Supplementary information, Data S1

Supplemental Experimental Procedures

Plasmids

Full-length cDNAs encoding human RHOBTB3, LIMD1, PHD2 and VHL (VHL30, VHLp24) were obtained by PCR using cDNA purified from HEK293T cells. Point mutations of HIF1α were performed by a PCR-based site-directed mutagenesis method using Pfu polymerase (Stratagene). DNA fragments amplified by PCR were verified by sequencing (Invitrogen, China). Expression plasmids for various proteins were constructed in the pCMV5 or pcDNA3.3 vector for transient transfection or in pBOBI for lentivirus packaging (stable expression). The lentivirus-based vector pLL3.7 was used for expression of siRNA in HEK293T. The 19-nucleotide sequence for siRNA against human *RHOBTB3* in pLL3.7 is 5'-GCCATGAATCTTCAGGCAA-3', 5'-GGAACAAGAAAGCTAATAA-3' for human *PHD2*, 5'-GCCUGAGAAUUACAGG AGA-3' for human *VHL*, and 5'-GAGCAGAGGATCAGGCCAT-3' for mouse *LIMD1*. The siRNAs against human *LIMD1* (si*LIMD1*-A) was constructed as described previously [58]. The details of the primer sequences used for constructing point mutants and deletion mutants are available upon request.

Antibodies and drugs

Mouse anti-HA (cat. sc-7392), anti-LIMD1 (cat. sc-271448), goat anti-PDK1 (cat. sc-7140), rabbit anti-VHL (cat. sc-5575, used to immunoblot endogenous human VHL),

and anti-SLC16A4 (cat. sc-50329) antibodies were purchased from Santa Cruz Biotechnology. Mouse anti-MYC (cat. #2276), Rabbit anti-HIF1α-OH-564 (cat. #3434), anti-PHD2 (cat. #4835), anti-HIS (cat. #2365), anti-GST (cat. #2625), anti-HK2 (cat. #2867), anti-HSP90 (cat. #4877), anti-LDHA (cat. #2012), and anti-GLUT1 (cat. 12939) antibodies were purchased from Cell Signaling Technology. Rabbit anti-HIF2α (cat. ab73895), anti-HIF3 alpha (cat. ab10134), anti-hydroxyproline (cat. ab37067), anti-PHD3 (cat. ab30782), Anti-PHD1 (cat. ab113077) and anti-PECAM1 (cat. ab28364) antibodies were purchased from Abcam. Rabbit anti-RHOBTB3 (cat. 19014-1-AP) and anti-HIF1α (cat. 20960-1-AP, used to detect endogenous mouse HIF1α) antibodies were purchased from Proteintech. Rabbit anti-CA9 (cat. NB100-417) was purchased from Novus. Mouse anti-VHL (cat. MABC14, used to immunoprecipitate endogenous human VHL from HEK293T) antibody was purchased from EMD Millipore. Mouse anti-FLAG (cat. F1804) and anti-β-ACTIN (cat. A1978) antibodies were purchased from Sigma. Mouse anti-HIF1α (cat. 601959, used to detect endogenous human HIF1α) antibody was purchased from BD Biosciences. MG-132 (sc-201270) was purchased from Santa Cruz Biotechnology. $CoCl_2$ (cat. C8661), Chloroquine diphosphate salt (cat. C6628) and FLAG peptide (cat. F3290) were purchased from Sigma. RHOBTB3 (cat. ab161438) protein was purchased from Abcam. Protease inhibitor cocktail was purchased from Roche.

Quantitative real-time PCR

Total RNAs from MEFs and tumors were extracted with Trizol reagent. The cDNA was then synthesized using M-MLV RTase system. The analyses were performed using Step One Plus, the fast Real-Time PCR System from ABI (Applied Biosystems). 18S rRNA was used as endogenous control for samples. Details of primers for each analyzed human and mouse gene are listed in the following tables:

Human genes

Gene	Accession Number	Primer Sequence (5'-3')	Product Length
18S rRNA	NR_003286.2	F: CGGCTACCACATCCAAGGAA R: GCTGGAATTACCGCGGCT	187 bp
HIF1A	NM_001243084.1	F: CCATTAGAAAGCAGTTCCGC R: TGGGTAGGAGATGGAGATGC	194 bp
RHOBTB3	NM_014899.3	F: GCTTGCCGATGTTGTCTTCG R: CAGGAGACACATGGCCTGG	232 bp
CA9	NM_001216.2	F: TAAGCAGCTCCACACCCTCT R: TCTCATCTGCACAAGGAACG	250 bp
VEGFA	NM_001171623.1	F: AGGAGGAGGGCAGAATCATCA R: CTCGATTGGATGGCAGTAGCT	76 bp
GLUT1	NM_006516.2	F: GATTGGCTCCTTCTCTGTGG R: TCAAAGGACTTGCCCAGTTT	129 bp
BNIP3	NM_004052.2	F: CAGGGCTCCTGGGTAGAACT R: CTCCGTCCAGACTCATGCTG	128 bp
VHL	NM_198156.2	F: GACCTGGAGCGGCTGACA R: TACCATCAAAAGCTGAGATGAAACA	101 bp
EPAS1	NM_001430.4	F: GTCTCTCCACCCCATGTCTC R: GGTTCTTCATCCGTTTCCAC	217 bp
PHD2	NM_022051.2	F: GCACGACACCGGGAAGTT R: CCAGCTTCCCGTTACAGT	176 bp
LDHA	NM_001165416.1	F: GGCCTGTGCCATCAGTATCT R: GCCGTGATAATGACCAGCTT	189 bp
HK2	NM_000189.4	F: TCCGTAACATTCTCATCGATTTCAT R: TGTCTTGAGCCGCTCTGAGAT	74bp

Mouse genes

Gene	Accession Number	Primer Sequence (5'-3')	Product Length
18S rRNA	NM_011296.2	F: CATTAAGGGCGTGGGGCGGAG R: CATGATGGTGATCACTCGC	118 bp
HIF1A	NM_010431.2	F: CAAGATCTCGGCGAAGCAA R: GGTGAGCCTCATAACAGAAGCTTT	113 bp
HIF2A	NM_010137.3	F: AGTAGCCTCTGTGGCTCCAA R: TCCAGGGCATGGTAGAACTC	230 bp
GLUT1	NM_011400.3	F: ACTGGGCAAGTCCTTTGAGA R: GTCTAAGCCAAACACCTGGGC	190 bp
LDHA	NM_001136069.2	F: GCTCCCCAGAACAAGATTACAG R: TCGCCCTTGAGTTTGTCTTC	131 bp

Protein expression

HIF1α (401-603aa) and its P564A mutant were cloned into pET-28a vector and were used for transformation the E. coli strain BL21 (DE3). The protein expression of transformed cells was induced with 0.1 mM IPTG at an optical density of 0.8 at 600 nm. After growing for 16 h at 20 ℃, the cells were collected and the expressed protein was purified with HIS-Select Nickel Affinity Gel (Sigma) through regular procedures. The protein-bound gel was then collected and stored in -80 ℃ with 20% glycerol for further use.

Immunohistochemistry

Sections were deparaffinized and rehydrated, and antigen retrieval was performed in

10 mM citric acid and 2 mM EDTA (pH 6.0). The sections were then blocked in 10% goat serum in PBS at 37 °C for 1 h, and were then incubated in different primary antibodies (1:100 in 10% goat serum, 4 °C, overnight), followed by incubation with fluorescent labeled-secondary antibodies (1:500 in PBS, room temperature, 2 h). The nuclei were stained with 1 μg/ml DAPI (Invitrogen, D21490) in PBS for 5 min at room temperature, and imaged on an Axio Imager A2 (Zeiss).

Immunofluorescence

Rhobtb3-/- MEFs were fixed on glass cover slips with 3.7% paraformaldehyde at room temperature after the treatment indicated in figure legends. Cells were washed three times with PBS followed by incubation with 0.5% NP-40 in PBS for 5 min. Subsequently, cells were blocked with PBS containing 5% (w/v) BSA for 20 min and incubated with anti-mouse HA antibody (sc-7392, Santa Cruz Biotechnology) or anti-rabbit VHL antibody (ab135576, Abcam) overnight at 4 °C. Excess antibodies were removed by washing the cells three times with PBS. This was followed by a 7-8 h incubation with secondary antibody (Alexa Fluor 488 and Alexa Fluor 594) in the dark at 4 °C. Subsequently the cells were further washed three times with PBS before mounting the cover slips on a glass slide with mounting media containing DAPI. Cells were later imaged on a Zeiss Laser Scanning Microscope (LSM) 780.

Supplemental References

81 Li Q, Lin S, Wang X *et al*. Axin determines cell fate by controlling the p53 activation threshold after DNA damage. *Nature cell biology* 2009; **11**:1128-1134.