Supplementary Information

The VHL protein-interacting deubiquitinating enzyme 2 deubiquitinates and stabilizes HIF-1α

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Supplementary Figures:

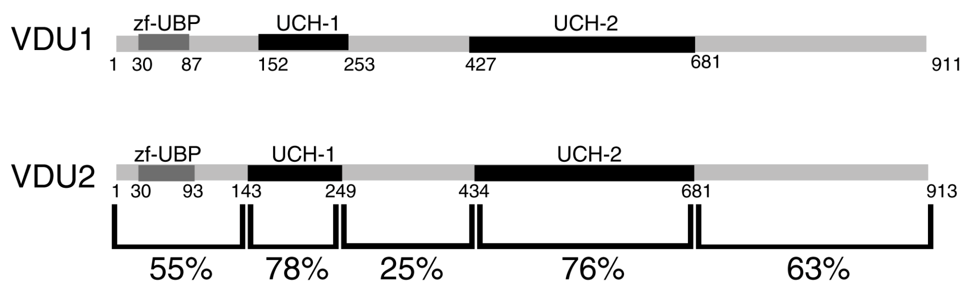

Fig. S1. The similarity and difference between VDU1 and VDU2 proteins. Schematic structures of VDU1 and VUD2 are shown here. Both proteins contain an N-terminal UBP-zinc finger (UBP-ZnF), and are highly conserved in the UCH1 and UCH2 domains. Percentages of amino acid identity in different regions of these two proteins are indicated in the diagram. VDU1 and VDU2 are identical in approximately 59% of amino acids overall with strong similarity in their N-termini and C-termini and a weaker similarity (25% identity) in the middle region between the UCH-1 and UCH2 domains.

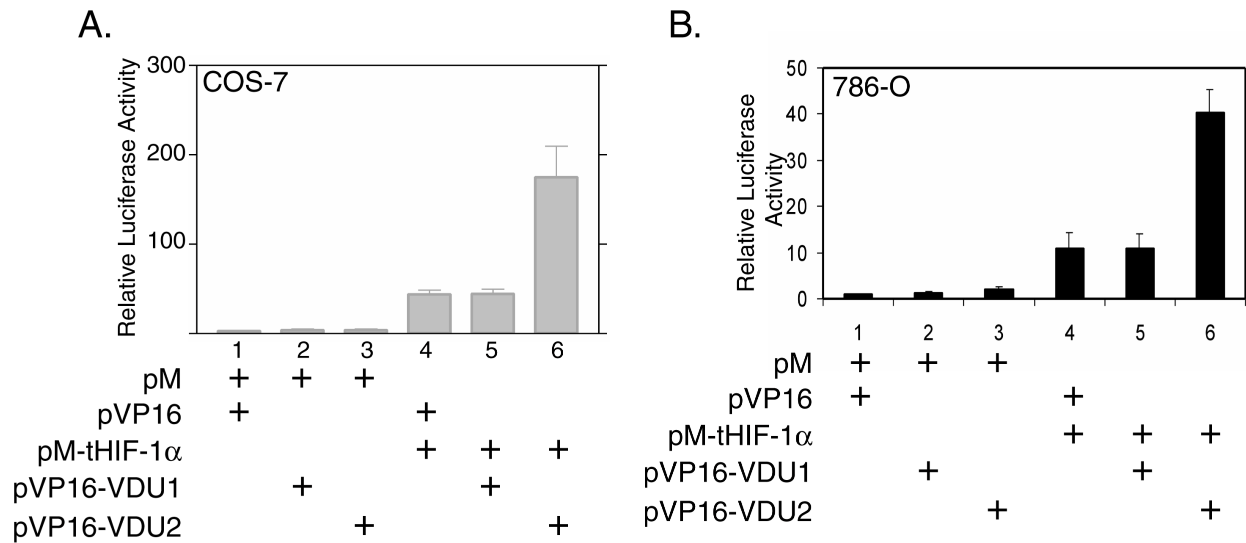

Fig. S2. VDU2 and HIF-1α interaction in a mammalian two-hybrid assay. In lane 5 or lane 6, pM-tHIF-1α (530-826) and pVP16-VDU1 or pVP16-VDU2 were co-transfected into COS-7 (**A**) or 786-O cells (**B**) together with reporter plasmid pG5CAT, respectively. Other lanes were served as negative controls including transfection with pM-BD and pVP16-AD empty vectors (lane 1), pM-BD and pVP16-VDU1 (lane 2), pM-BD and pVP16-VDU2 (lane 3), pVP16-AD and pM-tHIF-1α (lane 4).

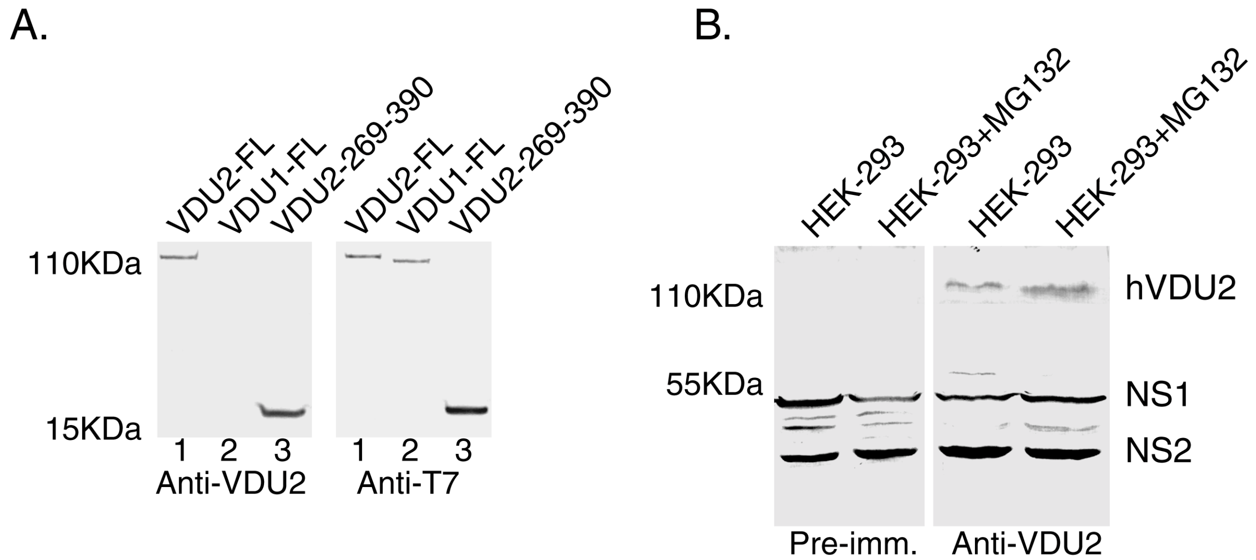

Fig. S3. Western blot analysis of *in vitro* translated proteins and endogenous VDU2 by anti-VDU2 antibody. (A) Proteins indicated on the top of the figure were translated *in vitro* with plasmid pET28c-VDU2, pET28c-VDU1, and pET28c-VDU2-269-390 using the TNT system (Promega), fractionated on a PAGE gel, and immunoblotted with anti-VDU2 antibody. Note that anti-VDU2 antibody has no cross reaction to the full-length VDU1. **(B)** The whole lysates from HEK-293 cells treated with or without MG132 were fractionated on PAGE gels and immunoblotted with pre-immunized control serum and anti-VDU2 antibody, respectively. Note that there are several non-specific bands labeled with NS and MG132 treatment increases the endogenous VDU2 protein level.

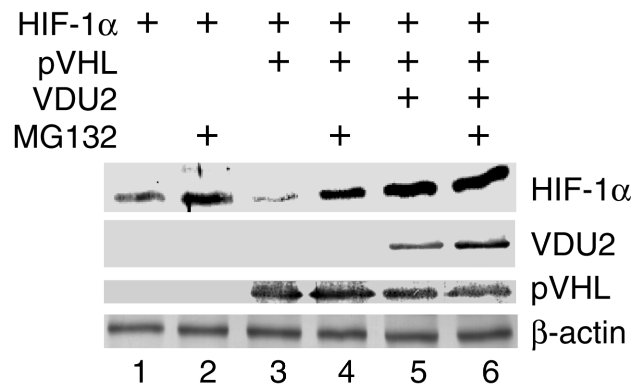

Fig. S4. Protection of HIF-1α from pVHL-mediated degradation by VDU2. Western blot analysis of extracts from 786-O cells transfected with HIF-1α (lanes 1 and 2), HIF-1α and pVHL (lanes 3 and 4), HIF-1α, pVHL and VDU2 (lanes 5 and 6), with (lanes 1, 3, and 5) or without (lanes 2, 4, and 6) MG132 treatment is demonstrated in the figure. Note that overexpression of pVHL significantly induced HIF-1α degradation and this phenomenon could be inhibited upon the coexpression of VDU2. Meanwhile, pVHL-induced HIF-1α degradation was also inhibited by MG132, a specific inhibitor of the 26S proteasome.

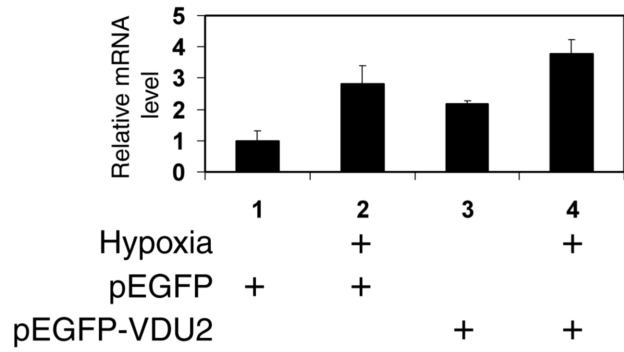

Fig. S5. The effects of overexpression of VDU2 on heme oxygenase 1 (HO-1) mRNA levels. The GFP-VDU2 or GFP stable-transfected HEK-293 cells were cultured under normoxic or hypoxic (1% O_2) conditions. HO-1 mRNA levels were determined by real-time PCR and normalized against β-actin levels.

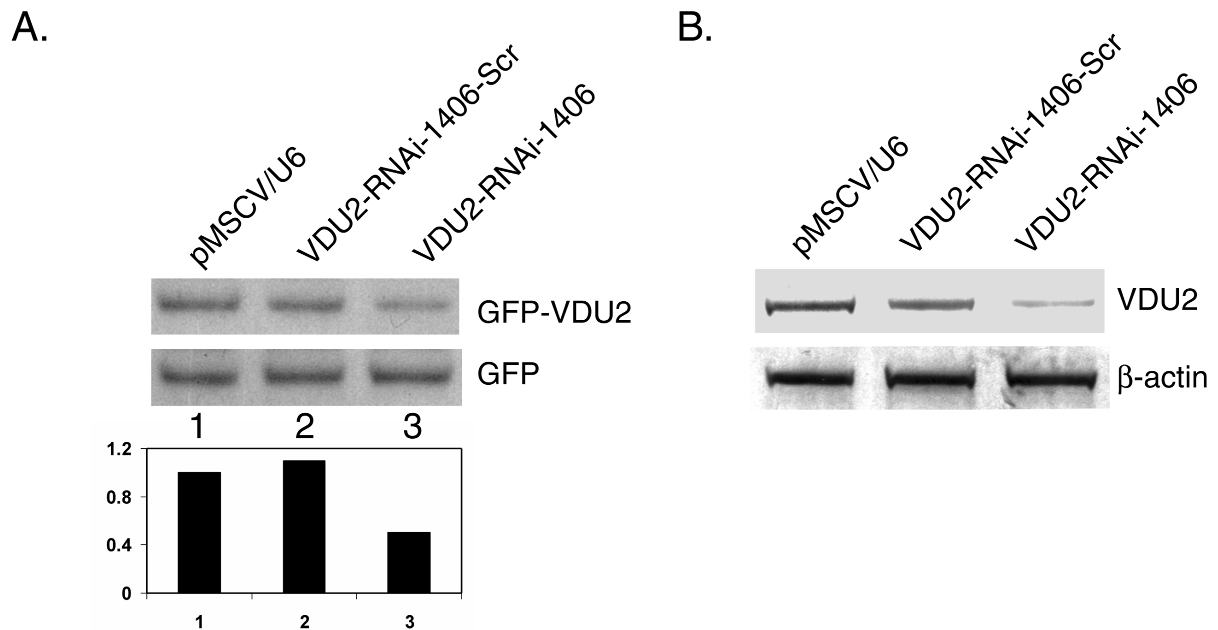

Fig. S6. Down-regulation of VDU2 with RNAi constructs. (A) HEK-293 cells were transfected with RNAi plasmids targeting VDU2 as indicated in the figure together with pEGFP-VDU2 and pEGFP at a ratio of 6:2:1. Cell lysates were collected and tested by immunoblotting with GFP antibody. The densities of GFP-VDU2 bands were quantified and normalized with the density of GFP bands. Note that whereas VDU2-RNAi-1406-Scr (scrambled siRNA negative control) had no effect, VDU2-RNAi-1406 dramatically reduced the GFP-VDU2 protein level. (B) HEK-293 cells were transfected with RNAi plasmids targeting VDU2 as indicated in the figure and cell lysates were collected and tested by immunoblotting with anti-VDU2 antibody. Note that whereas VDU2-RNAi-1406-Scr (scrambled siRNA negative control) had no effect, VDU2-RNAi-1406 dramatically reduced the endogenous VDU2 protein level.

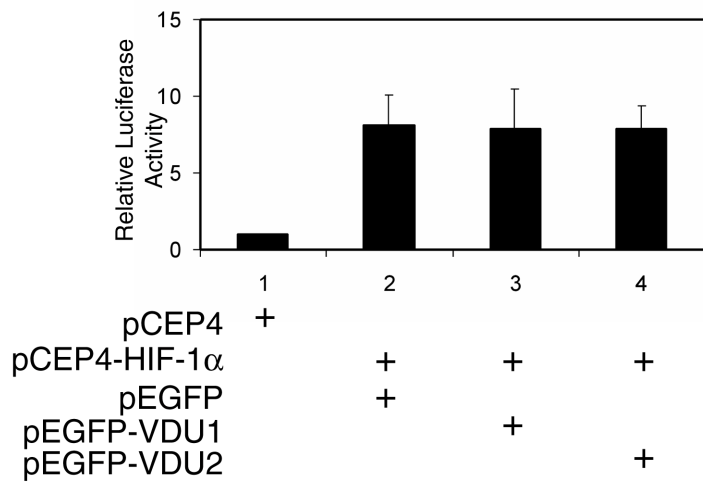

Fig. S7. Regulation of HIF-1α-mediated HRE-Luciferase reporter gene activity by VDUs in 786-O cells. Various combinations of expression plasmids as indicated with 300 ng of pRE-tk-LUC and 10 ng pRLSV40-LUC were transfected into 786-O cells. Twenty-four h after transfection, cells were harvested and measured in a dual-luciferase assay.

Supplementary Methods:

GST pull-down assay. The detailed method was described before (Li *et al.,* 2002a). In brief, BL21 cells transformed with GST-tHIF-1α were cultured at 30°C until A_{600} reached 0.8. The fusion proteins were then induced with IPTG (Isopropyl-β-D-thiogalactopyranoside), and purified using Glutathione Sepharose 4B MicroSpin Columns (Amersham Pharmacia). The amount of GST-fusion protein was quantified using the Bradford Assay (Bio-Rad). Equal amounts of GST-fusion protein and 5 µl of *in vitro* translated VDU1, VDU2, and VDU2 fragments were used for the pull-down assay. The samples were resolved on a 5% SDS-PAGE gel followed by Western blot analysis.

***In vitro* and *in vivo* co-immunoprecipitation**. As described previously (Li *et al.,* 2002a), the pre-cleared protein A/G-agarose (Santa Cruz) was mixed with 5 µl of each *in vitro* translation product, 10 µl of antibody and 470 µl of co-immunoprecipitation buffer. The mixture was incubated at 4°C overnight on a rotary shaker. The protein A/G-agarose was washed three times. The bound proteins were eluted by boiling for 3 min in 1× SDS loading buffer and then separated on an 8% SDS-PAGE gel. For *in vivo* co-immunoprecipitation assays, anti-HIF-1α monoclonal antibody or anti-VDU2 polyclonal antibody was added into the pre-cleared cell lysates (500 µg of total protein) and the mixtures were gently rocked for 2 hr at 4°C. 30 µl of protein A/G agarose was then added into the mixture followed by rotating at 4°C for overnight. The protein A/G agarose was spun down and washed thoroughly three times. The precipitates were resolved on an 8% SDS-PAGE gel and subjected to Western blot analysis using an anti-HIF-1α antibody or anti-GFP antibody.

Deubiquitination assay. The recombinant VDU1 or VDU2 protein was expressed in BL21(DE3) cells transformed with pET28c-VDU1 or pET28c-VDU2 and purified by using Ni-NTA spin columns (Qiagen). For the preparation of ubiquitinated HIF-1α, the *in vitro* translated HIF-1α was ubiquitinated in an *in vitro* ubiquitination system with ATP regeneration system and COS-7 extract as described before (Li *et al.,* 2002a and 2002b). The ubiquitinated HIF-1α was purified by immunoprecipitation using anti-ubiquitin antibody. The purified ubiquitinated HIF-1α protein was incubated with recombinant VDU1 or VDU2 protein (200 ng) in a deubiquitinating buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT) for the indicated times at 30°C. The reactions were resolved on SDS-PAGE and Western blot was performed. For *in vivo* ubiquitination assay, pCEP4-HIF-1α and HA-Ubiquitin plasmids together with or without pCMX-VHL19 and/or pEGFP-C3-VDU1 or pEGFP-C3-VDU2 were transiently co-transfected into HEK 293 cells with or without MG132 treatment.

DNA vector-based RNA interference. Small interfering RNA (siRNA) target sites were selected by scanning the cDNA sequence for AA dinucleotides via siRNA target finder (Ambion). Those 19-nucleotide segments that start with G immediately downstream of AA were recorded and then analyzed by BLAST search to eliminate any sequences with significant similarity to other genes. The siRNA inserts, containing selected 19-nucleotide coding sequences followed by a 9-nucleotide spacer and an inverted repeat of the coding sequences plus 6 Ts, were made to double-stranded DNAs with *Apa*I and *Eco*RI sites by primer extension, and then subcloned into plasmid pMSCV/U6 at the *Apa*I/*Eco*RI site. The corresponding oligonucleotides for the VDU2-RNAi-1406 insert are 5'- ACGTTCCAGGACTTATCACTTCAAGAGA

GTGATAAGTCCTGGAACGTTTTTTT-3' (forward) and 5'-AATTAAAAAAACGTT CCAGGACTTATCACTCTCTTGAAGTGATAAGTCCTGGAACGTGGCC-3' (reverse). A nonfunctional siRNA (VDU2-RNAi-1406-Scr), which contains scrambled nucleotide substitutions at the 19-nucleotide targeting sequence of VDU2-RNAi-1406, were constructed as a negative control (forward: 5'-ACGTTCCAGGACTTATCACTTCAAGAGAGTGATAA GTCCTGGAACGTTTTTTT-3' and reverse: 5'-AATTAAAAAAACGTTCCAGGAC TTATCACTCTCTTGAAGTGATAAGTCCTGGAACGTGGCC-3'). Twenty-four hours after transfection, the protein levels of GFP-VDU2 were evaluated by immunoblot analyses with anti-GFP antibody.

RNA isolation and RT-PCR.

Total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. 1 µg of RNA was subjected to reverse transcription using Superscript II (Invitrogen). The reactions were incubated at 42°C for 90 min. The VEGF primers, 5'-GGA GTG TGT GCC CAC CGA GGA GTC CAA C-3' (sense) and 5'-GGT TCC CGA AAC CCT GAG GGA GGC T-3' (antisense), the HO-1 primers, 5'- ATG ACA CCA AGG ACC AGA GC-3' (sense) and 5'-AGA CAG CTG CCA CAT TAG GG-3' (antisense), and the β-actin primers, 5'-GAT CAT TGC TCC TCC TGA GC-3' (sense) and 5'- TGT GGA CTT GGG AGA GGA CT-3' (antisense) were used in our experiments. Reactions each containing 5 µl of 10-fold diluted reverse transcription product, 25 µl of 2x SYBR green PCR mix, 1 µl of each primer (10 µM), and 18 µl water were performed on an iCycler iQ multicolor real-time PCR system (Bio-Rad) and cycling condition was 95°C for 10 min, followed by 50 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C

for 30 s. Each sample was run in triplicate. Data were analyzed by iCycler iQ software (Bio-Rad).

Determination of VEGF protein levels by ELISA.

Equal numbers of cells were plated on 6-well plates and grown overnight. Cells were transfected with different combinations of plasmids as indicated. Thirty-six hr after transfection, cell supernatants were collected, clarified by centrifugation at 2,000 rpm for 5 min, and stored at -70°C. Concomitantly, the total cell numbers in each well were determined after trypsinization. VEGF concentrations in the culture medium were determined in duplicate by ELISA using the reagents supplied with the Quantikine Human VEGF Immunoassay kit (R&D Systems). Differences in VEGF concentrations in different groups of cell medium were normalized for the total numbers of cells (Li *et al*., 2003).