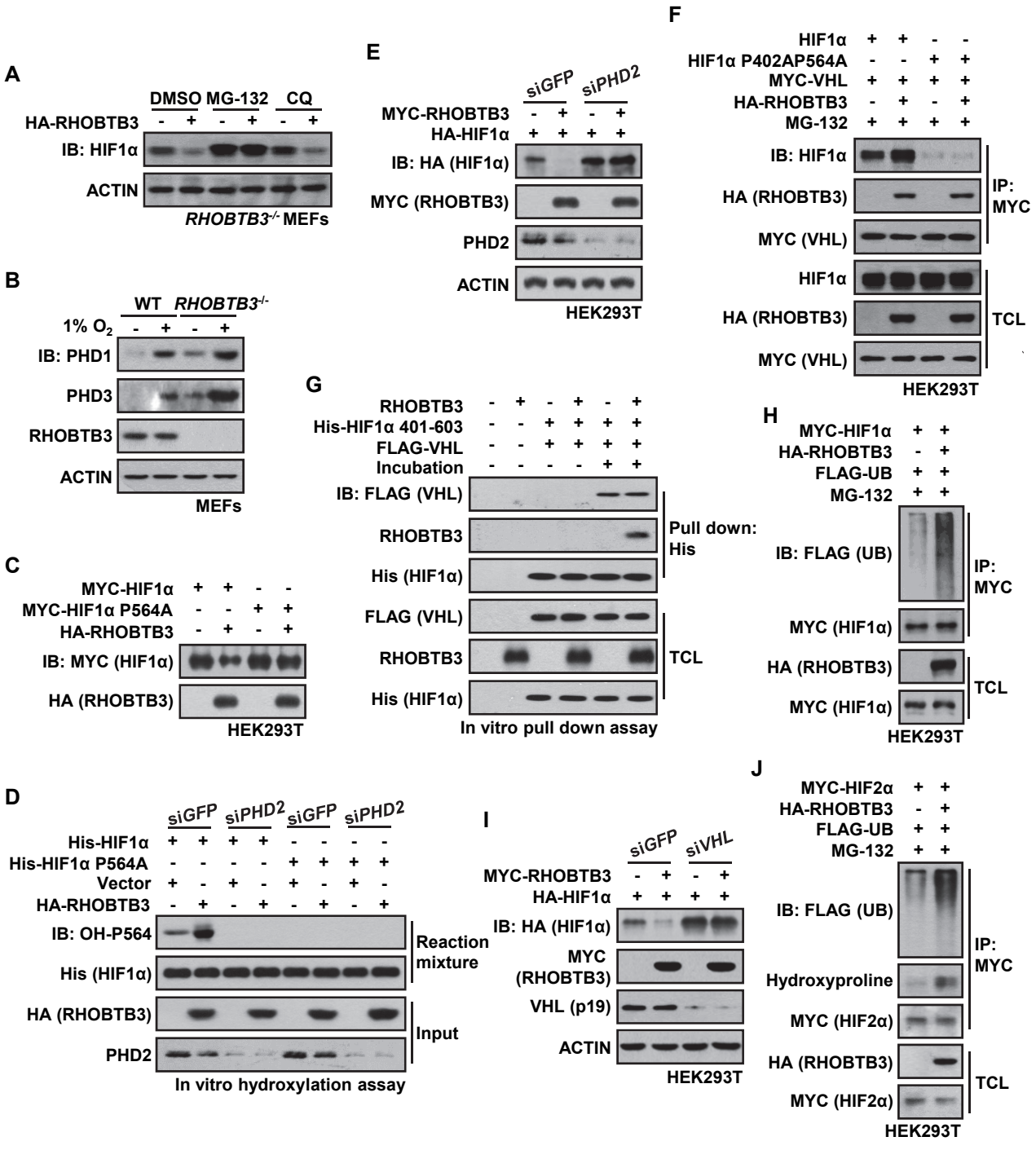
A
DMSO MG-132 CQ
HA-RHOBTB3 - + - + - +
IB: HIF1α
ACTIN
RHOBTB3-/- MEFs
B
WT RHOBTB3-/-
1% O2 - + - +
IB: PHD1
PHD3
RHOBTB3
ACTIN
MEFs
C
MYC-HIF1α + + - -
MYC-HIF1α P564A - - + +
HA-RHOBTB3 - + - +
IB: MYC (HIF1α)
HA (RHOBTB3)
HEK293T
D
siGFP siPHD2 siGFP siPHD2
His-HIF1α + + + + - - - -
His-HIF1α P564A - - - - + + + +
Vector + - + - + - + -
HA-RHOBTB3 - + - + - + - +
IB: OH-P564
His (HIF1α)
HA (RHOBTB3)
PHD2
Reaction mixture
Input
In vitro hydroxylation assay
E
siGFP siPHD2
MYC-RHOBTB3 - + - +
HA-HIF1α + + + +
IB: HA (HIF1α)
MYC (RHOBTB3)
PHD2
ACTIN
HEK293T
F
HIF1α + + - -
HIF1α P402AP564A - - + +
MYC-VHL + + + +
HA-RHOBTB3 - + - +
MG-132 + + + +
IB: HIF1α
HA (RHOBTB3)
MYC (VHL)
HIF1α
HA (RHOBTB3)
MYC (VHL)
IP: MYC
TCL
HEK293T
G
RHOBTB3 - + - + - +
His-HIF1α 401-603 - - + + + +
FLAG-VHL - - + + + +
Incubation - - - - + +
IB: FLAG (VHL)
RHOBTB3
His (HIF1α)
FLAG (VHL)
RHOBTB3
His (HIF1α)
Pull down: His
TCL
In vitro pull down assay
H
MYC-HIF1α + +
HA-RHOBTB3 - +
FLAG-UB + +
MG-132 + +
IB: FLAG (UB)
MYC (HIF1α)
HA (RHOBTB3)
MYC (HIF1α)
IP: MYC
TCL
HEK293T
I
siGFP siVHL
MYC-RHOBTB3 - + - +
HA-HIF1α + + + +
IB: HA (HIF1α)
MYC (RHOBTB3)
VHL (p19)
ACTIN
HEK293T
J
MYC-HIF2α + +
HA-RHOBTB3 - +
FLAG-UB + +
MG-132 + +
IB: FLAG (UB)
Hydroxyproline
MYC (HIF2α)
HA (RHOBTB3)
MYC (HIF2α)
IP: MYC
TCL
HEK293T

Supplementary information, Figure S2

(**A**) RHOBTB3 promotes HIF1α degradation in a proteasome-specific manner. *RHOBTB3*$^{-/-}$ MEFs were infected by lentivirus expressing HA-RHOBTB3 or blank vector as a control. At 16 h post-infection, cells were treated with 10 μM MG-132 or 200 μM chloroquine (CQ) and maintained under normoxia or exposed to hypoxia for 10 h, then lysed and analyzed by immunoblotting with antibodies indicated.

(**B**) The expression levels of PHD1 and PHD3 are increased in *RHOBTB3*$^{-/-}$ MEFs. *RHOBTB3*$^{-/-}$ MEFs and control (WT) MEFs were maintained in normoxia or exposed to hypoxia for 8 h. Cells were then lysed and analyzed by immunoblotting with antibodies indicated.

(**C**) RHOBTB3 has little effect on the stability of HIF1α P564A mutant. HEK293T cells in 6-well plates were transfected with 0.8 μg of MYC-HIF1α, 1.5 μg of MYC-HIF1α P564A, and 1 μg of HA-RHOBTB3 in different combinations. At 16 h post-transfection, cells were lysed and analyzed by immunoblotting with antibodies indicated.

(**D**) Knockdown of *PHD2* strongly attenuates RHOBTB3-enhanced hydroxylation of HIF1α in vitro. HEK293T cells infected by lentivirus carrying *GFP* or *PHD2* siRNA were transfected with HA-RHOBTB3 or vector as a control. At 16 h post-infection, cell lysates were then prepared and incubated with nickel affinity resin-bound bacterially expressed His-HIF1α (aa 401-603) or its P564A mutant for 90 min at 30 ° C. The mixtures were then added with an equal volume of 2 × SDS buffer, followed by immunoblotting with antibodies indicated.

(**E**) Ectopically expressed RHOBTB3 depends on PHD2 to downregulate HIF1α. HEK293T cells infected by lentivirus expressing siRNA to target *GFP* or *PHD2* were transfected with MYC-RHOBTB3, HA-HIF1α or vector as a control. At 16 h post-infection, cells were lysed, followed by immunoblotting with antibodies indicated.

(**F**) Ectopically expressed RHOBTB3 does not promote the interaction between VHL and the P402AP564A mutant of HIF1α. HEK293T cells were transfected with different combinations of HIF1α, HIF1α P402AP564A and HA-RHOBTB3. At 8 h post-transfection, cells were treated with 10 μM MG-132 for 10 h and the lysates were immunoprecipitated with antibody against MYC, followed by immunoblotting.

(**G**) In vitro-translated RHOBTB3 has no effect on the interaction between bacterially expressed ODD domain of HIF1α and VHL. The nickel affinity resin-bound bacterially expressed His-HIF1α (aa 401-603) was preincubated in the 50 μg of cytoplasmic extract (incubation) as described previously [70] or not. The resin was then incubated with in vitro-translated RHOBTB3 and immunoprecipitated FLAG-VHL overexpressed in HEK293T cells then eluted by FLAG peptide. The mixtures were then pulled down by centrifugation, followed by immunoblotting with antibodies indicated.

(**H**) RHOBTB3 promotes ubiquitination of HIF1α. HEK293T cells were transfected with different combinations of MYC-HIF1α, HA-RHOBTB3 and FLAG-UB. At 8 h post-transfection, cells were treated with 10 μM MG-132 for 10 h and the lysates were immunoprecipitated with antibody against MYC to pull down HIF1α, followed by immunoblotting as described in Figure 2G.

(**I**) Ectopically expressed RHOBTB3 fails to downregulate HIF1α in the absence of VHL. HEK293T cells infected by lentivirus carrying siRNA targeting *GFP* or *VHL* were transfected with MYC-RHOBTB3, HA-HIF1α or vector as a control. Cells were then lysed and the protein levels of HIF1α were analyzed as described in (**E**).

(**J**) RHOBTB3 promotes the ubiquitination and hydroxylation of HIF2α. HEK293T cells were transfected with different combinations of MYC-tagged HIF2α, FLAG-tagged UB and HA-tagged RHOBTB3. At 16 h post-transfection, cells were treated with 10 μM MG-132 for another 10 h, and were then lysed with RIPA buffer containing 1% SDS and boiled. The protein extracts were diluted with RIPA buffer without SDS to a final concentration of 0.2% SDS, and were subjected to IP with antibody against MYC for HIF2α, followed by immunoblotting with antibodies indicated.