Pro70 Hydroxylation of Actin Impairs Cell Motility Through Binding with VHL and Blocking His73 Methylation

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Abstract:

Protein hydroxylation is a post translational modification happens on various amino acids, which is catalyzed by the oxoglutarate and oxygen dependent dioxygenases. The best characterized hydroxylated protein is the hypoxia inducible factor (HIF), which is degraded by VHL/elongin C/elongin B/cullin 2/RBX1 (VCB/CR) E3 complex under normal oxygen conditions. Hypoxia or inhibitors (including FG4592 and MK8617) of PHDs stabilize HIF1a and regulate its downstream targets. Prolyl hydroxylase, including PHD2 and PHD3 has been reported in regulating actin polymerization and cell motility. Here, we found MK8617 regulated cell motility in Von Hippel Lindau (VHL) dependent manner. Through the protein hydroxylation proteome experiment upon MK8617 treatment, we identified Pro70 in actin could be hydroxylated and near to His73, which has been reported be methylated and stabilize actin polymerization. Through the biochemical assay, we found that hydroxylated actin (Pro70) binds to VHL and decrease the His73 methylation through blocking the interaction of actin with SETD3, the His73 methyltransferase, and further regulated actin polymerization and cell motility. In summary, our

study revealed that hypoxia and deficiencies in the VHL, in a HIF independent and prolyl hydroxylation dependent manner, regulate actin polymerization and cell motility.

Introduction:

Protein hydroxylation is a post-translational modification happens on various amino acids, including proline, lysine, asparagine, aspartate and histidine (1). The proline hydroxylation reaction is catalyzed by the 2-oxoglutarate- and oxygen-dependent dioxygenases (PHD1, PHD2, and PHD3) (2). A well-known example of hydroxylation is hypoxia inducible factor alpha (HIF-1α, at Pro 402 and 564), which regulate HIF-1α stability via the VHL-elongin C-elongin B-cullin 2-RBX1 (VCB-CR) E3 complex (3). Hypoxia-inducible factor (HIF) is a transcription factor involved in erythropoiesis, angiogenesis, glycolytic energy metabolism, and apoptosis (4). Besides HIF-dependent functions, VHL also play roles in the regulation of the extracellular matrix; microtubule stabilization and maintenance of the primary cilium; regulation of apoptosis; control of cell senescence; and transcriptional regulation (5).

Hypoxia or inhibitors (including FG-4592 and MK-8617) of PHDs stabilize HIF1a and regulate its' downstream targets (6). Small-molecule PHD enzyme inhibitors that efficiently activate HIF1a in the presence of oxygen (7-9). These small molecules could be therapeutically useful for a range of indications, most obviously for the treatment of anaemia that results from impaired erythropoietin production (7). Activation of HIF1a is beneficial in ischaemic or ischaemia–reperfusion settings, as HIF1a acts upstream of diverse gene products that should be protective in these contexts by promoting vasodilatation and angiogenesis, increasing glucose uptake, modulating mitochondrial metabolism and decreasing free radical production (10). PHD enzyme inhibitors might also be of benefit in wound healing, stroke, myocardial ischaemia, limb ischaemia, acute kidney injury, and cell motility (7).

Actin is a conserved cytoskeletal protein that forms filaments and plays an essential role in cell movement (11). Two forms of actin exist in cells, including monomeric (G-actin) and filamentous (F-actin), the polymerization of G-actin into F-actin provides intracellular mechanical support that facilitates cell migration (11,12). Actin undergoes various forms of posttranslational modifications, which regulate the balance between G-actin and F-actin (13), including phosphorylation of Tyr-53, ADP-ribosylation of Arg-177 and Arg-206, glutathionylation, carbonylation, or nitrosylation of Cys-374, which inhibit actin polymerization,

while transglutamination of Lys-50, methylation of His-73, ADP-ribosylation of Thr-148, and arginylation, which stabilize the F-actin (13). The His73 methylation has been reported to be catalyzed by the methyltransferase, SET domain protein 3 (SETD3), and regulate smooth muscle contractility (14,15).

Here, we found that MK-8617, the PHD enzyme inhibitor could regulate cell motility and actin oligomerization in VHL dependent manner. Through the hydroxylation modification proteome quantification analysis, we identified several hydroxylated-proline sites in actin, one of which is Pro70 and near to His73, which could be methylated and regulate actin polymerization. Through the biochemical assay, we found that, binding of hydroxylated Actin (Pro70) to VHL could block the interaction of actin with SETD3 and inhibit the His73 methylation, which subsequentially regulate actin polymerization and cell motility. Therefore, our studies revealed that hypoxia and deficiencies in the VHL, in a HIF-independent and prolyl hydroxylation–dependent manner, regulate actin polymerization and cell motility.

Results and Discussion:

MK-8617 Regulate Cell Motility and Actin Oligomerization in VHL Dependent Manner

MK-8617 have been used to treat angiogenesis and other disease (6,16). As protein hydroxylation has roles in cancer cell metastasis, we checked if MK-8617 affect the cell motility. Through the cell scratch assay, we found that MK-8617 could promote the cell motility in Hela cells, but not in the 786-O or RCCJF cells (Fig. 1A). As 786-O and RCCJF cells are CCRCC cell lines, which are VHL null cell line, while Hela cells harbor normal VHL expression, this result indicate that the effect of MK-8617 on cell motility is VHL dependent.

Actin exists in monomeric (G-actin) and filamentous (F-actin) forms in cells (12). Polymerization of G-actin into F-actin regulate cell migration (11). Therefore, we checked the F-Actin level by using TRITC–conjugated phalloidin immunostaining experiment. We found that MK-8617 promote the F-actin formation in Hela cells, but not in 786-O cells (Fig. 1B). This is consistent with the cell motility response upon MK-8617 treatment in Hela and 786-O cells (Fig. 1A). His73 methylation in actin has been reported to stabilize actin polymerization (14). We measured and found that the actin-His73 methylation level increased in Hela cells, while no change happened in 786-O cells upon MK-8617 treatment (Fig. 1C and 1D). This is also

observed in HEK293T and RCCJF cells (Fig. 1E and 1F). These results indicated that, MK-8617 regulate cell motility through regulating actin H73 methylation and actin polymerization, in VHL dependent manner (Fig. 1G).

Protein Hydroxylation Proteome Response upon MK-8617 Treatment

MK-8617 is a common Hypoxia-inducible factor (HIF)-prolyl hydroxylase inhibitor (HIF-PHI) (6), we then investigate the whole proteome and hydroxylation proteome changes upon MK-8617 treatment in MCF10A cells, which harbor high PHDs expression level. We performed quantitative proteomic experiments (using the tandem mass tag, TMT6 labeling approach) using the MCF10A cells upon MK-8617 treatment for time course (0/6/12/24/26/48 hours) (Fig. 2A). Under normal conditions, HIF1a is hydroxylated by the PHDs enzymes, and hydroxylated HIF1a is rapidly degraded by the von Hippel-Lindau–E3 ubiquitin ligase complex following ubiquitylation (2). Upon MK-8617 treatment, the degradation of HIF1a was inhibited, and this effect sustained for 48 hours (Fig. 2B and Table S1), which was consistent with the TMT-MS result (Fig. 2C). While the transcription of HIF1a has no change upon MK-8617 treatment (Fig. 2E). Besides HIF1a, we also found the protein, BNIP3, which is a reported proapoptotic gene, as a HIF1a downstream target, could be induced upon MK-8617 treatment at both transcriptional and protein level (Fig. 2D, 2E Table S1).

To characterize the whole hydroxylation-proteome change upon MK-8617 treatment, we performed cluster analysis of the TMT-MS result, which showed that most of the proteins' hydroxylation level decreased upon 6 hours MK-8617 treatment and reached the lowest level upon 12 hours treatment (Fig. 2F). The hydroxylation motif was enriched in a poly-Proline sequence through the motif analysis base on the hydroxylated peptides identified by TMT-MS experiments (Fig. 2G).

Among all the hydroxylated proteins, Actin (including alpha and beta actin) could be hydroxylated on several proline sites, including the Pro70 (72 in ACTA)/102/109/164/258/264/307 (Fig. 3A, 3B and Table S2). Among the proline sites, the Pro307 has been reported to be hydroxylated by PHD3 and play roles in the cell motility (17). To check which PHDs enzyme hydroxylated actin, we performed co-immunoprecipitation assay, and found that actin could interact with PHD2 and PHD3, which indicated that actin could be hydroxylated by PHD2 and PHD3 (Fig. 3C). One of the hydroxylated proline sites in actin

identified by the mass spectrometry is Pro 70, with the hydroxylation level decreased upon 6- and 12-hours MK-8617 treatment (Fig. 3D and 3F). The total actin protein amount has no change upon MK-8617 treatment (Fig. 3E).

Pro70-Hydroxylation Bind to VHL and Inhibit His73 Methylation

We showed that MK-8617 could downregulate Pro70 hydroxylation (Fig. 3D) and upregulate His73 methylation (Fig. 1C-1F), which indicate the correlation between actin Pro70 hydroxylation and His73 methylation. Histidine 73 in actin is methylated by SETD3, we found that the interaction of actin and SETD3 could be enhanced by the Pro70 to Ala mutation (Fig. 3G). This result indicated the crosstalk between Pro70 hydroxylation and His73 methylation.

We synthesized the hydroxylated peptide (Hyp70) and non-hydroxylated peptide (P70) (Fig. 3H). We found that only hydroxylated-Pro70 peptides bind to VHL but not the non-hydroxylated peptides (Fig. 3I). We further performed the in vitro methylation assay using the peptides, the result showed that Pro70 non-hydroxylated peptide has no effect on H73 Methylation either with or without VHL. However, the Pro70 hydroxylated peptide could block the H73 methylation when incubate with VHL compared to the without VHL condition (Fig. 3J). These results suggested that, the hydroxylation on Pro70 of actin could bind to VHL, then block the interaction of actin with SETD3, and subsequentially leads to the downregulation of His73 methylation.

VHL Regulate Cell Motility Through Regulating H73 Methylation

Knockdown VHL in Hela cells using shRNA, leads to the increase of actin polymerization and H73 methylation level compared to the control cells (Fig. 4A, 4B and 4C). VHL knockdown also leads to the increase of cell motility (Fig. 4D). Meanwhile, we overexpress the VHL in 786-O cells, which leads to the decrease of actin polymerization compared to the control cells (Fig. 4A). Consistently, the cell motility will be blocked by the overexpress of VHL in 786-O cells (Fig. 4E). These results indicated that, VHL regulated cell motility through binding to hydroxylated actin (Pro70) and inhibiting the actin His73 methylation.

In summary, these results showed a new mechanism about how VHL regulate cell motility (Fig. 4F). PHD2 or PHD3 hydroxylated actin at Pro70, the hydroxylated actin binds to VHL and block the interaction between actin and SETD3, which subsequentially inhibit the His73 methylation. The downregulated H73 methylation leads to the decreased F-actin formation and cell motility.

Discussion

The best-characterized function of VHL is that VHL mediates degradation of the hydroxylated hypoxia-inducible factor (HIF) as a component of E3 ligase complex under normal oxygen conditions (3). The proline hydroxylation reaction is catalyzed by the 2-oxoglutarate- and oxygen-dependent dioxygenases PHD1, PHD2, and PHD3 (2). Inhibitors targeted to PHD enzymes have been used in anemia treatment, however, these inhibitors targeting to PHD proteins will have pleiotropic effects, because these inhibitors will inhibit other 2-OG-dependent oxygenases (7). Here, we identified Actin as a direct PHD2 and PHD3 hydroxylation substrate through the Co-immunoprecipitation assay (Fig. 3C). And found that the hydroxylation level of hydroxylated-Actin at Pro70 decreased upon MK-8617 treatment (Fig. 3B and 3D). Further analysis found that hydroxylated actin (Hyp70 peptide) binds to VHL (Fig. 3I).

Actin exists in both a monomeric globular state (G-actin) and polymerized filamentous state (F-actin), and the switch between the two states is highly dynamic (12). The actin filaments play crucial roles in countless cellular functions, including muscle contraction, cell signaling, as well as cell integrity and motility (18). Numerous studies and reviews describe the actin PTMs shedding light on a crucial aspect of actin biology (13), and the crosstalk between PTMs, create a "PTM code" on proteins, which can be recognized to regulate downstream events (19).

The hydroxylated proline site, Pro70 is near to His 73, a critical site, which could be methylated by SETD3, and regulate F-actin formation and cell motility. We observed that VHL binds to hydroxylated actin (Hyp70) and block the binding of actin with SETD3, which subsequentially prevents actin methylation on histidine 73, leading to decrease of F-actin formation (Fig. 3). Indeed, hydroxylation regulated cell motility is VHL-dependent, shown as MK-8617 can regulate this phenotype only in the VHL expressed cells (Hela or 293T cells), but not in the 786-O/RCCJF cells, which has no VHL expression (Fig. 1). Here, we identified that VHL knock down causes higher cell motility in Hela cells and this can be inhibited by VHL overexpression in 786-O cells (Fig. 4). By identifying actin P70 as a hydroxylation target, we provide the mechanistic link between Pro70 hydroxylation and H73 methylation, and the regulation of

hydroxylation or VHL in the regulation of cell motility. In summary, we unraveled a novel molecular mechanism of F-actin formation and cell motility regulated by VHL, actin Pro70 hydroxylation and H73 methylation.

Figures:

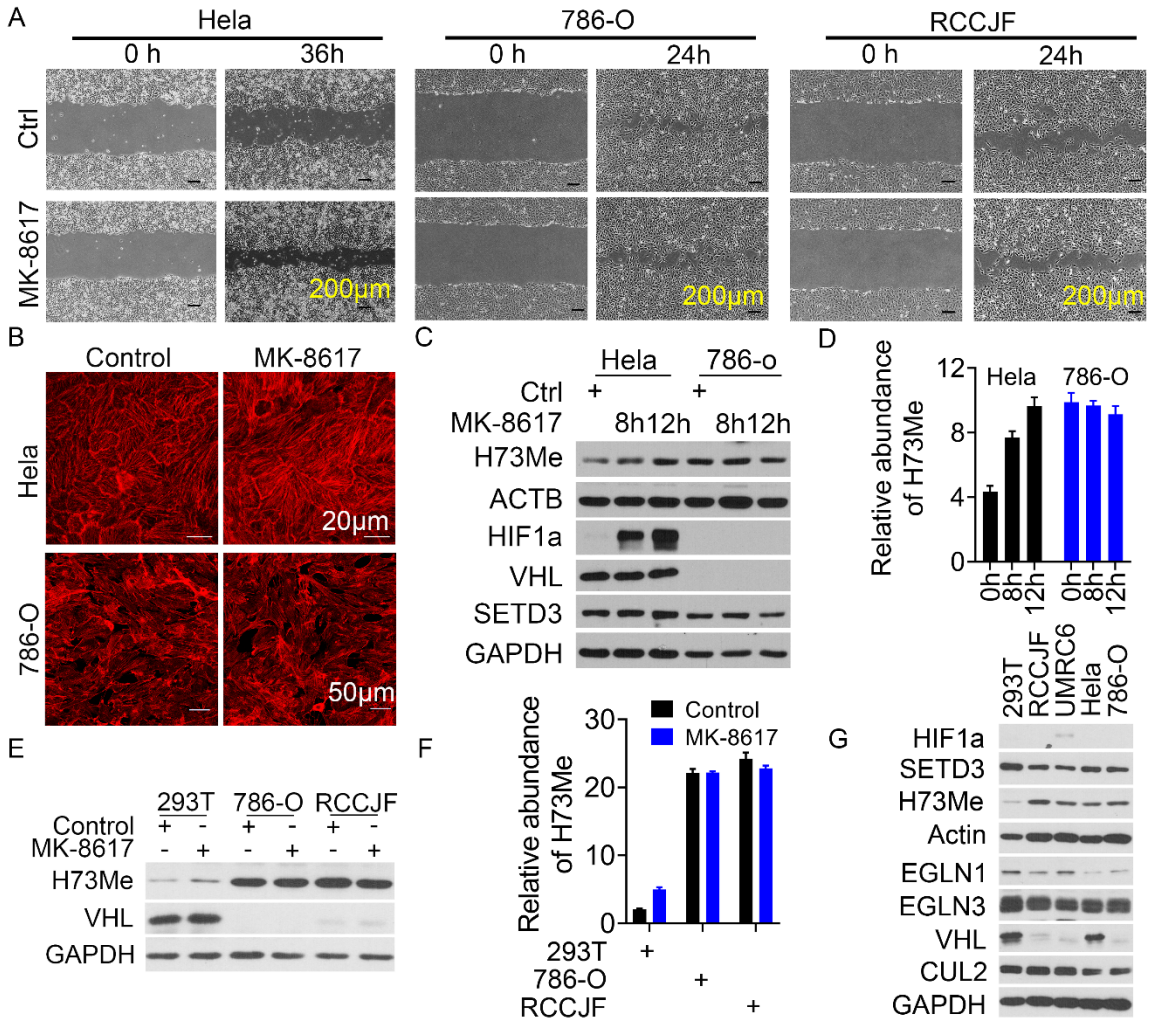

Fig. 1. MK-8617 Regulate Cell Motility and Actin H73 Methylation

A. MK-8617 promote cell motility in Hela cells but not in 786-O and RCCJF cells. Scratch assays were performed with Hela, 786-O and RCCJF cells under control and MK-8617 treatment (5μM). Representative images at indicated time points from two independent experiments. Scale bar, 200 μm.

B. MK-8617 increases β-actin polymerization in Hela cells but not in 786-O cells. HeLa and 786-O cells were treated with DMSO or MK-8617 (5 μM) for 24 h. Cells were fixed, permeabilized, stained with TRITC–conjugated phalloidin, and imaged by fluorescence microscopy. Scale bar, 50 μm.

(C-D). MK-8617 regulate H73Me-actin in Hela cells but not in 786-O cells. Hela and 786-O cells were treated with MK-8617 (5 μM) for indicated time points. Cell lysis were subjected to western blot (C) and H73Me level were quantified (D). GAPDH were used as the control.

(E-F). MK-8617 regulate H73Me-actin in Hela cells but not in 786-O and RCCJF cells. Hela, 786-O and RCCJF cells were treated with MK-8617 (5 μM) for 12 hours. Cell lysis were subjected to western blot (E) and H73Me level were quantified (F). GAPDH were used as the control.

G. Expression profile of HIF1a, SETD3, H73Me, Actin, EGLN1, EGLN3, VHL and CUL2 were measured in indicated cell lines. The same protein amount of indicated cells were subjected to western blot.

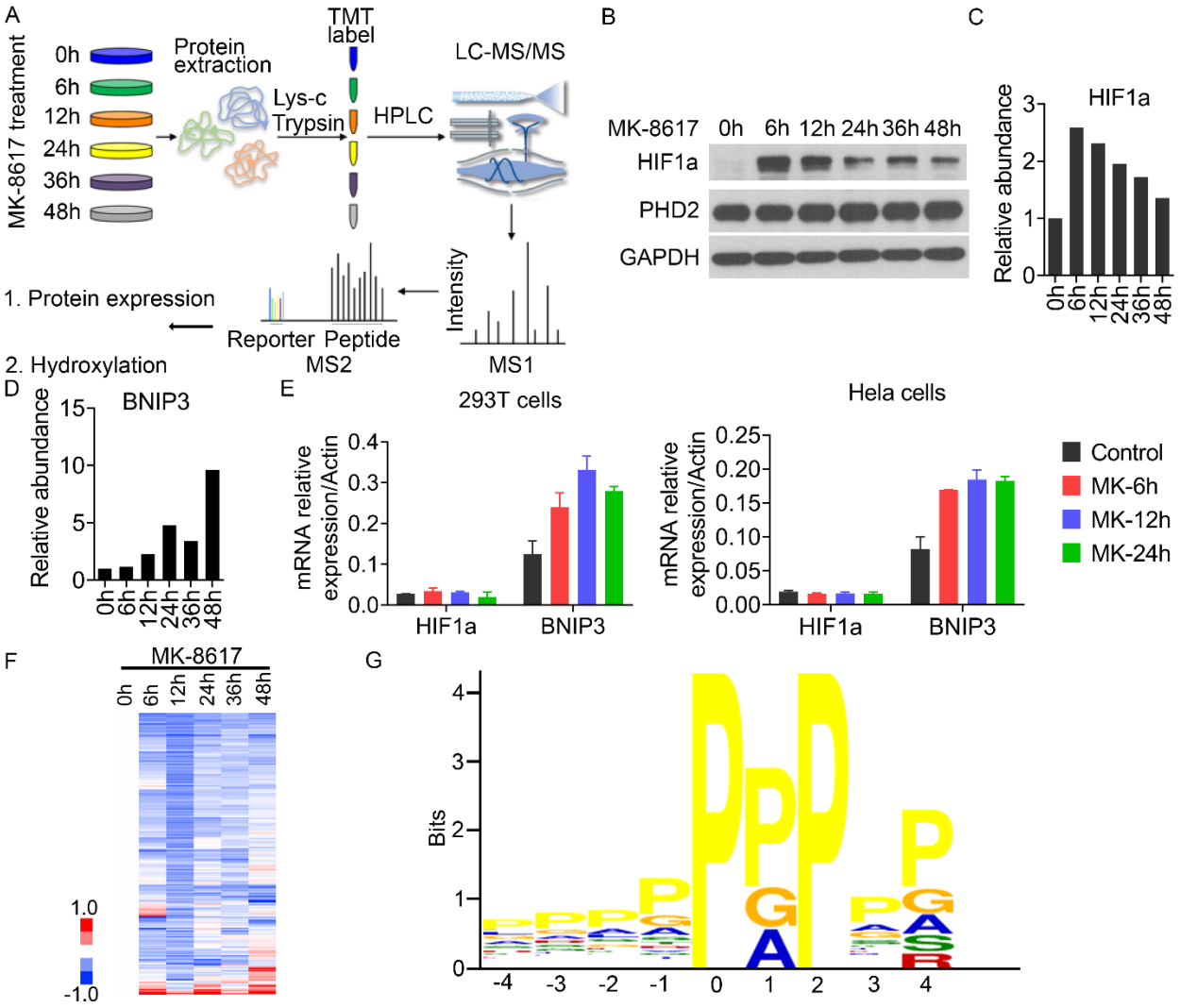

Fig. 2. Whole Hydroxylation Proteome Response upon MK-8617 Treatment

A. Overview of the workflow of the quantitative proteomic experiments. MCF10A cells were treated with MK-8617 (5 μM) upon the indicated time point. Cell lysis were collected and subjected to TMT6-mass spectrometry analysis.

B. HIF1a level were measured using western blot upon MK-8617 (5 μM) treatment as indicated time points. GAPDH as the internal control.

(C-D). TMT results of HIF1a and BNIP3 in MCF10A cells upon MK-8617 (5 μM) treatment of the indicated time points.

E. Real-time RT-PCR analyses of HIF1a and BNIP3 in HEK293T and Hela cells upon MK-8617 (5 μM) treatment as indicated time point. Data are shown as mean ± SEM.

F. Hierarchical clustering of protein hydroxylation profiles of MCF10A cells upon MK-8617 (5 μM) treatment as the indicated time point. The whole hydroxylation proteome decreased upon MK-8617 treatment.

G. Motif analysis of hydroxylation peptide were analyzed using website tool: "https://meme-suite.org/meme/tools/meme". The hydroxylation peptides were identified using TMT6-MS.

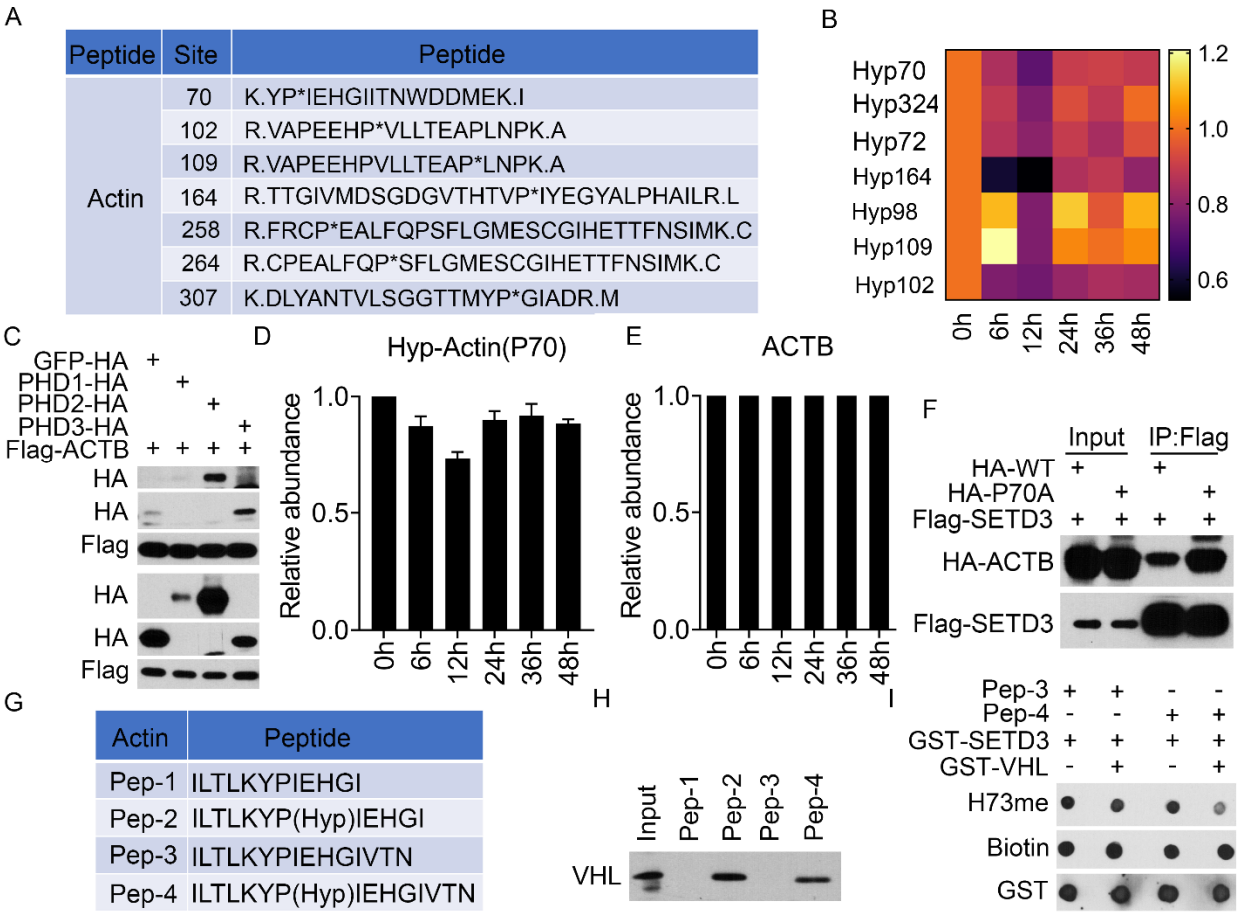

Fig. 3. Pro70-Hydroxylation Bind to VHL and inhibit His73 Methylation

A. Actin hydroxylation proline site and peptide identified by TMT6-MS experiment.

B. The level of hydroxylated-proline site in actin identified by TMT6-MS experiment upon MK-8617 (5 μM) treatment as the indicated time point.

C. Actin interact with PHD2 and PHD3. Flag-tagged actin was co-transfected with HA tagged GFP, PHD1, PHD2 and PHD3 into HEK293T cells. The lysates were subjected to

immunoprecipitation using the anti-Flag beads. PHD1-3 were probed using an antibody against the HA-tag.

(D-E). TMT results of Hyp70-actin and actin in MCF10A cells upon MK-8617 (5 μM) treatment of the indicated time points.

F. Pro70 mutant actin block the interaction of SETD3 and actin. HA-tagged actin (wild type and P70A mutant) was co-transfected with Flag-tagged SETD3 into HEK293T cells. The lysates were subjected to immunoprecipitation using the anti-Flag beads. HA-actin (WT and P70A mutant) were probed using an antibody against the HA-tag.

G. The peptides sequence synthesized, including non hydroxylated and hydroxylated (Hyp70) peptide.

H. VHL interact with hydroxylated Pro70-actin peptide but not the non-hydroxylated peptide. The peptides were incubated with cell lysis from HEK293T cells, and were subjected to streptavidin beads.

I. VHL blocked H73 methylation in the hydroxylated (Pro70) peptide. In vitro methylation assay with synthesized WT and Hydroxylated (Pro70) peptides.

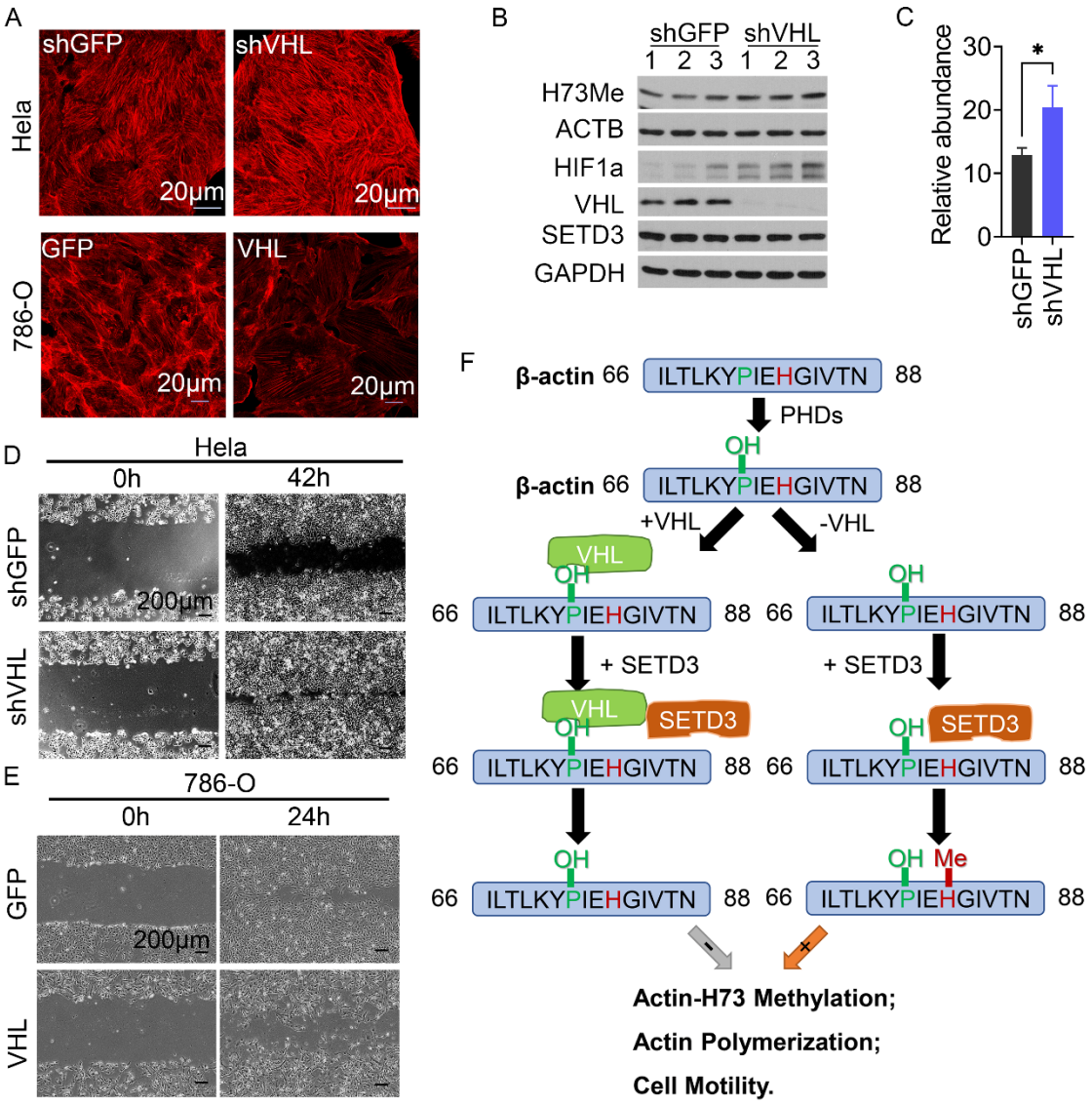

Fig. 4. VHL regulate cell motility through regulating H73Me

A. VHL regulate H73Me-actin level. VHL knockdown increases β-actin polymerization in Hela cells and VHL overexpress decrease β-actin polymerization in 786-O cells. Cells were fixed, permeabilized, stained with TRITC–conjugated phalloidin, and imaged by fluorescence microscopy. Scale bar, 20 μm.

(B-C). VHL knockdown increases H73Me and HIF1a level in Hela cells. Cell lysis were subjected to western blot (B) and H73Me level were quantified (C). GAPDH were used as the control. Data are shown as mean ± SEM, n=3 each group.

(D-E). VHL regulate cell motility. VHL knockdown increases cell motility in Hela cells and VHL overexpress decrease cell motility in 786-O cells. Representative images at indicated time points from two independent experiments. Scale bar, 200 μm.

F. The model of the mechanism about hydroxylation and VHL in regulating actin-H73Me, actin polymerization and cell motility. PHD2 or PHD3 hydroxylated actin at Pro70, the hydroxylated actin binds to VHL and block the interaction between actin and SETD3, which subsequentially inhibit the His73 methylation. The downregulated H73 methylation leads to the decreased F-actin formation and cell motility

Experimental procedures:

Chemicals and Antibodies

MK-8617 was purchased from Selleckchem (S8443), used at 5 μM final concentration; TRITC–conjugated phalloidin was purchased from (P1951), used at 1ug/ml, Isopropyl-β-D-thiogalactopyranoside (IPTG) (Dioxane-free) (Fisher Scientific, BP1755). β-Actin (13E5) Rabbit mAb (Cell Signaling, 4970s), Anti-H73Me antibody was provided by Dr. Or Gozani, Stanford University School of Medicine; Anti-GAPDH (Thermo Fisher Scientific, AM43000), HIF-1α Antibody (Cell Signaling, 3716s), VHL antibody (Cell Signaling, 68547s), SETD3 Antibody (Bethyl laboratories, A304-072A), PHD-2/Egln1 (D31E11) Rabbit mAb (Cell Signaling, 4835s), EGLN3/PHD3 Antibody (Novus Biologicals, NB100-139), Anti-CUL-2 Antibody (C-4) (Santa Cruz, sc-166506), Anti-Flag (Sigma, F7425), Anti-Flag (Sigma, F1804), Anti-HA (Cell Signaling Technology, 3724s), Donkey anti Rabbit IgG (GE healthcare, NA9340), Goat anti Mouse IgG (Millipore, AP181P), Monoclonal Anti-HA−Agarose (Sigma Aldrich, A-2095), ANTI-FLAG M2 Affinity Gel (Sigma Aldrich, A2220), GST (26H1) Mouse mAb (Cell Signaling, 2624s), HRP-Conjugated Streptavidin (Thermo Fisher, 21130).

Cell Culture

Hela (ATCC,), and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, sigma, D5796) supplemented with 10% fetal bovine serum (FBS, sigma, 12306C) and antibiotics (Gibco, 15240112) at 37 ℃ in a 5% CO2 incubator.

786-O, and RCCJF cells were cultured in RPMI-1640 medium (sigma, R8758) supplemented with 10% fetal bovine serum (FBS, sigma, 12306C) and antibiotics (Gibco, 15240112) at 37 ℃ in a 5% CO2 incubator.

HEK293T cells were cultured to 50–70% confluency in 10 cm dishes, and were then transfected using Lipofectamin-2000 (Sigma). The Plenti or pLKO.1 plasmid, VSVG, and delta8.9 were co-transfected at 8 μg, 6 μg, and 4 μg, respectively. The medium was changed 6 hours after the transfection. Viruses were collected twice at 24 h and 48 h after the transfection. Subsequently, 1 mL of virus was added to each well of Hela or 786-O cells in 6-well plates with Polybrene at a final concentration of 8 μg ml-1. After splitting the cells once, cells were infected with virus again using the same procedure. The medium was replaced after 48 h with a fresh growth medium containing 2 μg ml-1 blasticidin or puromycin.

Tandem Mass Tags (TMT) Mass Spectrometry

After the compound treatment, the cells were lysed using 1% SDS lysis buffer (1% SDS, 10 mM HEPES, pH 7.0, 2 mM MgCl2, 20 U ml-1 universal nuclease with added protease inhibitors and phosphatase inhibitors). Protein lysates were reduced by 2 mM dithiothreitol, and alkylated by adding iodoacetamide to a final concentration of 50 mM, followed by incubation in the dark for 20 min. The lysates were extracted by methanol-chloroform-precipitation and the proteins were solubilized in 8 M urea, then digested with Lys-C. The samples were then diluted to a final concentration of 2 M urea by the addition of 100 mM ammonium bicarbonate (pH 7.8). Proteins were digested overnight with sequencing-grade trypsin at a 1:100 (enzyme/substrate) ratio. Digestion was quenched by the addition of trifluoroacetic acid to a final concentration of 0.1%. For TMT MS samples, peptides were desalted using SepPak C18 columns (Waters) according to the manufacturer's instructions. Peptides were fractionated by using an off-line two-dimensional SCX-RP-HPLC (strong-cation-exchange reversed-phase HPLC) protocol. The sample were desalt using stage tips and subjected to MS analysis.

The MS samples were analyzed by LC-MS/MS experiments on an LTQ Velos Pro Orbitrap mass spectrometer (Thermo) using a top-20 CID (collision-induced dissociation) method. MS/MS spectra were searched against a composite database of the human protein database and its reversed complement using the Sequest algorithm. Search parameters allowed for a static modification of 57.02146 Da for cysteine and a dynamic modification of oxidation (15.99491 Da) on methionine, respectively. Search results were filtered to include <1% matches to the reverse database by the linear discriminator function using parameters including Xcorr, dCN, missed cleavage, charge state (exclude 1C peptides), mass accuracy, peptide length and fraction of ions matched to MS/MS spectra. Peptide quantification was performed by using the Core Quant algorithm (20).

Immunoblot Analysis

For immunoblot analysis, the cells were lysed using 1% SDS lysis buffer (1% SDS, 10 mM HEPES, pH 7.0, 2 mM MgCl2, 20 U ml-1 universal nuclease with added protease inhibitors and phosphatase inhibitors). Protein concentrations were measured using the BCA assay (23227, Thermo Fisher). The lysates were mixed with the 4X reducing buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue). Samples were boiled for 10 min and the same amounts of proteins were subjected to electrophoresis using the standard SDS-PAGE method. Proteins were then transferred to a 0.22 μm nitrocellulose membrane (GE, 10600001), and were blocked with a TBST buffer (25 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.05% Tween-20) containing 3% non-fat dried milk and incubated overnight with primary antibodies (1:1,000 dilution) at 4 °C and for 1h at room temperature with peroxidase-conjugated secondary antibodies (1:10000 dilution). Blots were developed using enhanced chemiluminescence and were exposed on autoradiograph films and were developed using standard methods.

The results were quantified using Image J and analyzed using Prism GraphPad Software.

In Vitro Methylation Assay

In vitro methyltransferase reactions were performed with 5 μg SETD3, 10 μg substrate peptide (hydroxylated or non-hydroxylated peptide), with/out VHL protein and 80 μM SAM (S-

adenosylmethionine) in a buffer that contained 50 mM Tris pH 8.0, 20 mM KCl and 5 mM $MgCl_2$ and were incubated at 30 °C overnight. Reactions were resolved by dot blot analysis.

CO-Immunoprecipitation Assay

HEK293T cells were rinsed once with ice-cold PBS 24-36 hours after the transfection and lysed in the ice-cold BLB lysis buffer (10 mM KPO4, pH 7.6, 6 mM EDTA, pH 8.0, 10 mM MgCl2, 0.5 % NP-40, 0.1 % BriJ-35, 0.1 % DOC, adjust pH to 7.4) or IP buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) added with EDTA-free protease inhibitors (Roche). The soluble fractions from cell lysates were isolated by centrifugation at 13,000 rpm for 10 minutes. For immunoprecipitations, 20 μl of a 50% slurry of anti-FLAG (A2220, Sigma) or anti-HA beads (A2095, Sigma) were added to each lysate and incubated with rotation for overnight at 4°C. Immunoprecipitants were washed three times with BLB lysis buffer. Immunoprecipitated proteins were denatured by the addition of 100 μl of sample buffer and boiling for 10 minutes and subjected to immunoblot analysis.

For the peptide and VHL co-immunoprecipitation assay, peptide (hydroxylated or no-hydroxylated peptide) were incubated with streptavidin beads for 1 hour, then incubated with HEK293T cell lysis for 2 hours. After 3 times washing, the sample were subjected to western blot analysis.

Immunostaining Assay

Cells were plated on micro cover glasses (Electron Microscopy Sciences 72226-01). After confluence, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 10 min. The cells were permeabilized (0.2% Triton X-100, 10 min), blocked in 3% BSA (Sigma, A9418), then incubated with Phalloidin–Tetramethylrhodamine B isothiocyanate (P1951) for one hour at room temperature. After the incubation with primary antibodies, slides were washed for three times, then stained with DAPI (Sigma, D9542), and sealed with mounting medium (FluorSave reagent, Millipore, 345789). Photomicrographs were taken of representative 10×, 20× or 40× magnification fields using confocal microscope (Confocal Zeiss LSM880 Airyscan).

Cell Scratch Assay

Cells were seeded onto 6 well plate and cultured overnight to reach 50% confluence. The cell layer was scratched with a sterile 1-ml pipette tip, labeled, and photographed at the indicated time points with microscope.

qPCR analysis of target gene expression

Hela or HEK293T cells were lysed with TRIzol (Thermo Fisher Scientific) after MK-8617 treatment for indicated time point. Total RNA was extracted according to the manufacturer's instructions. The RNA was reverse transcribed with SuperScript™ III One-Step RT-PCR System (Thermo Fisher). Real-time RT-PCR experiments were performed using a Power SYBR™ Green PCR Master Mix (Thermo Fisher). The following primers were used for the qPCR reactions: Actin: F 5'-AAATCGTGCGTGACATTAAGGA-3' and R 5'-GCCATCTCTTGCTCGAAGTC-3'; HIF-1a: F 5'- TATGAGCCAGAAGAACTTTTAGGC-3' and R 5'- CACCTCTTTTGGCAAGCATCCTG-3'; BNIP3: F 5'-TCAGCATGAGGAACACGAGCGT-3' and R 5'- GAGGTTGTCAGACGCCTTCCAA-3'.

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