Fig. 4D. The right lane that had a mutated Phd3 band indicates Phd3(H196A)FF mice. The left lane having mutated Phd3 and Villin-Cre bands indicates the Phd3(H196A)IEC-KI mice. It is known that PHD3 hydroxylates ATF4, leading to ATF4 destruction (4). So, we determined the effect of PHD3(H196A) knockin on expression of ATF4 in small intestine and colon epithelial cells. The results show that ATF4 protein level is enhanced in intestinal epithelial cells from Phd3(H196A)IEC-KI mice (Fig. 4E), implying that the mutated PHD3 is deficient of hydroxylase activity. Finally, we determined the protein level of p53 in these cells and found that PHD3(H196A)−knockin had no effect p53 protein levels (Fig. 4F).

PHD3 inhibits the expression of NANOG through p53

Bunch of studies have implicated a critical role of p53 in stem cells through regulating expression of genes related to these cells (33). Among these genes, NANOG is a major one (34). The expression of NANOG was demonstrated to be regulated negatively by p53 (35). Therefore, we asked whether PHD3 influenced the expression of NANOG through p53. In agreement with previous results, overexpression of p53 decreased (Fig. 5A) and knockdown of p53
enhanced the expression of NANOG (Fig. 5B). As expected, overexpression of PHD3 inhibited (Fig. 5C) and knockdown of PHD3 stimulated (Fig. 5D) the expression of NANOG. To verify that PHD3 regulate the expression of NANOG through p53, we introduced p53 siRNA oligos into the cells. We found that knockdown of p53 reversed the expression of NANOG suppressed by overexpression of PHD3 (Fig. 5E). These results imply that PHD3 inhibits NANOG expression through p53. In addition, we found that overexpression of PHD3(H196A) also restrained the expression of NANOG as PHD3 did (Fig. 5F). The results suggest that PHD3 attenuates the expression of NANOG in a hydroxylase-independent manner.

We also determined the effect of PHD3 on other p53 downstream genes including p21, BAX, PUMA and NOXA in RKO cells. The results show that overexpression of PHD3 induced the expression of p21 and PUMA (Fig. 5G). And knockdown of PHD3 decreased the expression of p21, PUMA and NOXA (Fig. 5H).

**PHD3 inhibits properties of colon cancer stem cells (CSC)**

Because PHD3 induced the expression of p53 and inhibited the expression of NANOG (Fig. 5), we presumed that PHD3 was capable of suppressing the properties of colon CSCs. To know this, we determined the effects of PHD3 on sphere formation of RKO cells. Sphere-forming assay has been widely used to identify stem cells (36). Our results show that overexpression of PHD3 suppressed sphere formation of the cells (Fig. 6A). As expected, overexpression of PHD3 inhibited the expression of colon CSC genes NANOG and LGR5 of the cells (Fig. 6B). Overexpression of PHD3(H196A) also repressed sphere formation (Fig. 6A) and attenuated the expression of NANOG and LGR5 (Fig. 6B). On the contrary, knockdown of PHD3 promoted sphere formation (Fig. 6C) and enhanced the expression of NANOG and LGR5 (Fig. 6D). Next, we determine whether PHD3 affect sphere formation through p53. We found that knockdown of p53 reversed the sphere formation inhibited by overexpression of PHD3 (Fig. 6E). Similarly, knockdown of p53 also prevented PHD3(H196A) from suppressing sphere formation of the cells (Fig. 6F). Together, these results suggest that PHD3 functions to inhibit the properties of colon CSCs through p53.

**DISCUSSION**

We have demonstrated in this manuscript that PHD3 stabilizes p53 through inhibiting the interaction between p53 and MDM2, independent of its hydroxylase activity. The PHD3-induced stabilization of p53 leads to attenuation of the expression of NANOG and suppresses the properties of colon CSCs. Our results reveal a new mechanism of regulation of p53 expression through PHD3 and highlight a role of PHD3 in suppressing colon CSCs.

Stability of p53 is regulated by the ubiquitin-proteasome system and MDM2 acts as the E3 ubiquitin ligase that mediates p53 degradation (37). Binding to the central domain of MDM2 is essential for p53 ubiquitination and destruction (23-25, 38). We found that PHD3 also bound the central domain of MDM2 (Fig. 2) as p53 did. Thus, PHD3 may block the accessing of p53 to the central domain of MDM2, which prevents MDM2 from mediating p53 destruction. Interestingly, our results showed that the hydroxylase-deficient PHD3(H196A) also stabilized p53 (Fig. 3). It bound MDM2 and blocked the interaction of p53 and MDM2 as the wild-type PHD3 did (Fig. 3E, F). We generated PHD3(H196A)–knockin mice...
and found that loss of prolyl hydroxylase activity of PHD3 had little influence on protein level of p53 in vivo (Fig. 4). Considering the fact that PHD3-knockout decreased p53 in mice intestinal epithelial cells (Fig. 1G), our data suggest that PHD3 induces p53 independent of its hydroxylase activity. In hypoxia, HIFα is accumulated and initiates the expression of PHD3 (28). Hypoxia also induces expression of p53 (29-32). The hypoxia-induced PHD3 may reduce MDM2-p53 interaction and stabilize p53. Indeed, we found that knockdown of PHD3 attenuated the hypoxic induction of p53 (Fig. 3G). Thus, the hypoxia-induced PHD3 may contribute the hypoxic induction of p53.

Rodriguez et al (39) reported recently that PHD3 hydroxylates p53, leading to stabilization of p53. They found that PHD3 hydroxylated p53 to promote the interaction between p53 and deubiquitinases, leading to p53 stabilization. They showed that hypoxia resulted in a reduction of p53 in Hela and HepG2 cells, implying that the hydroxylase activity is required for p53 expression. We found that the prolyl hydroxylase-deficient PHD3(H196A) also enhanced p53 protein in colon epithelial RKO and CCD841 cells as the wild-type PHD3 did. The cause for the discrepancy is not clear. One possible reason is that different cells were examined in these two works. Hypoxic induction of p53 is cell-type specific. For instance, Rodriguez et al (39) demonstrated that hypoxia did not enhance p53 in Hela and HepG2 cells. Some other reports showed that hypoxia induced accumulation of p53 in lymphoblastoid GM02184B cells (29) and RKO cells (30). Hypoxic induction of p53 is complex. Pan et al (31) found that hypoxia alone could not induce p53. An et al (32) reported that HIF-1α is required for stabilization of p53 in hypoxia. It is known that the regulation of expression of p53 is complicated and may be at multiple levels, including transcription, translation, and post-translation. So, it is not surprising for the discrepancies of these works. PHD3 may stabilize p53 in different mechanisms. It is noted that PHD3 had little effect on the expression of p53 in HCT116 cells (Fig. 1G), suggesting that PHD3 stabilizes p53 in a cell type-specific manner.

P53 suppresses self-renewal during the oncogenic process and it is a barrier to the formation of CSCs (40-42). P53 mediates the stem cell function and suppresses self-renewal via regulating the expression of NANOG (33). NANOG is a crucial factor that confers certain CSCs properties (34), including colon CSCs (43, 44). PHD3 stabilizes p53 and suppresses the expression of NANOG (Fig. 5). Thus, PHD3 may function to inhibit colon CSCs. In fact, our data show that PHD3 functions to suppress the properties of colon CSCs.

PHDs initially are recognized as hydroxylases that catalyze the proline hydroxylation of proteins. Recent studies have indicated that PHDs may also have hydroxylase-independent functions. For instance, Chan et al (45) demonstrated that PHD2 regulated angiogenesis independent of its hydroxylase activity. Fu and Taubman (26) reported that PHD3 competed with cIAP1 for IKKγ binding, leading to inhibition of cIAP1-IKKγ interaction, IKKγ ubiquitination, and IKK/NF-κB signaling. We found that PHD3 stabilized the tight junction protein occludin (46) in a hydroxylase-independent mechanism. These results suggest that PHD3 works through hydroxylase-dependent and -independent mechanisms.

In this study, we have shown that PHD3 stabilizes p53 and inhibits colon CSC properties independent of its hydroxylase
activity. These results broaden the functional scope of PHD3 and expand our understanding of PHD3 as a suppressor of cancer cells.

EXPERIMENTAL PROCEDURES

Animals

The C57BL/6J mice were purchased from Shanghai Experimental Animal Center. The intestinal epithelia-specific Phd3 knock-out mice (Phd3IEC-KO) were generated by intercrossing the Phd3\textsuperscript{floxflox} (Phd3\textsuperscript{FF}) mice with Villin-Cre ones as described (46). The Phd3(H196A)\textsuperscript{floxflox} (Phd3(H196A)\textsuperscript{FF}) mice were created at Shanghai Research Center for Model Organisms. Intestinal epithelial Phd3(H196A)-knockin (Phd3(H196A)IEC-KI) mice were generated by intercrossing the Phd3(H196A)\textsuperscript{FF} mice with Villin-Cre mice. Animals were housed in specified pathogen-free conditions. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, CAS.

Cell culture

Human colon cancer RKO, human embryonic kidney 293T, human breast cancer MCF7, human renal cancer ACHN and human cervical cancer Hela cells were grown in DMEM. Human normal colon epithelial CCD841 cells were grown in RPMI-1640 medium. Human colon cancer HCT116 cells and human renal cancer Caki cells were grown in M5A medium. All medium were supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin. The cells were cultured at 37°C in an incubator with 5% CO2.

Antibodies and reagents

The antibodies against PHD3(NB100-303) and HIF-2\textalpha(NB100-122) were from Novus Biologicals. The p53(DO-1), p53(DO-7), MDM2(SMP14), ATF4, GST, His, HA and Myc antibodies were from Santa Cruz Biotechnology. The HIF-1\textalpha(610959) and NANOG(AB80892) antibodies were from BD and Abcam, respectively. β-actin antibody was from Sigma (St. Louis, MO). EGF was purchased from Life Technologies. B27 and bFGF was purchased from Invitrogen and Peprotech, respectively.

Construction of vectors

The vectors encoding myc-tagged human PHD3 and PHD3(H196A) were as described (9). The lentivirus encoding Flag-tagged human PHD3 was purchased from Genechem (Shanghai, China). The HA-p53 vector was from Dr. Zhixue Liu (Institute for Nutritional Sciences, CAS) and p53 vector was from Dr. Hai Jiang (Shanghai Institute for Biochemistry and Cell Biology, CAS). The vector encoding glutathione S-transferase (GST)-MDM2 fusion protein was constructed by inserting PCR-generated DNA fragments encoding regions of MDM2 into pGEX-4T-1. The His-PHD3 vector was generated as described (9). The BL21-Gold(DE3)pLysS Escherichia coli cells were transformed with pGEX-4T-1 or pET28a vectors and treated with isopropyl-D-thiogalactoside (0.1 mM) for 4 h.

Small interfering RNA (siRNA) and short hairpin RNA (shRNA)

Small interfering RNA (siRNA) duplexes were synthesized by GenePharma (Shanghai, China). The cells were transfected with siRNA oligos using LipofectAMINE-2000. siP53-1: 5’GUACCACCAUCACUACAATT3’. siP53-2: 5’GUAAUCUACUGGGACGGAATT3’. siPHD3-1: 5’GUGAUGGUCUGCUAGCAATT3’. siPHD3-2: 5’GGAGAGGUCUAAGGCAUGTT3’.
Control:
5’UUCUCCGAACGUGUCACGU TT3’.

Quantitative real-time PCR (qPCR)
qPCR was performed as described (9). Beta-actin was used as the internal control. The primers are as follows.

TP53:
5’GAGAGCTGAATGAGGCCTTG3’(F),
5’TTATGGCGGGAGGTAGACTG3’(R).

NANOG:
5’GGTGGCAGAAAAACAACTGG3’(F),
5’CATCCCTGGTGGTAGGAAGA3’(R).

LGR5:
5’GGTCGCTCTCTACCTTGCTCA3’(F),
5’GCCACAGGGCAGTTTAGGAT3’(R).

p21:
5’TGGGGATGTCCGTCAGAACC3’(F),
5’TACCCTCCAGTGGTGTCTC3’(R).

PUMA:
5’CGGAGCAGCACCTGGAGTCG3’(F),
5’TTCGAGTCGTCCGCAGTTAGAT3’(R).

NOXA:
5’CGCAAGAACGCTCAACCGAG3’(F),
5’CTGCCGGAAGTTCAGTTTGTCT3’(R).

BAX:
5’ACCAAGAAGCTGAGCGAGTGT3’(F),
5’CCAGTTGAAGTTGCCGTCAG3’(R).

β-actin:
5’GATCATTTGCCTCCTCGAG3’(F),
5’ACTCCTGCTTGTGATCCAC3’(R).

In vitro p53 ubiquitination assay
The MDM2/p53 Ubiquitination Kit (Cat. # K-200B) from BostonBiochem was used in the experiment. The in vitro ubiquitination assay of p53 was performed as described (47). In brief, 293T cells were transfected to express myc-PHD3. 100 or 400 µg cellular proteins were immunoprecipitated with 0.25 or 1 µg of Myc antibody at 4 °C for 3 h. Forty microliters of protein A/G PLUS agarose beads were added, and the incubation continued at 4 °C overnight. The beads were washed, spun and re-suspended in a reaction volume of 30 µl. The mixture was incubated at 37 °C for 1 h. After the reaction completed, the supernatant was used for p53 ubiquitination analysis in a western blot. The beads were boiled in loading buffer, resolved in SDS-PAGE and immunoblotted with Myc antibody.

Isolation of intestinal epithelial cells
The intestine was removed and washed free of fecal material with solution A (96 mM NaCl, 27 mM sodium citrate, 1.5 mM KCl, 0.8 mM KH2PO4, 5.6 mM Na2HPO4, 5,000 U/L penicillin, 5 mg/L streptomycin, 0.5 mM DTT, and 2 mM phenylmethylsulfonyl fluoride, pH 7.4). Square pieces of tissue were placed in solution A (10 ml) at 37°C for 10 min with gentle shaking. This removed the mucus, bacteria, and other lumen contents. The tissue fragments were then incubated in solution B (0.1 mM EDTA, 115 mM NaCl, 25 mM NaHCO3, 2.4 mM K2HPO4, 0.4 mM KH2PO4, 0.5 mM DTT, 5 mg/l streptomycin, 2.5 mM glutamine, 5,000 U/l penicillin, and 2 mM phenylmethylsulfonyl fluoride, pH 7.4) at 37°C for 30 min. The disruption of the mucosa and elution of cells was stopped by adjusting to 1 mM CaCl2. The cells recovered in the suspension were collected by centrifugation and lysed in RIPA buffer for immunoblotting.

Tumor sphere formation assay
The transfected cells were seeded in 96-well ultra-low attachment plates at a density of 2×10³ cells/well in 200 µl serum-free DMEM/F12 medium containing 2% B27, 20 ng/ml EGF, 10 ng/ml bFGF, and 5 µg/ml insulin. The cells were incubated in a humidified atmosphere at 37 °C with 5% CO2. After 120 h, spheres (>50 µm in diameter) were counted under a microscope.

Statistical analysis
Statistical analysis was made using the unpaired two-tailed Student’s t-test or two-way analysis of variance (ANOVA)
with GraphPad Prism 5.0. The data represent mean±SEM from 3 independent experiments except where indicated. \( P < 0.05 \) is considered statistically significant.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

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| Primer | Sequence (5’→3’) |
|-------|-----------------|
| P1    | GTTCGCCTATCAGCTTTGGG |
| P2    | GCATAAAGCCCACATCGAAG |
| P3    | AGGATTCACTAGCATACAAA |
| P4    | GCATAAAGCCCACATCGAAG |
| Villin-Cre | ATTTGCGCTGATTACCGGTGTC |

**Table 1** Primers for determining wild-type (P3 and P4) and mutated (P1 and P2) allele of Phd3 and Villin-Cre

**FIGURE LEGENDS**

**Figure 1** PHD3 stabilizes p53.

(A) Overexpression of PHD3 increased p53. CCD841 and RKO cells were transfected with control or myc-PHD3 plasmid. After 24 h, the cells were harvested for western blot. (B) Overexpression of PHD3 had little effect on mRNA levels of *TP53*. The cells were treated as in A. (C) Knockdown of PHD3 decreased p53. The cells were transfected with control or PHD3 siRNA oligos. After 48 h, the cells were harvested. (D) RKO and CCD841 cells were transfected with control or siPHD3 oligos as indicated. After 48 h, the cells were harvested for determination of *TP53* mRNA level by qPCR. (E) CCD841 and RKO cells were transfected as indicated. After 24 h, CHX (10 µg/ml) was added to inhibit new protein synthesis and the cells were harvested at different time intervals. The right panel shows the relative p53 level at different time point. (F) CCD841 cells were transfected with control or siPHD3 oligos. After 48 h, the cells were treated with CHX (10 µg/ml) for different time intervals. The right panel shows the relative p53 level. (G) Proteins extracted from small intestinal and colonic epithelial cells of Phd3+/F and Phd3IEC-KO mice were subjected to western blot. The p53[DO-7] antibody was used to detect mouse p53. (H) The cells were transfected with control or myc-PHD3 plasmid as indicated. After 24 h, the cells were harvested. The western-blots were quantified using ImageJ and the results are from one experiment. ns, no significance.
Figure 2 PHD3 stabilizes p53 via MDM2.
(A) RKO cells were transfected with myc-PHD3 vector. After 24 h the cells were harvested and cellular proteins (350 µg) were used for immunoprecipitation assay. 50 µg of proteins were used in Input (14%). (B) The endogenous PHD3 and MDM2 bound each other. Cellular proteins (500 µg) of RKO and CCD841 cells were used to do the immunoprecipitation assay with 50 µg of the proteins as Input (10%). (C) PHD3 bound MDM2 directly. Equal amounts of bacterial lysates (containing His-PHD3) were incubated with the glutathione-Sepharose beads that had already captured GST-MDM2 or GST proteins. The beads were washed, and His-PHD3 retained on beads was determined by immunoblotting. (D) PHD3 inhibited p53-MDM2 interaction. RKO cells were transfected as indicated. After 24 h, the cells were treated with MG132 (10 µM) for 3 h, followed by immunoprecipitation experiment. (E) RKO cells were transfected as indicated. After 48 h, the cells were treated with MG132 (10 µM) for 2 h. The cells were then harvested for immunoprecipitation experiment. (F) The E. coli supernatant containing GST-MDM2 protein was incubated with beads at 4 °C for 2 h. The beads were washed and incubated at 4 °C with RKO cell lysates containing p53 and different amount of His-PHD3 protein. After 3 h, the beads were washed and subjected to immunoblotting. (G) Overexpression of PHD3 reduced ubiquitination of p53. RKO cells were transfected with myc-PHD3, HA-Ub and p53 vectors as indicated. After 24 h, MG132 (10 µM) was added and the cells were incubated for another 3 h, followed by immunoprecipitation. (H) Knockdown of PHD3 enhanced p53 ubiquitination. RKO cells were transfected as indicated. After 48 h, MG132 (10 µM) was added and the cells were incubated for another 3 h. The cells were harvested for immunoprecipitation. (I) In vitro p53 ubiquitination was performed as described in Methods. (J) Equal amounts of bacterial lysates containing His-PHD3 were incubated with the beads that had already captured GST, GST-MDM2 or mutated GST-MDM2. The beads were washed, and His-PHD3 retained on the beads was determined by immunoblotting. The asterisk indicates the band that the arrow pointed.

Figure 3 PHD3(H196A) induced p53.
(A) RKO cells were transfected with control or Myc-PHD3 vector as indicated. After 24 h, DMOG (2 mM) was added and the cells were incubated for another 4 h, followed by immunoblotting. (B) RKO and CCD841 cells were transfected with control, Myc-PHD3 or Myc-PHD3(H196A) vector. After 24 h, the cells were harvested for immunoblotting. (C, D) RKO cells were transfected with control, Myc-PHD3 or Myc-PHD3(H196A) vector. After 24 h, CHX (10 µg/ml) was added to inhibit new protein synthesis and the cells were harvested at different time intervals. The right panel shows the relative p53 level at different time point. Relative p53 was shown in D. (E) RKO cells were transfected with myc-PHD3(H196A) vector. After 24 h, the cells were harvested and immunoprecipitation was performed to determine the interaction between PHD3(H196A) and MDM2. (F) RKO cells were transfected with control or PHD3(H196A) vector. After 24 h, MG132 (10 µM) was added and the cells were incubated for another 3 h before harvesting the cells. Immunoprecipitation was performed to determine the effect of Myc-PHD3(H196A) on the interaction between p53 and MDM2. (G) RKO and CCD841 cells were transfected with control or PHD3 siRNA oligos as indicated. After 48 h, the cells were incubated in normal or 0.1% O2 for 10 h, followed by western blot. The western-blots were quantified using ImageJ and the results of quantification
are from one experiment.

**Figure 4 PHD3(H196A)-knockin did not affect the expression of p53.**

(A) RKO cells were transfected with Flag-tagged mouse PHD3 or PHD3(H196A). After 24 h, the cells were harvested for immunoblotting. (B) Structure of wild-type and mutant allele of Phd3 of mice. (C) Determination of the mutated Phd3 (left panel) and wild-type Phd3 (right panel) by PCR. Primers 1 and 2 are used for determining the mutated Phd3. Primers 3 and 4 are used for determining the wild-type Phd3. The homozygous Phd3(H196A) had a band of 1184 bp and wild-type Phd3 had a band of 656 bp. The primers for determining wild-type and mutated Phd3 were shown in Table 1. (D) The genotyping of Phd3(H196A)F/F and Phd3(H196A)IEC-KI mice. The primers for determining Villin-Cre was shown in Table 1. (E, F) The proteins extracted from small intestine and colon epithelial cells of Phd3(H196A)F/F and Phd3(H196A)IEC-KI mice were subjected to immunoblotting with ATF4 (E) and p53 (F) antibodies. The p53(DO-7) antibody was used to detect mouse p53.

**Figure 5 PHD3 induces NANOG.**

(A) RKO cells were transfected with control or HA-p53 vector. After 48 h, the expression of NANOG was determined by western blot and quantitative PCR. (B) RKO cells were transfected with control or p53 siRNA oligos as indicated. After 48 h, the expression of NANOG was determined. (C) RKO cells were transfected with control or myc-PHD3 vector. After 24 h, the mRNA and protein levels of NANOG were determined. (D) RKO cells were transfected with control or PHD3 siRNA oligos. After 48 h, the cells were harvested for determination of expression of NANOG. (E) RKO cells were transfected as indicated. After 48 h, the expression of NANOG was determined. (F) RKO cells were transfected as indicated. After 24 h, the cells were harvested for determination of mRNA and protein levels of NANOG. (G) RKO cells were transfected as indicated. After 24 h, the cells were harvested for qPCR. (H) RKO cells were transfected as indicated. After 48 h, the cells were harvested for qPCR. Western-blots were quantified using ImageJ. The quantification of western blot is from one experiment. ns, no significance. *, P < 0.05; ***, P < 0.001.

**Figure 6 PHD3 inhibits CSC properties of colon cancer cells.**

(A) RKO cells were transfected with control, Myc-PHD3 or Myc-PHD3(H196A) vector. The sphere formation was determined as described in Experimental Procedures. The right panel shows that number of spheres per well. (B) RKO cells were transfected with control, myc-PHD3 or myc-PHD3(H196A) vector. The mRNA levels of NANOG and LGR5 were determined as described in Methods. (C) RKO cells were transfected with control or PHD3 siRNA oligos. Sphere formation was determined. The right panel shows that number of spheres per well. (D) RKO cells were transfected with control or PHD3 siRNA oligos as indicated. The mRNA levels of NANOG and LGR5 were determined. (E) RKO cells were transfected with Myc-PHD3 in the absence or presence of p53 siRNA oligos. The right panel shows that number of spheres per well. (F) RKO cells were transfected with Myc-PHD3(H196A) vector with or without p53 siRNA oligos. The right panel shows that number of spheres per well. ns, no significance. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 1

A

CCD841 RKO
Myc-PHD3 – + – +
p53
IB: Myc
β-actin
p53/actin 1 3.2 1 1.8

B

CCD841 RKO
Relative TP53 mRNA level
Myc-PHD3 – + – +
p53
PHD3
β-actin
p53/actin 1 0.5 1 0.4

C

CCD841 RKO
siPHD3 – 1 2 – 1 2
p53
PHD3
β-actin
p53/actin 0 0.0

D

RKO CCD841
siPHD3 – 1 2 – 1 2
Relative TP53 mRNA level

E

Control Myc-PHD3
Hour 0 2 4 6 8 0 2 4 6 8
p53
IB: Myc
β-actin
p53
IB: Myc
β-actin

F

RKO CCD841
Min 0 30 60 90
p53
PHD3
β-actin
p53
PHD3
β-actin

G

Small intestine Colon
Myc-PHD3 Phd3FF Phd3EC-KO
p53
IB: Myc
β-actin
p53
IB: Myc
β-actin
p53

H

MCF7 Hela ACHN Caki-1 HCT116 293T
Myc-PHD3 – + – + – + – +
p53
IB: Myc
β-actin
p53/actin 1 1.7 1 1.7 1 1.9 1 1.5 1 0.9 1 0.9
Figure 2

A

| Myc-PHD3 | + | + |
|-----------|---|---|
| IP        | IgG Myc |
| MDM2      |   |   |
| IB: Myc   | 55| 34|
| β-actin   | 43 |

B

| RKO       | IP | IgG PHD3 |
|-----------|----|----------|
| MDM2      |   | PHD3     |
| IB: Myc   | 55| 34       |
| β-actin   | 43 |

C

| GST-MDM2  | Input |
|-----------|-------|
| GST-MDM2  |       |
| GST-MDM2  |       |
| GST-MDM2  |       |

D

| MDM2      | + | + |
|-----------|---|---|
| IP        | p53 |
| MDM2      |   |   |
| IB: Myc   | 55| 34|
| p53       | 43 |

E

| siPHD3    | IP | p53 |
|-----------|----|-----|
| MDM2      |   |   |
| p53       | 55 |

F

| GST-MDM2  | + | + |
|-----------|---|---|
| His-PHD3  |   |   |
| p53       |   |   |
| IB: His   | 55| 34|
| IB: GST   | 130|
| β-actin   | 43 |

G

| MG132     | + | + |
|-----------|---|---|
| HA-Ub     |   |   |
| His-p53   |   |   |
| IB: Myc   | 55| 34|
| p53       | 43 |

H

| E1/E2/ATP | + | + |
|-----------|---|---|
| His-p53   |   |   |
| GST-MDM2  |   |   |
| GST-MDM2  |   |   |
| MDM2      |   |   |

I

| His-PHD3  | + | + |
|-----------|---|---|
| GST-MDM2  |   |   |
| GST-MDM2  |   |   |
| GST-MDM2  |   |   |

J

| GST-MDM2  | + | + |
|-----------|---|---|
| GST-MDM2  |   |   |
| GST-MDM2  |   |   |
| GST-MDM2  |   |   |

16
Figure 3

A

| Treatment | DMOG | Myc-PHD3 | Myc-PHD3 (H196A) |
|-----------|------|----------|------------------|
| HIF1α     | -    | -        | -                |
| p53       | -    | -        | +                |
| IB:Myc    | -    | +        | +                |
| β-actin   | -    | -        | +                |
| p53/actin | 1    | 1.9      | 2                |

B

| Cell Line | CCD841 | RKO |
|-----------|--------|-----|
| Myc-PHD3  |        |     |
| Myc-PHD3 (H196A) | |     |
| p53       | Con | WT | H196A |
| IB:Myc    | - 55| - 34| - 43  |
| β-actin   | - 55| - 34| - 43  |
| p53/actin | 1   | 2  | 2.2   |

C

| Condition | Control | Myc-PHD3 | Myc-PHD3 (H196A) |
|-----------|---------|----------|------------------|
| CHX       | +       | +        | +                |
| Time (h)  | 0 2 4 6| 0 2 4 6  | 0 2 4 6          |
| p53       |        | -        | -                |
| IB:Myc    | - 55   | - 34     | - 43             |
| β-actin   | - 55   | - 34     | - 43             |
| p53/actin | 1 2.3  | 2.2 1.8  | 1 1.8 0.9        |

D

Graph showing relative p53 level over time (h) for Control, Myc-PHD3, and Myc-PHD3 (H196A).

E

| Input | IP   | MDM2 | Myc-PHD3 (H196A) |
|-------|------|------|------------------|
|       | IgG  | p53  |                  |
|       | MDM2 | p53  |                  |
| MDM2  | p53  | p53  |                  |
| β-actin| p53  | p53  |                  |
| Input | IP   | p53  |                  |

F

| Input | IP | MDM2 | p53 |
|-------|----|------|-----|
|       | IgG| p53  |     |
|       | MDM2| p53 |     |
| MDM2  | p53| p53 |     |
| β-actin| p53| p53 |     |
| Input | IP | p53  |     |

G

| Cell Line | CCD841 | RKO |
|-----------|--------|-----|
| O2        | 21%    | 21% |
| siPHD3    | 0.1%   | 0.1%|
| p53       | - -    | - - |
| PHD3      | - -    | - - |
| HIF1α     | - -    | - - |
| HIF2α     | - -    | - - |
| β-actin   | - 17  | - 17|
| p53/actin | 1 2.7 | 1 1.7|

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Figure 4

A. Mouse

Control
Flag-PhD3
Flag-PhD3(H196A)

HIF1α
HIF2α
p53
Flag
β-actin

B. Wild-type allele

Mutant allele

Homology arm
Primer location
Loxp
Loxp2272
FRT
Mutated site (H196A)

C. Marker

Phd3 mutant

Primers P1, P2

1184 bp

Primers P3, P4

656 bp

D. Primers

Phd3(H196A)fl/fl
Phd3(H196A)fl/cre

Primers P1, P2

1184 bp

Primers P3, P4

PHD3

p53

β-actin

E. ATF4

Small intestine

β-actin

Colon

ATF4

β-actin

F. PHD3

Small intestine

β-actin

Colon

β-actin

43
Figure 5

A

HA-p53 − +

Relative NANOG mRNA level

HA-p53 − +

B

si-p53 − 1 2

Relative NANOG mRNA level

si-p53 − 1 2

C

Myc-PHD3 − +

Relative NANOG mRNA level

Myc-PHD3 − +

D

siPHD3 − 1 2

Relative NANOG mRNA level

siPHD3 − 1 2

E

Myc-PHD3 − + − − − +

Relative NANOG mRNA level

Myc-PHD3 − + − − − +

F

Myc-PHD3

Relative NANOG mRNA level

Myc-PHD3

G

Control  myc-PHD3

Relative mRNA level

Control  myc-PHD3

H

Control  siPHD3-1  siPHD3-2

Relative mRNA level

siPHD3-1  siPHD3-2
Figure 6

A. pcDNA3.1, Myc-PHD3, Myc-PHD3(H196A)

B. Relative mRNA level

C. Control, siPHD3-1, siPHD3-2

D. Relative mRNA level

E. Control, Myc-PHD3, Myc-PHD3 + si-p53-1, Myc-PHD3 + si-p53-2

F. Control, Myc-PHD3(H196A), Myc-PHD3(H196A) + si-p53-1, Myc-PHD3(H196A) + si-p53-2

Scale bar=50μm
Prolyl hydroxylase 3 stabilizes the p53 tumor suppressor by inhibiting the p53–MDM2 interaction in a hydroxylase-independent manner
Yiming Xu, Qiang Gao, Yaqian Xue, Xiuxiu Li, Liang Xu, Chenwei Li, Yanqing Qin and Jing Fang

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