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# Label-free Interactome Analysis Revealed an Essential Role of CUL3-KEAP1 Complex in Mediating the Ubiquitination and Degradation of PHD2

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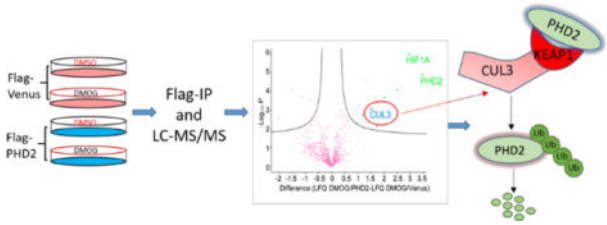
## Abstract

Prolyl Hydroxylase Domain-Containing Protein 2 (PHD2/EGLN1) is a key regulatory enzyme that plays a fundamental role in the cellular hypoxic response pathway, mediating proline hydroxylation-dependent protein degradation of selected target proteins. However, the regulation of PHD2 homeostasis at the protein level is not well understood. Here, we performed label-free quantitative interactome analysis through immunoprecipitation coupled with mass spectrometry analysis. To minimize the side effects caused by ectopic overexpression, in HeLa cells we stably overexpressed Flag-tagged PHD2 while suppressing the endogenous PHD2 by using an shRNA targeting its 3' UTR region. We identified and validated Cullin 3 as a novel PHD2 interactor in vivo. Through candidate screening, we further identified CUL3-KEAP1 E3 ubiquitin ligase complex as the major enzyme that regulates PHD2 degradation. Overexpression of either CUL3, KEAP1 or both significantly increases PHD2 ubiquitination and reduces PHD2 protein abundance. The knockdown of CUL3 or KEAP1 decreased PHD2 ubiquitination and inhibited PHD2 degradation. Accordingly, loss of the CUL3-KEAP1 complex under hypoxia promoted PHD2 stabilization and led to significantly reduced abundance of the PHD2 target, hypoxia-inducible factor 1A (HIF1A). Thus, CUL3-KEAP1 is an essential pathway that regulates PHD2 ubiquitination and degradation in cells.

## Graphical Abstract

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SUPPORTING INFORMATION:

The following supporting information is available free of charge at ACS website http://pubs.acs.org

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository[51] with the data set identifier PXD014757.

**Keywords**

PHD2/EGLN1; Cul3; KEAP1; ubiquitination; degradation; hypoxia; LCMS

## INTRODUCTION

In comparison with normal tissues, uncontrolled cell proliferation is one of the fundamental traits of human tumors[1]. During tumorigenesis, these fast-growing cancer cells require much more nutrient and oxygen than the normal vasculature can supply. Consequently, the regional hypoxia environment was formed around the tumor, and as a response to hypoxia, the cells were pushed to enhance the expression of factors promoting angiogenesis and new blood vessels were formed. Though these tumor-formed vessels not always function normally as vessels in normal tissues, they could maintain the consecutive growth of tumor[2]. In addition to angiogenesis, hypoxia plays important roles in other aspects of tumorigenesis, including but not limited to glycolysis, immune evasion, tissue invasion and metastasis, genomic instability and cancer stem cell[3]. It is not only a disease-associated condition but also related to normal tissue development, such as placental development and fetal programming[4].

Hypoxia response in animal cells is mediated by a group of transcriptional factors called hypoxia-inducible factors (HIF alphas), including HIF1A and HIF2A[5]. These HIFs regulate transcription through forming dimers between each α subunit and the HIF-1β subunit to directly bind to the promoter of their targets genes[6]. Under normoxia and with $Fe^{2+}$, 2-oxoglutarate as co-factors, two prolines (Pro402 and Pro564) of HIF1α are hydroxylated by a family of enzymes called proly-4-hydroxylase domain proteins (PHDs)[7–9]. Hydroxylated HIF1α is recognized and bound by the von Hippel Lindau (VHL) E3 ubiquitin ligase complex and degraded by the proteasome[10–12]. There are mainly three PHDs identified in human cells, namely PHD1 (EGLN2), PHD2 (EGLN1) and PHD3 (EGLN3), of which PHD2 is the main hydroxylase of HIF1A[13].

PHD2 plays an important role in tumorigenesis and hypoxia-related pathological processes, such as ischemia, wound healing, asthma and obesity[7, 13]. Most recent studies identified PHD2 as a potential therapeutic target in clear cell ovarian cancer patients and played roles in the regulation of T cell and immune activity[14, 15]. Although PHD2 is known to be the major hydroxylase that regulates HIF1A, other targets of PHD2 have been recently identified including NDRG3, AKT, and BRD4[16–18]. However, the regulation of PHD2 homeostasis and protein abundance has not been well studied.

To systematically study the regulation of PHD2 in cells, we performed a quantitative interactome study using label-free quantification in HeLa cells. Cell lines stably expressing Flag-tagged PHD2 and control vectors were generated. Immunoprecipitation and mass spectrometry analysis were performed to identify potentially new PHD2 interacting proteins. Through subsequent validation and functional studies, we determined an essential role for CUL3-KEAP1 E3 ubiquitin ligase to mediate the ubiquitination and degradation of PHD2.

## MATERIALS AND METHODS

### Cell lines and reagents

HEK293T, HEK293FT, and HeLa cells were all cultured in Dulbecco's Modified Eagle's medium (DMEM) (11965092, Gibco), supplemented with 10% FBS (F8067, Sigma) and 1% penicillin-streptomycin (Corning). The cells were maintained in a 37 °C incubator supplied with 5% $CO_2$. For hypoxia treatment, the cells were cultured in a hypoxia chamber (BioSperix) in the regular 37 °C incubator supplied with 1%$O_2$/5% $CO_2$/94% $N_2$. The main chemicals used in this study included DMOG (A4506, APEXBIO), puromycin (EMD Millipore), blasticidin (R21001, Gibco).

### Plasmids and transfection

pcDNA3-myc-CUL3 was a gift from Yue Xiong (Addgene plasmid # 19893)[19], Flag-KEAP1 was a gift from Qing Zhong (Addgene plasmid #28023)[20], FLAG-PHD2-pLenti6 and FLAG-Venus-pLenti6 were gifts from William Kaelin (Addgene plasmid #36949 and #36948)[21], HA-PHD2-pcDNA3 was a gift from William Kaelin (Addgene plasmid # 18963)[22]. Control shRNA plasmid and shRNA plasmid of PHD2 (TRCN0000001042) were purchased from the University of Minnesota Genomics Center. Regular transfection in HEK293T cells was performed with poly(ethyleneimine) (Sigma) while X-tremeGENE™ HP DNA Transfection Reagent (Sigma) was used for lentivirus packaging in HEK293FT cells.

### siRNAs and transfection

MISSION® siRNA Universal Negative Control #1 (Sigma) was used as the control siRNA. CUL3 siRNA was synthesized by Dharmacon with the sequence 5'-AACAACUUUCUUCAAACGCUA-3', which has been validated in a previous publication[23]. KEAP1 siRNA (SI03246439) was ordered from Qiagen and also validated previously[24]. siRNA transfection was performed with DharmaFECT 1 Transfection Reagent (Dharmacon) following the manufacturer's instructions.

### Establishment of stable PHD2-expression HeLa cell line and control HeLa cell line

Lentivirus expressing Flag-PHD2 or PHD2 shRNA was produced in HEK293FT cell line by co-transfection with FLAG-PHD2-pLenti6/ pCMV-dR8.2/ pCMV-VSV-G or shPHD2/ psPAX2/pMG2.D. Twenty-four hours after transfection, the medium was replaced with fresh medium, and after another 24 hours, the lentivirus was harvested by filtering with a 0.45 μm syringe filter. HeLa cells growing in 60 mm dishes were firstly infected with 1 ml FLAG-PHD2 lentivirus supernatant in the presence of 8 μg/ml polybrene for 12 hours. Then the cells were replenished with fresh medium, the blasticidin was added into the medium at the final concentration of 5 μg/ml. Cells were further cultured until becoming resistant to blasticidin and were further infected with lentivirus containing PHD2 shRNA. Twenty-four hours after the second infection, the cells started to be cultured in medium containing 2 μg/ml blasticidin and 1 μg/ml puromycin. The flag-venus control cell line was generated in a similar way as Flag-PHD2 cell line using lentivirus expressing Flag-Venus-pLenti6. The control cell line was further transfected with control shRNA plasmid.

### Immunoprecipitation (IP) and Western blotting (WB)

Cells were harvested by washing with cold PBS buffer and lysed in cell lysis buffer (150 mM NaCl, 0.5% NP-40, 50 mM Tris-HCL, 10% glycerol, pH 7.5, protease inhibitor cocktail (Roche)) on ice for at least 15 minutes. Then the cell lysates were collected and transferred into a pre-cold 1.5 ml Eppendorf tube and kept on ice for another 10 minutes. Finally, the cell lysates were clarified by centrifugation at 4 °C for 10 minutes. 10% of the supernatant was set aside as the input and the rest was incubated with either anti-FLAG M2 affinity gel (A2220, Sigma) or Anti-HA Magnetic Beads (Thermo Fisher) for 6 hours. After incubation, the M2 gel or the Anti-HA Beads was washed with cell lysis buffer containing 300 mM NaCl for three times. The M2 gel was then eluted with 3X Flag peptide (A6001, ApexBio) while Anti-HA Beads was eluted by boiling in 1X SDS-PAGE loading buffer. The M2 gel eluate was further mixed with 4X SDS-PAGE loading buffer and boiled. The proteins were separated in homemade SDS-PAGE gel and transferred onto PVDF membrane. Blocking was done with 5% skim milk (BD) in TBST (TBS+0.1% Tween-20). After blocking, the membrane was incubated with primary antibody overnight and washed with TBST for at least 3 times, then incubated with HRP-linked secondary antibody (Cell Signaling Technology, #7074 and 7076) for more than 2 hours and washed with TBST. The signal was developed with Luminata Crescendo Western HRP Substrate（WBLUR0500, Millipore）and captured with X-ray film. Primary antibodies used in the current study included Anti-Flag (F3165, Sigma), Anti-HA, Anti-myc, HIF1α (04–1006, Millipore), Ubiquitin (MAB1510, Millipore), ubiquitin (MAB1510, Millipore), α-Tubulin (T6199, Sigma).

### Immunofluorescence staining

293T cells were seeded on coverslips in a 24-well plate. 24 hours later, cells were co-transfected with HA-PHD2 and Flag-KEAP1 plasmids. 24 hours after transfection, cells were washed with PBS twice, fixed with 4% paraformaldehyde for 30 min at room temperature (RT), permeabilized with 0.15% Triton X-100 for 15 min at RT, and blocked with 2% BSA for 30 min. Cells were then incubated simultaneously with FLAG-tag antibody (Rabbit mAb, #14793, Cell Signaling Technology) and HA-tag antibody (Mouse mAb, BioLegend) at 4 °C overnight. After washing with PBS 3 times, cells were incubated with FITC-labeled goat anti-rabbit IgG and TRITC-labeled goat anti-mouse IgG at RT for 2 h. Nuclei were stained with DAPI, coverslips were mounted with ProLong™ Diamond Antifade Mountant (Thermo Fisher), and photographs were taken with a Deltavision PersonelDV microscope (Applied Precision).

### Mass spectrometry (MS) sample preparation and data analysis

HeLa cells expressing Flag-Venus or Flag-PHD2 were treated with 2 mM DMOG or the equal volume DMSO for 4 hours before harvesting for Flag-IP. For each sample, one-third of the eluates were used for WB to check the IP efficiency and the rest were resolved in SDS-PAGE. All bands in each lane were cut out and subject to reduction/alkylation with tris(2-carboxyethyl)phosphine (TCEP) and iodoacetamide (IA), followed by in-gel trypsin digestion as previously described[25, 26]. Peptides were desalted with self-packed C18 Stage Tip[27]. For LC-MS/MS analysis, the peptides were dissolved in HPLC buffer A (0.1% formic acid) and loaded onto a capillary HPLC column (25 cm in length, 360 μm O.D. and 75 μm

I.D.) packed with ReproSil-Pur Basic C18 resin (1.9 μm particle size and 100 Å pore size) with a Proxeon Easy nLC 1000 Nano-UPLC system connected to an Orbitrap Fusion mass spectrometer (ThermoFisher). The peptides were eluted off the column with a one-hour gradient of 7% to 32% HPLC buffer B (0.1% formic acid in acetonitrile). The full MS was acquired with a mass range of 300–1500 m/z at 60,000 resolution (200 m/z). Data-dependent MS/MS spectra were acquired in the ion trap and in a top-speed mode (3 seconds for each cycle) with an isolation window of 1.6 m/z and higher energy-collision dissociation (HCD) (35% collision energy). The MS raw data were analyzed with MaxQuant software version 1.5.2.8[28] and searched against the UniProt human database (73928 sequences, downloaded on 3/15/2019). Protein quantification data from MaxQuant was further analyzed with Perseus (version 1.6.1.1)[29]. Briefly, proteins were first filtered to remove reversed decoy sequence and potential contaminants. Proteins quantified with LFQ in biological replicates were log10 transformed and the missing values were replaced based on the normal distribution in the total matrix. Two-sample Student's t-test was used to determine the statistical significance of the quantitative difference between groups with permutation-based FDR (false discovery rate) of 0.05 and the results were displayed in the volcano plot.

### *In vivo* ubiquitination assay

Twenty-four hours after siRNAs transfection, Flag-PHD2-expressing HeLa cells were treated with 5 μM MG132 for 24 hours. The cells were then harvested by trypsinization, washed with PBS, pelleted and resuspended in SDS- lysis buffer (150 mM NaCl, 1% SDS, 50 mM Tris-HCl, pH7.5) and boiled for 10 minutes. The cell lysate was cooled to room temperature and diluted with dilution buffer (150 mM NaCl, 50 mM Tris-HCL, pH7.5) to reduce the concentration of SDS to 0.2%, sonicated thoroughly, and then centrifuged at 21000g. Ten percent of the supernatant was reserved as the input and the rest was incubated with Flag M2 beads overnight at 4 °C. Finally, the M2 beads were washed with wash buffer (150 mM NaCl, 0.2% SDS, 50 mM Tris-HCl, pH7.5) for 4 times and eluted with 1X SDS-PAGA sample loading buffer by boiling for 6 minutes. The input was mixed with a 4X loading buffer and boiled the same way. The samples were analyzed with Western blotting.

### Quantitative real-time PCR (qRT-PCR)

Total RNA extraction was done with TRIzol™ Reagent (Invitrogen) manually. For each sample, 2 μg raw RNA was reverse-transcribed into cDNA with M-MLV Reverse Transcriptase (Promega) in a 25 μl reaction system. The reverse-transcription product was further diluted to 150 μl and 2 μl of which was used as a template for each qRT-PCR reaction. The reaction was performed on CFX96 Touch™ Real-Time PCR Detection System (BioRad) and the reagent was Luna® Universal qPCR Master Mix (New England BioLabs, M3003). For relative quantification, the expression level of each gene was normalized to the expression of GADPH. The primers used in this paper included: *GAPDH*, 5′-GGTGGTCTCCTCTGACTTCAACA-3′ and 5′-GTTGCTGTAGCCAAATTCGTTGT-3′; *PHD2*, 5′-CCCAACGGGCAGACGAAGCC′ and 5′- CTTCCCGGTGTCGTGCAGGG-3′; *CUL3,* 5′-GCCTTTCCGGTGCGAGAAGA-3′ and 5′-TTGTTGTACATACACACGGTCCAT-3′*; KEAP1,* 5′-GTCCCCTACAGCCAAGGTCC-3′ and 5′-CTCAGTGGAGGCGTACATCA-3′.

# RESULTS

## Interactome analysis identified new PHD2 interacting proteins

In order to identify new PHD2 interacting proteins and potential regulatory enzymes, we established a pair of stable HeLa cell lines, expressing either Flag-tagged PHD2 (Flag-PHD2) or Flag-tagged Venus, a variant of exogenous yellow fluorescent protein (YFP) that was previously used as a control[21]. The protein expression in the stable cell lines were confirmed with Western blotting both with Flag-tag antibody and PHD2 antibody (Fig.S1A) To further modulate the expression level of PHD2, we knocked down the endogenously expressed PHD2 in the Hela cell line that stably expressed Flag-PHD2 by stably expressing a short hairpin RNA (shPHD2) that targeted the 3' UTR region of *PHD2* mRNA, while the Flag-Venus cell line was transfected with control shRNA. We tested the expression level of PHD2 in these two new stable cell lines using Western blotting. Our data showed that the endogenous PHD2 protein was efficiently reduced (Fig.S1B).

We applied the cell lines for label-free, quantitative interactome analysis outlined in Fig. 1A. To validate the specificity of our immunoaffinity purification workflow, we performed the preliminary analysis with Western blotting to detect HIF1A, a well-established PHD2 target, and an interacting protein. Analysis of immunoprecipitated proteins confirmed the interaction between PHD2 and HIF1A through Western blotting and further showed that the interaction between these two proteins was apparently enhanced after DMOG treatment following a substrate-trapping mechanism as previously described[30], suggesting the stable cell lines we established were effective to identify PHD2 interacting proteins (Fig. 1B). For quantitative interactome analysis, we treated the stable cells either with DMSO or prolyl-hydroxylase inhibitor dimethyloxalylglycine (DMOG) to stabilize the interactions between PHD2 and some of its binding partners. Immunoprecipitation with anti-Flag agarose beads was performed in parallel between the control cells and the cells stably expressing Flag-PHD2. Each set of experiments was analyzed with biological triplicates for label-free quantification and statistical analysis. Data were processed by MaxQuant software for protein identification and the label-free quantification (LFQ)[28]. The data produced from MaxQuant was further analyzed by Perseus software[29]. Correlation analysis showed excellent reproducibility among biological triplicates (Fig. S2).

Using Student's t-test and permutation-based FDR analysis, we quantified the interacting proteins between control and PHD2-expressing cells that were treated with either DMSO or DMOG (Fig. 1C and D, Table S1 and S2). Immunoprecipitation under DMOG treatment indeed yielded more identifications of PHD2 interacting proteins. From the two treatment conditions, we confidently identified 21 PHD2 interacting proteins, out of which 8 proteins have been previously known to interact with PHD2 including HIF1A, HIF2A, FKBP5, and HSP90AB1[31–34]. We further performed bioinformatics analysis to identify a significantly enriched domain or protein complexes among the PHD2 interacting proteins (Table S3). Our analysis revealed known protein complexes that interact with PHD2 including VHL-HIF1A complex, HIF1A-ARNT complex and HIF1A-EGLN3 complex. Interestingly, we noticed that a number of Cullin3 (CUL3)-involved ubiquitin-E3 ligase complexes were also enriched in our dataset. CUL3 is a member of the Cullin-RING ubiquitin ligases family and mediates

the ubiquitination and degradation of many proteins involved in human diseases including Aurora B, NRF2 and pancreatic duodenal homeobox 1(Pdx1)[35]. We hypothesized that a CUL3 ubiquitin ligase complex may be involved in mediating the ubiquitination and degradation of PHD2.

## PHD2 interacts with CUL3 and KEAP1

Because the regulation of PHD2 at the protein level has not been reported, we aimed first to validate the interaction between CUL3 and PHD2. We performed co-immunoprecipitation experiments expressing myc-tagged CUL3 and HA-tagged PHD2 in 293T cells. Western blotting following HA-IP confirmed the interaction between CUL3 and PHD2 and we found that this interaction was independent of DMOG treatment (Fig. 2A). The interactions between CUL3 and its substrates are typically mediated by substrate-specific adaptor proteins containing the BTB/POZ domain[36]. To identify potential adaptor protein that is involved in the interaction, we performed a candidate screening and identified Kelch-like ECH-associated protein 1 (KEAP1), a well-characterized CUL3 adaptor protein containing a Kelch and a BTB domain[37], that was involved in this process. The interaction between KEAP1 and PHD2 was first validated with co-immunoprecipitation. Using both 293T and HeLa cells, we overexpressed HA-PHD2 or the empty vector and performed immunoprecipitation followed by Western blotting to detect endogenous KEAP1 (Fig.2B and Fig. S4A). For reciprocal IP, we overexpressed Flag-KEAP1 and performed immunoprecipitation/Western blotting to detect endogenous PHD2 (Fig.2C and Fig. S4B). Our data convincingly demonstrated the interaction between KEAP1 and PHD2 in vivo.

To further identify the potential domains that mediate KEAP1-PHD2 interaction, we constructed a panel of GFP-tagged KEAP1 truncation plasmids (Fig 2D). HEK293T cells were co-transfected with HA-PHD2 and individual KEAP1 plasmids. Immunoprecipitation of GFP-tagged KEAP1 isoforms followed by Western blotting analysis with anti-HA antibody showed that KEAP1 interacted with PHD2 through the Kelch repeats (Fig.2D), which agreed well with the current knowledge on the domain specificity of KEAP1 when interacting with its ubiquitination substrates[38].

Finally, to provide more evidence for the interaction between PHD2 and KEAP1 we analyzed the distribution of these two proteins in 293T cells by using immunofluorescence microscopy. It showed that ectopically expressed HA-PHD2 and Flag-KEAP1 colocalized with each mainly in the cytoplasm (Fig. 2E).

## CUL3 and KEAP1 regulate the ubiquitination of PHD2

After confirming the interaction between CUL3-KEAP1 and PHD2, we aimed to test whether CUL3-KEAP1 could regulate the ubiquitination and degradation of PHD2. As the mechanism of PHD2 degradation is not yet known, we first performed a protein degradation assay. Our data showed that when protein synthesis was blocked, the inhibition of proteasome activity by MG132 inhibited PHD2 degradation, suggesting that PHD2 degradation was mediated through proteasome activity in cells (Fig.3A). To determine if CUL3 and KEAP1 mediate PHD2 ubiquitination, we performed in vivo ubiquitination assay with immunoaffinity purification and Western blotting. We first overexpressed CUL3 and

KEAP1 in 293T cells stably expressing PHD2. Flag-tagged PHD2 was immunoprecipitated and then analyzed by Western blotting with the anti-Ub antibody. Our data showed that overexpression of either CUL3 or KEAP1 individually increased the ubiquitination level of PHD2 while overexpression of both proteins significantly increased PHD2 ubiquitination (Fig. 3B). On the other hand, knockdown of either CUL3 or KEAP1 with siRNAs reduced the ubiquitination of PHD2 in HeLa cells, while concomitant knockdown of both genes significantly reduced the PHD2 ubiquitination (Fig.3C). Accordingly, overexpression of either CUL3, KEAP1 or both reduced the endogenous protein level of PHD2, while knockdown of CUL3, KEAP1 or both increased PHD2 protein abundance in HeLa cells (Fig.3D and E). In addition, overexpression of CUL3 together with KEAP1 could promote the turnover of HA-PHD2 in HeLa cells (Fig.3G)

To examine the possibility that the knockdown of CUL3 and KEAP1 may regulate PHD2 protein abundance through the regulation of its mRNA level, we performed real-time qPCR analysis. We found that KEAP1 knockdown alone indeed decreased the level of PHD2 mRNA slightly, but neither the knockdown of CUL3 nor the concomitant knockdown of CUL3 and KEAP1 affected the mRNA level of PHD2. Taken together, these data suggested that the CUL3-KEAP1 complex mainly regulates PHD2 protein abundance through poly-ubiquitination and proteasome-mediated protein degradation (Fig.3F).

### Knockdown of CUL3-KEAP1 inhibits the induction of HIF1A under hypoxia

To determine the physiological effects of CUL3-KEAP1-mediated degradation of PHD2, we aimed to test if the regulation of PHD2 by CUL3-KEAP1 complex may affect the abundance of PHD2 target HIF1A. As PHD2-mediated hydroxylation and ubiquitination of HIF1A lead to its rapid degradation, we hypothesized that the CUL3-KEAP1 complex may regulate HIF1A abundance through the regulation of PHD2 level. To this end, HeLa cells were treated with siRNAs to target CUL3, KEAP1 or both under normoxia and hypoxia. Our data showed that either the knock-down of KEAP1 alone or double knockdown of both CUL3 and KEAP1 increased PHD2 abundance and inhibit the hypoxia-induced stabilization of HIF1A (Fig.4A). The knock-down of CUL3 alone, however, could not inhibit the induction of HIF1A despite the increase of PHD2 abundance. These data suggest that CUL3-KEAP1-mediated poly-ubiquitination and degradation of PHD2 regulates HIF1A level in cells, but alternative CUL3-dependent mechanisms may also regulate HIF1A protein abundance independent of PHD2 and proline hydroxylation pathways.

## DISCUSSION

As a widespread protein post-translational modification, proline hydroxylation has been identified and characterized in many proteins, playing important roles in human disease[39–42]. As an irreversible modification, proline hydroxylation is mediated by prolyl hydroxylase enzymes including P4H and PHD families[42–44]. HIF1A is a well-known proline hydroxylation substrate and the modification of HIF1A by PHD2 tightly regulates the protein level of HIF1A in the cells by regulating its ubiquitination and proteasome-dependent protein degradation[13]. Despite the functional significance, how PHD2 was regulated at the protein level was not fully understood. A recent study suggested that

peptidyl-prolyl cis/trans isomerase FK506-binding protein 38 (FKBP38) was able to regulate the stability of PHD2, but the detailed mechanism was not clear[45]. In this study, by combining quantitative interactome analysis and biochemical assays, we identified CUL3 and KEAP1 as novel PHD2 interacting proteins and further demonstrated that CUL3-KEAP1 mediates PHD2 ubiquitination and proteasome-dependent protein degradation (Fig. 4B).

Cullin E3 ubiquitin ligase family plays important role in oxygen-sensing and hypoxia response. Cullin2 mediates the ubiquitination and degradation of HIF1A through pVHL protein in an oxygen-dependent manner[36]. More recently, Cullin3 has been reported to mediate the ubiquitination and degradation of PHD1 (EGLN2) with an adaptor protein called Speckle Type BTB/POZ protein (SPOP)[46]. Despite the sequence similarities, SPOP was not found to mediate the degradation of PHD2 (EGLN1). Interestingly, in this study, we identified that CUL3 mediates the poly-ubiquitination and degradation of PHD2 through another adaptor protein, KEAP1.

CUL3-KEAP1 ubiquitin ligase is well-known for its regulation on NF-E2-Related Factor 2 (NRF2), which plays very important roles in anti-oxidative and anti-inflammatory response[47, 48]. They also mediate the downregulation of NF-κB signaling by targeting IKKβ[38]. Somatic mutations on KEAP1 has been known as driver cancer mutations that prevent NRF2 repression[49, 50]. Here we demonstrated that CUL3-KEAP1 regulated the abundance of PHD2 through poly-ubiquitination and degradation. The loss of CUL3-KEAP1 complex increased PHD2 protein abundance and led to a significantly reduced level of HIF1A under hypoxia (Fig. 4B). Given the importance of PHD2 in regulating hypoxia response, our findings integrated the role of KEAP1 in anti-oxidation and the regulation of hypoxia response pathways. Future studies are necessary to systematically elucidate the overall impact on the cellular hypoxia-response during this process and the regulation of these pathways by the CUL3-KEAP1 complex in cancer and inflammation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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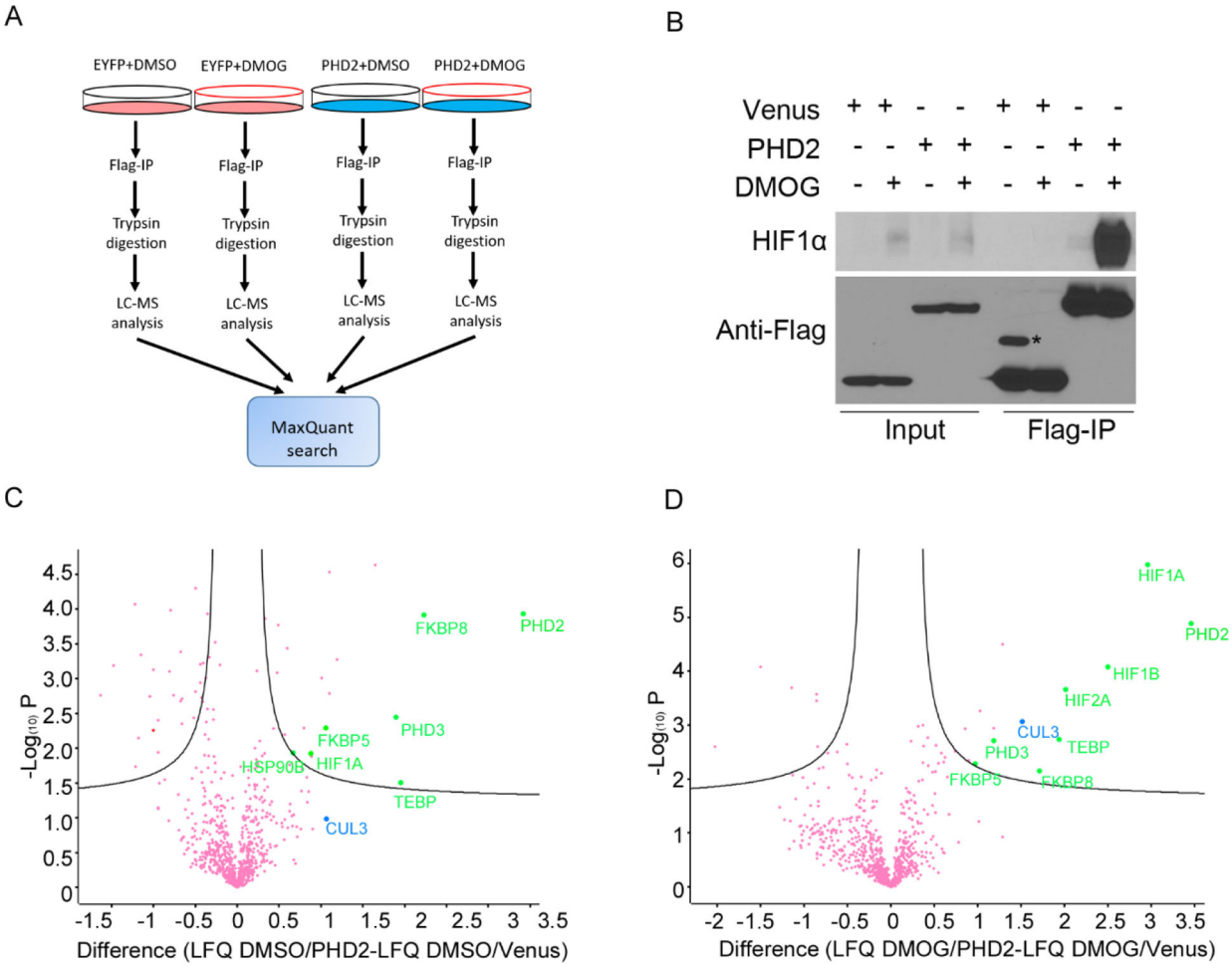


**Figure 1.**

Interactome study of PHD2 with label-free quantitative proteomics analysis in HeLa cells. (A) A schematic representation of a sample analysis workflow. (B) Interaction between PHD2 and HIF1α in the HeLa cell line that stably expresses Flag-PHD2 and shPHD2. The indicated cells were treated either with DMSO or 2mM DMOG for 4 hours and then prepared for IP with Flag-M2 agarose and WB with the indicated antibodies.”*” indicates unspecific band. Volcano plot analysis showing the PHD2 interacting proteins identified from HeLa cells either treated with DMSO (C) or DMOG (D). Known PHD2 binding proteins were shown in red and new interacting proteins were shown in green.

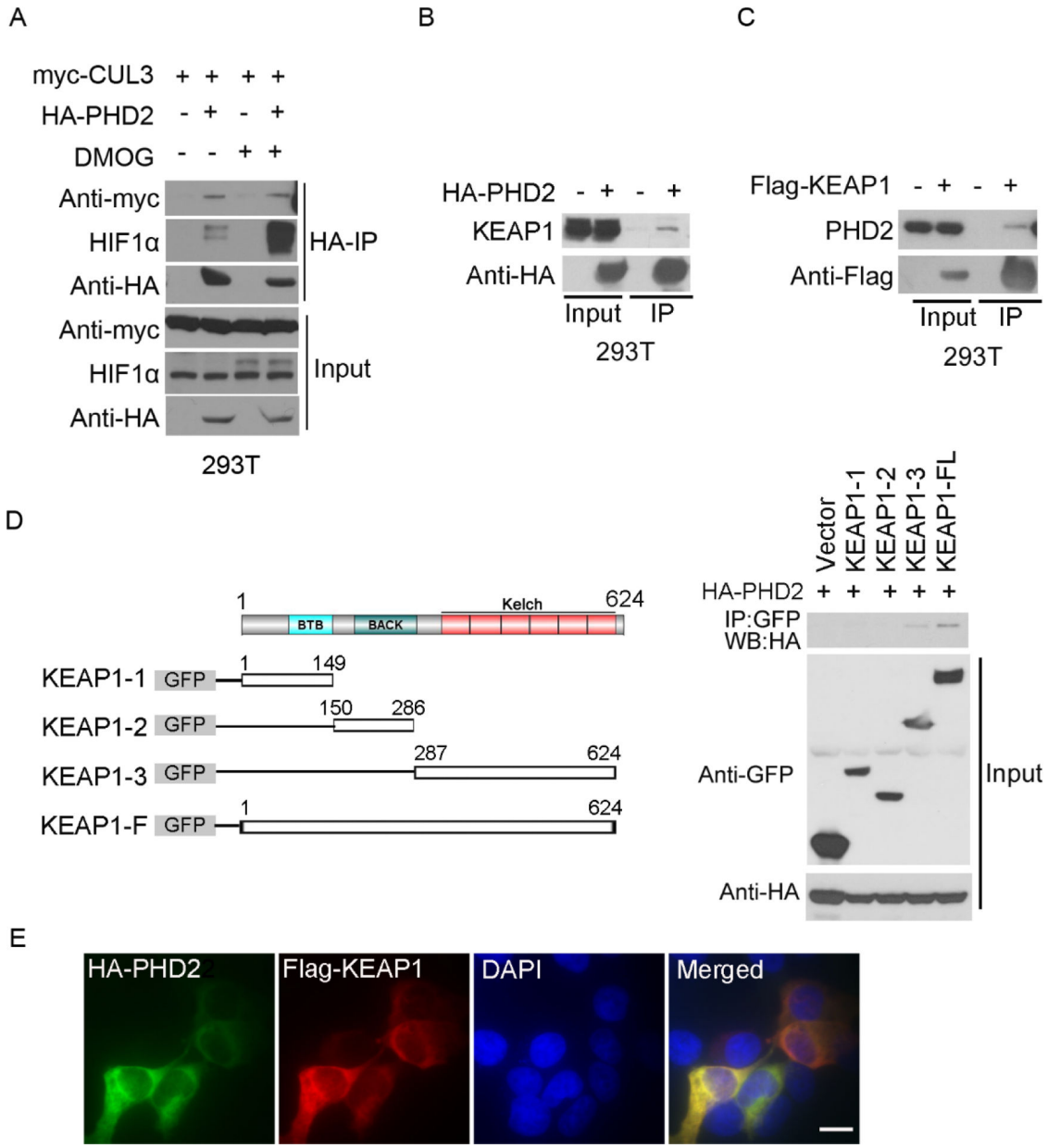


**Figure 2.**

Interaction of PHD2 with CUL3/KEAP1. (A) 293T cells transfected with the indicated plasmids were treated with DMSO or DMOG for 4 hours and then prepared for IP with anti-HA magnetic beads and WB with the indicated antibodies. (B) 293T cells were transfected either with HA-PHD2 or the empty vector plasmid and then prepared for IP with anti-HA Magnetic Beads and WB with the indicated antibodies. (C) 293T cells were transfected either with Flag-KEAP1 or the empty vector plasmid and then prepared for IP with Flag-M2 agarose and WB with the indicated antibodies. (D) Mapping the KEAP1 domains mediating its interaction with PHD2. Left, a schematic diagram showing different KEAP1 domains. Right, 293T cells transfected with the indicated plasmids were prepared for IP with anti-GFP agarose and WB with the indicated antibodies. (E) Immunofluorescence staining

showing the distribution of ectopically expressed PHD2 and KEAP1 in 293T cells. Scale bar, 50μm.

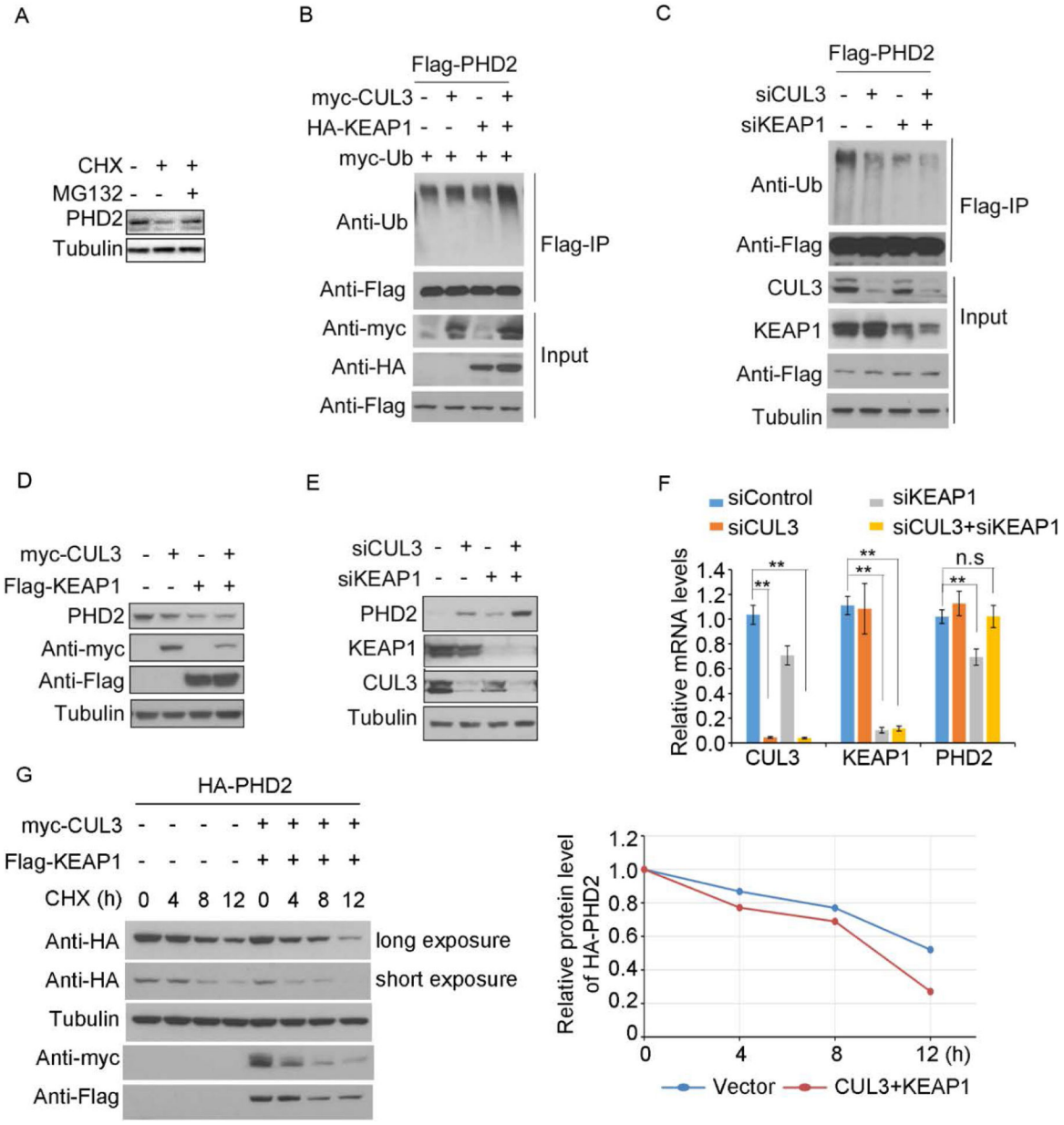


**Figure 3.**

Regulation of PHD2 ubiquitination by CUL3/KEAP1 in vivo. (A) Extracts of HeLa cells treated with the indicated chemicals for 24 hours were immunoblotted with PHD2 antibody. (B) 293T cells expressing Flag-PHD2 were transfected with the indicated plasmids and then treated with 5 μM MG132 for 12 hours. The cell lysates were prepared for Flag-IP and immunoblotted with the indicated antibodies. (C) HeLa cells expressing Flag-PHD2 were transfected with the indicated siRNAs and then treated with 5 μM MG132 for 24 hours. The cell lysates were analyzed as in (B). (D) Cell lysates from 293T cells transfected with the indicated plasmids were immunoblotted with the indicated antibodies. HeLa cells transfected with the indicated siRNAs were prepared either for WB with the indicated antibodies (E) or for total RNA extraction and qRT-PCT with the indicated primers (F). (G)

Left, HeLa cells were transfected with the indicated plasmids for 24h and then treated with 80 μM CHX for the indicated time points. Then the cells were harvested for WB with the indicated antibodies. Right, protein levels of HA-PHD2 in the left panel were quantified with ImageJ software by normalizing the amount of Tubulin. In (F), bar blot show mean and SD, n=3, **P<0.01, Student's t-test.

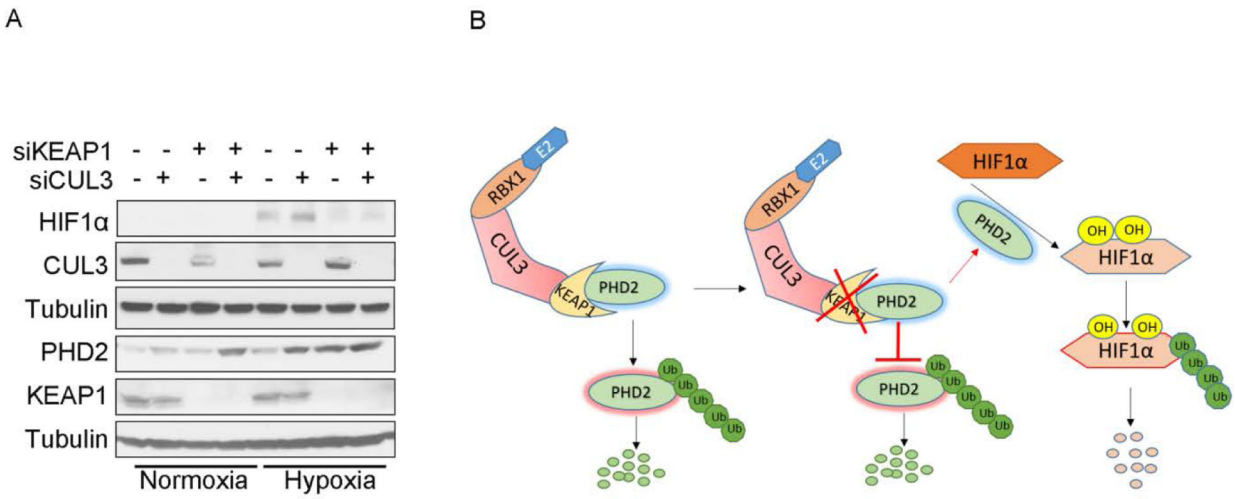


**Figure 4.**

Regulation of HIF1α protein by CUL3/KEAP1 under hypoxia in HeLa cells. (A) HeLa cells were transfected with the indicated siRNAs for 48 hours and then either treated with hypoxia (1% oxygen) or continue to be cultured under normoxia for 12 hours. After treatment, cell lysates were prepared for WB with the indicated antibodies. (B) A schematic illustration to summarize the regulation of CUL3/KEAP1 on the poly-ubiquitination and degradation of PHD2.