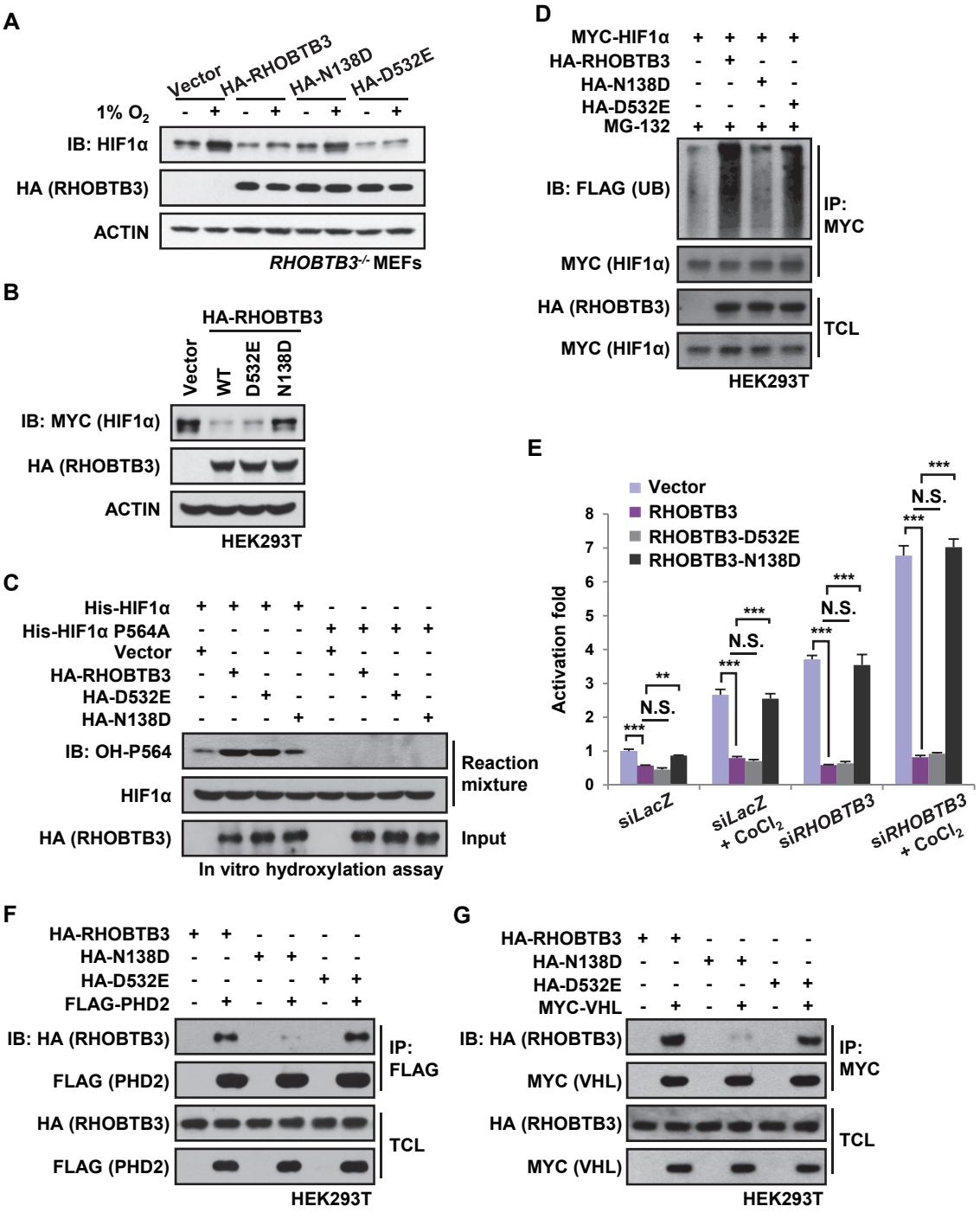
A
Vector
HA-RHOBTB3
HA-N138D
HA-D532E
1% O2
IB: HIF1α
HA (RHOBTB3)
ACTIN
RHOBTB3-/- MEFs
B
HA-RHOBTB3
Vector
WT
D532E
N138D
IB: MYC (HIF1α)
HA (RHOBTB3)
ACTIN
HEK293T
C
His-HIF1α
His-HIF1α P564A
Vector
HA-RHOBTB3
HA-D532E
HA-N138D
IB: OH-P564
HIF1α
HA (RHOBTB3)
Reaction mixture
Input
In vitro hydroxylation assay
D
MYC-HIF1α
HA-RHOBTB3
HA-N138D
HA-D532E
MG-132
IB: FLAG (UB)
MYC (HIF1α)
HA (RHOBTB3)
MYC (HIF1α)
IP: MYC
TCL
HEK293T
E
Vector
RHOBTB3
RHOBTB3-D532E
RHOBTB3-N138D
Activation fold
N.S.
siLacZ
siLacZ + CoCl2
siRHOBTB3
siRHOBTB3 + CoCl2
F
HA-RHOBTB3
HA-N138D
HA-D532E
FLAG-PHD2
IB: HA (RHOBTB3)
FLAG (PHD2)
HA (RHOBTB3)
FLAG (PHD2)
IP: FLAG
TCL
HEK293T
G
HA-RHOBTB3
HA-N138D
HA-D532E
MYC-VHL
IB: HA (RHOBTB3)
MYC (VHL)
HA (RHOBTB3)
MYC (VHL)
IP: MYC
TCL
HEK293T

Supplementary information, Figure S5

(**A**) The N138D mutant of RHOBTB3 fails to downregulate endogenous HIF1α. *RHOBTB3*$^{-/-}$ MEFs were infected with blank lentivirus (vector) or lentivirus expressing various HA-RHOBTB3 proteins. At 36 h post-infection, cells were maintained in normoxia or exposed to hypoxia (1% O_2) for 8 h, followed by lysis and immunoblotting with antibodies indicated.

(**B**) RHOBTB3 N138D mutant fails to downregulate ectopically HIF1α. HEK293T cells were transfected with different combinations of MYC-tagged HIF1α and HA-tagged RHOBTB3 (WT or mutant). At 16 h post-infection, cells were lysed, followed by immunoblotting with antibodies indicated.

(**C**) RHOBTB3 N138D mutant fails to promote hydroxylation of HIF1α. *RHOBTB3*$^{-/-}$ MEFs were infected with blank lentivirus (vector) or the virus expressing HA-tagged RHOBTB3 (WT or mutant). Following lysis, cell lysates were 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.

(**D**) RHOBTB3 N138D mutant fails to promote ubiquitination of HIF1. HEK293T cells were transfected with different combinations of MYC-tagged HIF1α, FLAG-tagged UB and HA-tagged RHOBTB3 (WT or mutant). 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 HIF1α, followed by immunoblotting with antibodies indicated.

(**E**) RHOBTB3 N138D mutant does not downregulate the transcriptional activity of HIF1α. HEK293T cells were transfected with HA-tagged RHOBTB3 (WT or mutant). After 12 h, cells were treated with 200 μM $CoCl_2$ for another 8 h, and then lysed; the luciferase activities driven by the hypoxic response element (HRE-Luc) were measured and normalized to a Renilla luciferase (dual luciferase assay system). Data are presented as mean ± SEM, n = 3 for each group, ***p < 0.001 (ANOVA followed by Tukey); N.S., not significant.

(**F, G**) RHOBTB3 N138D mutant fails to interact with PHD2 (**F**) or VHL (**G**). HEK293T cells were transfected with different combinations of HA-RHOBTB3 (WT and mutants), FLAG-PHD2 (**F**) and MYC-VHL (**G**). Protein extracts were immunoprecipitated and immunoblotted with antibodies indicated.