Multi-omics Profiling to Assess Signaling Changes upon VHL Restoration and Identify Putative VHL Substrates in Clear Cell Renal Cell Carcinoma Cell Lines

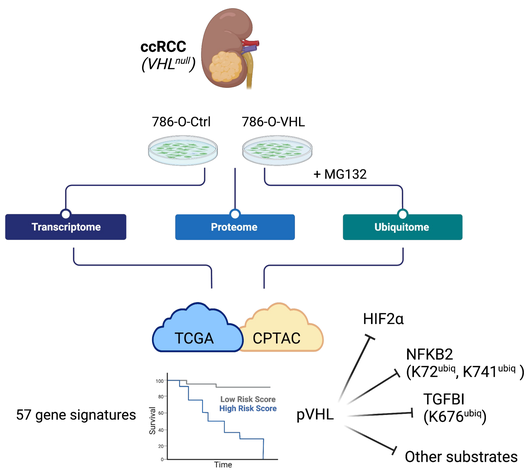
Xuechun Wang[1], Jin Hu[2], Yihao Fang[3], Yanbin Fu[4], Bing Liu[5*], Chao Zhang[6*], Shan Feng[3*], Xin Lu[2*]

[1]Department of Biological Sciences, Boler-Parseghian Center for Rare and Neglected Diseases, Harper Cancer Research Institute, University of Notre Dame, Notre Dame, IN 46556, USA
[2]Mass Spectrometry & Metabolomics Core Facility, Key Laboratory of Structural Biology of Zhejiang Province, Westlake University, Hangzhou, Zhejiang, China, 310024
[3]Department of the Applied and Computational Mathematics and Statistics, University of Notre Dame, Notre Dame, IN 46556, USA
[4]Department of Plastic and Reconstructive Surgery, Shanghai Institute of Precision Medicine, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, 200125
[5]Department of Urology, Eastern Hepatobiliary Surgery Hospital, Shanghai, China, 201805
[6]Shanghai YangZhi Rehabilitation Hospital (Shanghai Sunshine Rehabilitation Center), Shanghai Key Laboratory of Signaling and Disease Research, School of Life Sciences and Technology, Tongji University, Shanghai, China, 200092

Abstract

Inactivation of von Hippel-Lindau (VHL) is critical to clear cell renal cell carcinoma (ccRCC) and VHL syndrome. VHL loss leads to stabilization of hypoxia-inducible factor α (HIFα) and other substrate proteins, which together drive various tumor-promoting pathways. There is inadequate molecular characterization of VHL restoration in VHL-defective ccRCC cells. The identities of HIF-independent VHL substrates remain elusive. We reinstalled VHL expression in 786-O and performed transcriptome, proteome and ubiquitome profiling to assess the molecular impact. The transcriptome and proteome analysis revealed that VHL restoration caused downregulation of hypoxia signaling, glycolysis, E2F targets and mTORC1 signaling, and upregulation of fatty acid metabolism. Proteome and ubiquitome co-analysis together with the ccRCC CPTAC data enlisted 57 proteins that were ubiquitinated and downregulated by VHL restoration and upregulated in human ccRCC. Among them, we confirmed the reduction of TGFBI (ubiquitinated at K676) and NFKB2 (ubiquitinated at K72 and K741) by VHL re-expression in 786-O. Immunoprecipitation assay showed the physical interaction between VHL and NFKB2. K72 of NFKB2 affected NFKB2 stability in a VHL-dependent manner. Taken together, our study generates a comprehensive molecular catalog of VHL-restored 786-O model, and provides a list of putative VHL-dependent ubiquitination substrates including TGFBI and NFKB2 for future investigation.

Workflow



Workflow of multi-omics profiling to assess signaling changes upon VHL restoration with integrations of ccRCC TCGA and CPTAC databases. 57-signature proteins generated and lysine ubiquitinated sites identification.

Reference

1. Wang, Xuechun, Jin Hu, Yihao Fang, Yanbin Fu, Bing Liu, Chao Zhang, Shan Feng, and Xin Lu. 2022. "Multi-Omics Profiling to Assess Signaling Changes upon VHL Restoration and Identify Putative VHL Substrates in Clear Cell Renal Cell Carcinoma Cell Lines" Cells 11, no. 3: 472. https://doi.org/10.3390/cells11030472

Results

VHL restoration in 786-O depleted HIF2α and abrogated orthotopic tumor formation

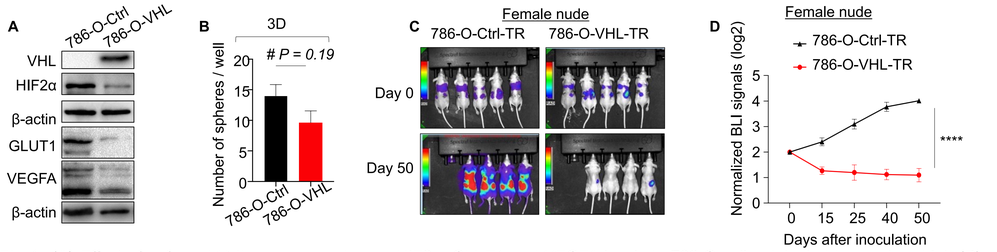


Fig 1 (A) Effect of enforced VHL overexpression on HIF2α, GLUT1 and VEGFA levels in 786-O cells, detected by western blot. **(B)** Comparison of 786-O-Ctrl and 786-O-VHL in forming 3D tumor spheres (C) Normalized BLI signals for female nude mice orthotopically injected with 786-O-Ctrl-TR (n=5) or 786-O-VHL-TR (n=4). BLI signals were normalized to the Day 0 signals and log2 transformed. (D) BLI images of Day 0 and Day 50 (endpoint).

VHL restoration downregulated HIF-driven pathways

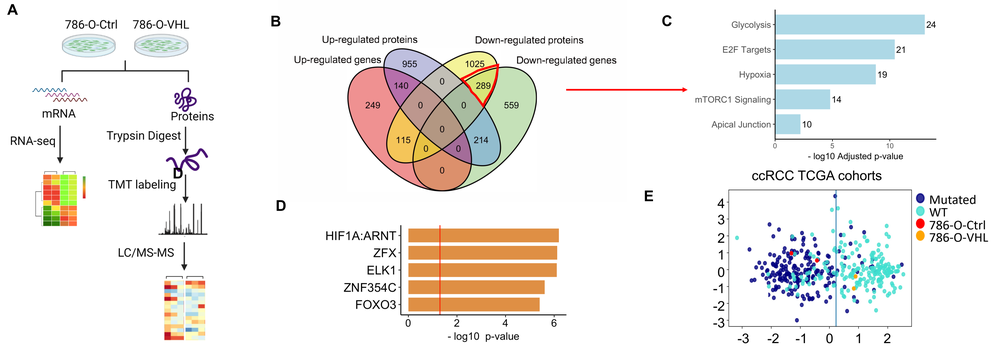


Fig 2 (A) Workflow of RNA-seq and mass spec proteomic profiling in 786-O-Ctrl and 786-O-VHL.**(B)** Venn diagram showing the overlap between significantly upregulated or downregulated mRNA and proteins between 786-O-Ctrl and 786-O-VHL (FDR < 0.05).**(C)** Top enriched pathways for 289 genes downregulated at both mRNA and protein levels by VHL restoration**. (D)**Top 5 transcription factor binding sites in the promoter region of downregulated mRNA and proteins analyzed by oPOSSUM. **(E)** LDA classification of the ccRCC TCGA tumors (n=403) to the two-dimensional transcriptome space with VHL status annotated.

Ubiquitome profiling identified potential VHL substrates

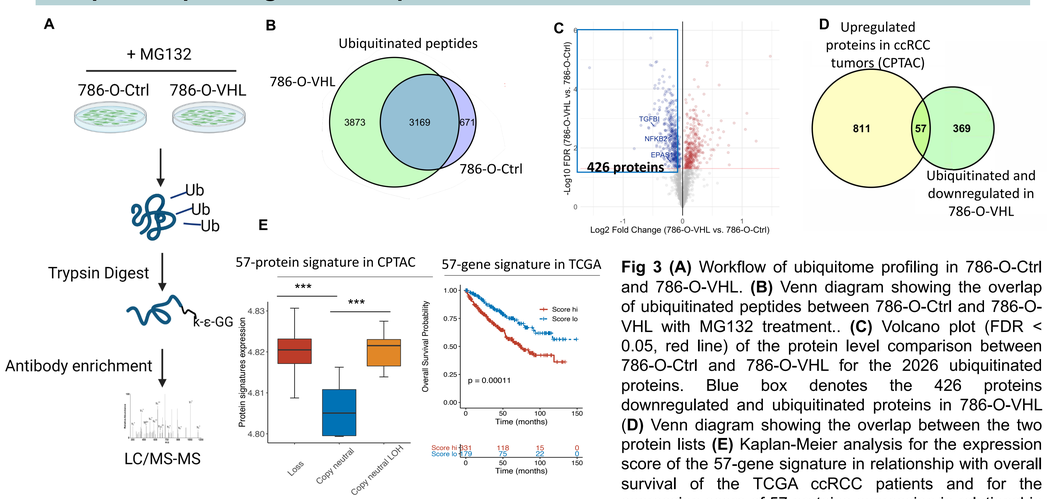


Fig 3 (A) Workflow of ubiquitome profiling in 786-O-Ctrl and 786-O-VHL. **(B)** Venn diagram showing the overlap of ubiquitinated peptides between 786-O-Ctrl and 786-O-VHL with MG132 treatment.. **(C)** Volcano plot (FDR < 0.05, red line) of the protein level comparison between 786-O-Ctrl and 786-O-VHL for the 2026 ubiquitinated proteins. Blue box denotes the 426 proteins downregulated and ubiquitinated proteins in 786-O-VHL **(D)** Venn diagram showing the overlap between the two protein lists **(E)** Kaplan-Meier analysis for the expression score of the 57-gene signature in relationship with overall survival of the TCGA ccRCC patients and for the expression score of 57 proteins expression in relationship with chr3 copy number conditions.

TGFBI and NFKB2 are putative VHL targeted proteins

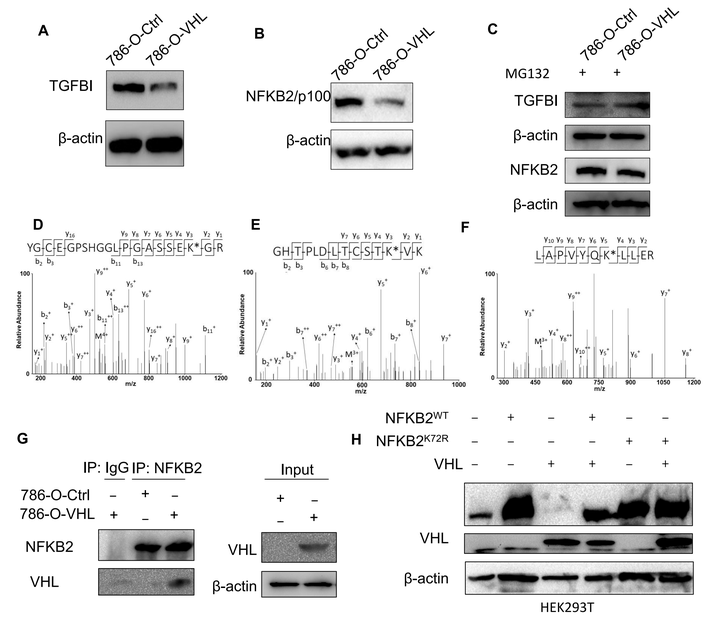


Fig 4 (A) Western blot of TGFBI in 786-O-Ctrl and 786-O-VHL cells..**(B)**. Western blot of NFKB2/p100 in 786-O-Ctrl and 786-O-VHL cells. **(C).**Western blot of TGFBI and NFKB2 in 786-O-Ctrl and 786-O-VHL cells after MG132 treatment. **(D).**The MS/MS result of the TGFBI peptide LAPVYQKubLLER containing the ubiquitinated lysine K676, the Δm between y5 and y4 corresponds to the mass of Lys residue plus diGly. **(E).**The MS/MS result of the NFKB2 peptide YGCEGPSHGGLPGASSEKubG containing the ubiquitinated lysine K72 and **(F)** GHTPLDLTCSTKubVK containing K741. **(G).**Immunoassay of the endogenous VHL-NFKB2 association in 786-O-Ctrl and 786-O-VHL cells, assessed by immunoprecipitation(IP) with immunoglobulin G (IgG), as a control, or with anti-NFKB2, followed by immunoblot analysis with anti-NFKB2 or anti-VHL.**(H)** Western blot of NFKB2 and VHL in HEK293T cells transiently transfected with NFKB2 (wild type), NFKB2 (K72R mutant), and/or VHL plasmids.

Conclusions

Our study is the first to integrate proteome and ubiquitome of VHL-restored cells to identify VHL substrates. To enhance the clinical relevance of the findings, we also integrate the ccRCC TCGA and CPTAC data during analysis. We generated 57 potential VHL substrates with clinical prognostic significance. Among the 57 proteins, we have focused on two VHL substrate candidates, transforming growth factor beta induced (TGFBI) and nuclear factor kappa B subunit 2 (NFKB2), both of which are likely important players in promoting ccRCC progression. Overall, through multi-omics studies we generate a list of putative non-HIFα targets for VHL, uncover potentially additional functional mechanisms of VHL and illuminate new therapeutic opportunities of VHL-deficient ccRCC.

Acknowledgments

UNIVERSITY OF NOTRE DAME | COLLEGE OF SCIENCE
BOLER-PARSEGHIAN Center for Rare & Neglected Diseases
HARPER CANCER RESEARCH INSTITUTE