Supplementary Material (ESI) for Chemical Communications

-Supporting Information-

Photoactivable peptides for identifying enzyme-substrate and protein-protein interactions.

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Contents: Overview of the photo cross-linking strategy; Synthetic schemes, procedures and characterization of the probes **CP** and **HCP**; Expression and purification of proteins (PHD2 and the VCB complex); Biological validation of **HCP** (FRET-based VCB binding assay); Cell cultures and preparation of whole cell lysates, photo cross-linking experiments; Affinity enrichment experiments; MALDI-TOF analysis of photo-affinity tagging; SDS-polyacrylamide gel electrophoresis (SDS-PAGE); Western blot analysis of photo-affinity tagging; Protein digestion; Nano LC-MS/MS; Table of proteins identified from SDS-PAGE gel.

Overview of the photo cross-linking strategy

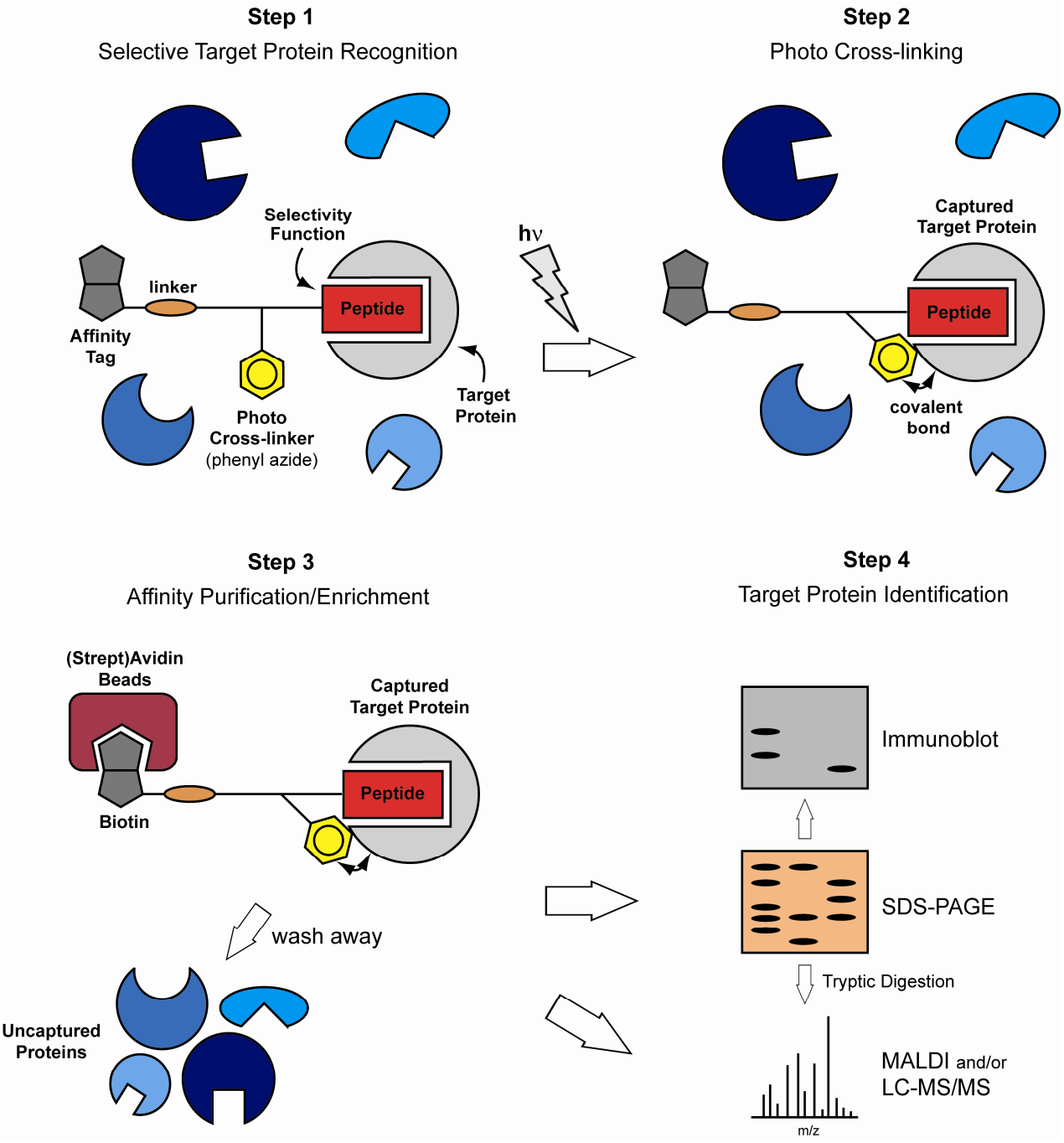


Fig. S1 The photo cross-linking strategy applied to peptide-protein interactions.

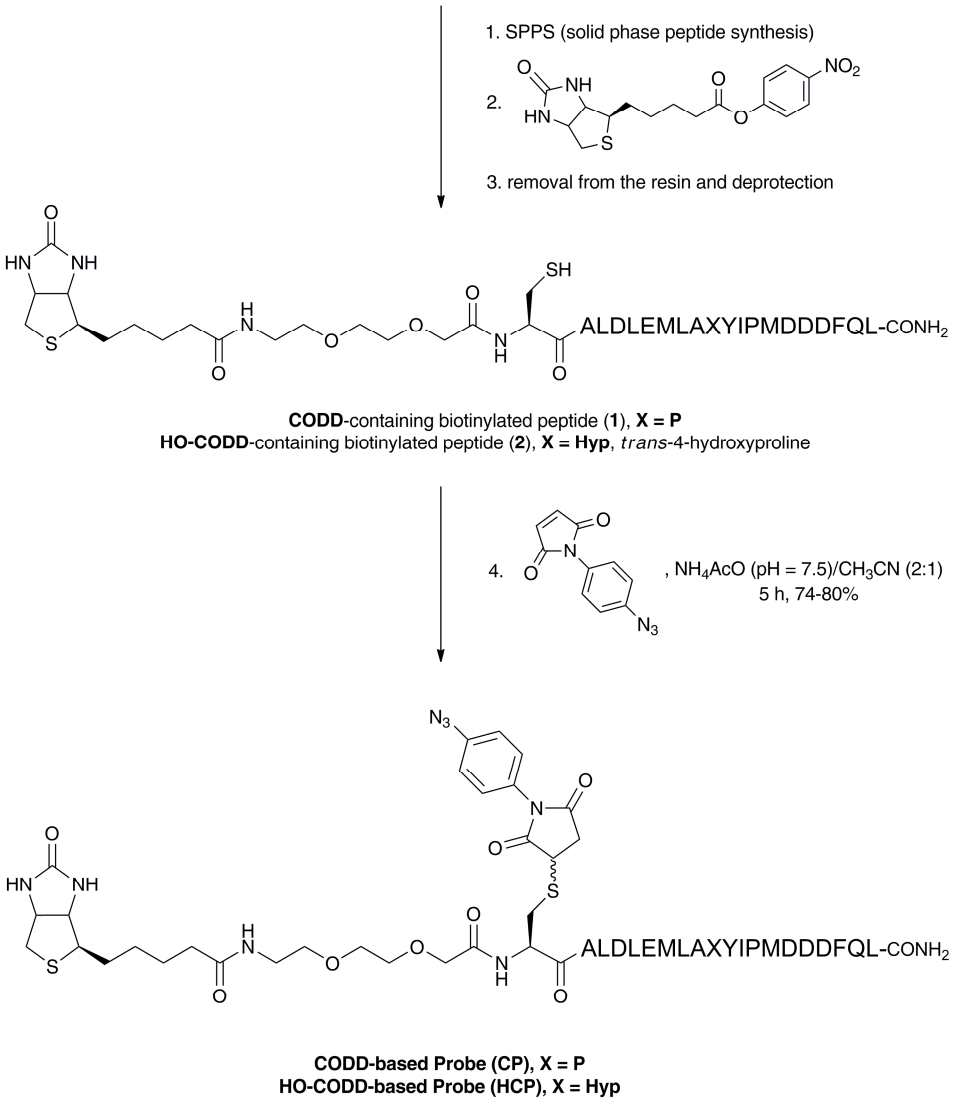
General description of the synthesis of the probes

The routes for the synthesis of the peptide probes (**CP** and **HCP**) are shown in Schemes S1 and S2. The CODD- and HO-CODD-containing intermediate peptides (**1** and **2**) were prepared by the solid phase peptide synthesis (SPPS) (automated peptide synthesizer CS-Bio CS336) using a PL-AMS resin (Polymer Laboratories), a Rink amide linker, and a standard 9-fluorenylmethoxycarbonyl (Fmoc)/ *N*,*N*′-diisopropylcarbodiimmide (DIC)/1-hydroxybenzotriazole (HOBt) strategy. Fmoc removal was carried out by treatment with a mixture 20% piperidine/20% DMSO/60% DMF. The peptides were *N*-terminally biotinylated using a biotin *p*-nitrophenyl ester (Novabiochem), and the final cleavage from the resin (CF_3COOH:phenol:H_2O:triisopropylsilane 88:5:5:2) gave peptides as *C*-terminal primary amides. Without purification the (HO)-CODD-containing biotinylated intermediate peptides (**1** and **2**) reacted with 1-(4-azidophenyl)-1*H*-pyrrole-2,5-dione (**5**) at room temperature in a 2:1 mixture NH_4AcO buffer (pH = 7.5)/CH_3CN to give **CP** and **HCP** (Scheme S1).

5 was prepared from commercially available *tert*-butyl 4-aminophenylcarbamate (Scheme S2) (for an alternative synthesis of **5** see reference 1). Reaction with maleic anhydride gave the (*Z*)-4-(4-(*tert*-butoxycarbonylamino)phenylamino)-4-oxobut-2-enoic acid (**3**) which in turn was converted into the *tert*-butyl 4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenylcarbamate (**4**) via reaction with sodium acetate in acetic anhydride at 95 °C. Deprotection of **4** with CF_3COOH in dry CH_2Cl_2 followed by the treatment with $NaNO_2$ and NaN_3 in a mixture AcOH:H_2O (1:1) provided **5** (Scheme S2).

The CODD peptide (DLDLEMLAPYIPMDDDFQL) was from Peptide Protein Research Ltd., Fareham, U.K. The hydroxylated CODD (HO-CODD) peptide (DLDLEMLAHypYIPMDDDFQL, where Hyp represents the *trans*-4-hydroxyproline) was prepared as reported.[2] The *N*-terminally biotinylated CODD and HO-CODD peptides were prepared as reported.[3]

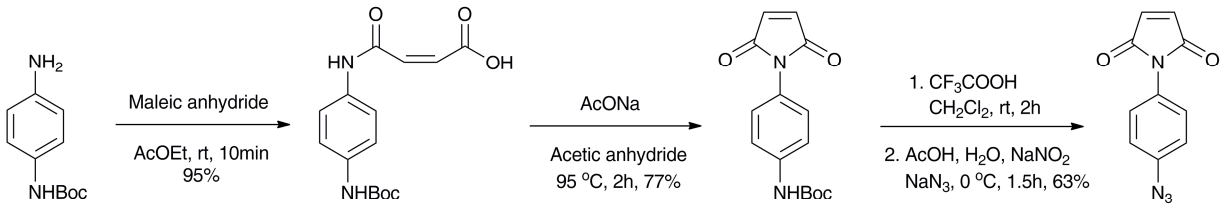
Scheme S1



CODD-containing biotinylated peptide (**1**), **X = P**
HO-CODD-containing biotinylated peptide (**2**), **X = Hyp**, *trans*-4-hydroxyproline

CODD-based Probe (CP), X = P
HO-CODD-based Probe (HCP), X = Hyp

Scheme S2

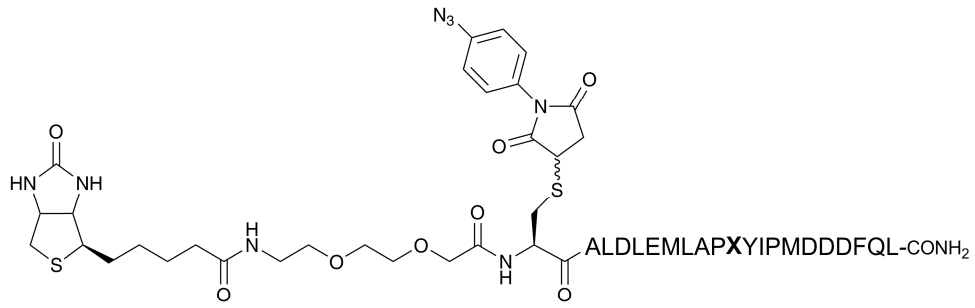


3 **4** **5**

Synthetic methods and analysis

^{1}H-NMR spectra were recorded on a Bruker AV400 (400 MHz) spectrometer and referenced to residual solvent peaks. Chemical shifts are quoted in parts per million (ppm). Assignments were made on the basis of chemical shifts, coupling constants (*J*), ^{13}C, COSY, and HMQC data. Resonances are described as s (singlet), d (doublet) and br. s. (broad singlet). Coupling constants are given in Hz and are reported to the nearest 0.5 Hz. ^{13}C-NMR spectra were recorded on a Bruker AV400 spectrometer and a Bruker AV500 spectrometer (fitted with an inverse cryoprobe for ^{13}C observation) and referenced to $CDCl_3$ or DMSO-*d*6. Infra-red (IR) spectra were recorded on a Bruker Tensor 27 FT-IR spectrophotometer as KBr disks. Selected absorption maxima (νmax) are given in wavenumbers (cm^{-1}). High resolution mass spectra (HR-MS) were recorded using a Bruker MicroTOF. Microanalysis experiments were performed by Mr. Stephen Boyer (London Metropolitan University). Melting points were recorded using a Gallenkamp Hot Stage apparatus. All moisture or oxygen sensitive reactions were carried out under a nitrogen atmosphere. Oven-dried glassware was used throughout. Anhydrous solvents were obtained from solvent stills in the Chemistry Research Laboratory and were activated by passing over a short column of activated alumina. Cooling was performed in ice-water baths (0 °C). Reagents were from Aldrich, Fluka, Lancaster or TCI fine chemical suppliers. All other chemicals were used as received. Thin layer chromatography (TLC) was performed on Merck DC-Kieselgel 60 F254 0.2 mm pre-coated plates with fluorescence indicator. Visualization of spots was achieved using UV light (254 nm) and by developing in an acidic solution of ceric ammonium nitrate (CAN) and ammonium heptamolybdate (Seebach's Magic Stain), followed by heating.

General procedure for the preparation of the peptide probes CP and HCP



CODD-based Probe (**CP**) **X** = **P**
HO-CODD-based Probe (**HCP**) **X** = **Hyp**, *trans*-4-hydroxyproline

Example: CODD-based Probe (CP)

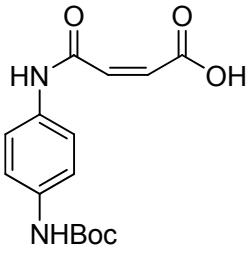
To a solution of the CODD-containing biotinylated peptide (**1**, 25.1 mg, 9.4 μmol, 1 eq.) in 30 mL of a 2:1 mixture NH_4AcO buffer (pH = 7.5)/CH_3CN were added 107.7 μL of an 100 mM DMSO solution of the 1-(4-azidophenyl)-1*H*-pyrrole-2,5-dione (**5**) (10.8 μmol, 1.15 eq.). The resultant mixture was stirred at room temperature under nitrogen atmosphere for 5 h. After the completion of the reaction, checked by observing the disappearance of the starting material by MALDI-TOF, the crude mixture was lyophilized and the resultant residue was re-suspended in 50 % aqueous-acetonitrile (500 μL) and purified using a Vydac® 218TP C18 Reversed Phase (250 mm x 22 mm, 10-15μm) pre-equilibrated in 10% aqueous-acetonitrile before running the gradient shown in Table S1. Elution was monitored using a Waters Micromass Quattro micro mass spectrometer (in positive mode) equipped with a Waters 2777 sample manager and a Waters 1525μ Binary HPLC pump system. Fractions with masses corresponding to anticipated product were collected (5-10 mL) and lyophilized to give 21.8 mg of the desired **CP** (yield 80%).

Table S1. LC-MS gradient used to purify **CP** and **HCP**	
Time (minutes)	% Acetonitirile
0	10
10	30
15	40
25	85
35	100
40	100
45	10
60	10

CODD-based probe (**CP**): *m/z* 1449.79 (M + $2H^+$)/2, calculated *m/z* 1449.13 (M + $2H^+$)/2; *m/z* 967.15 (M + $3H^+$)/3, calculated *m/z* 966.42 (M + $3H^+$)/3.

HO-CODD-based probe (**HCP**): *m/z* 1458.28 (M + $2H^+$)/2, calculated *m/z* 1457.12 (M + $2H^+$)/2; *m/z* 972.48 (M + $3H^+$)/3, calculated *m/z* 971.75 (M + $3H^+$)/3; yield 74%.

(*Z*)-4-(4-(*tert*-Butoxycarbonylamino)phenylamino)-4-oxobut-2-enoic acid (3)



To a stirred solution of commercially available *tert*-butyl 4-aminophenylcarbamate (1.40 g, 4.57 mmol, 1 eq.) in ethyl acetate (10 mL) was added a solution of commercially available maleic anhydride (0.66 g, 4.57 mmol, 1 eq.) in ethyl acetate (3 mL). The resulting suspension was stirred for 10 minutes at room temperature. Solids were filtered off, resuspended in CH_2Cl_2 (20 mL) and dried *in vacuo* to afford the compound **3** as a yellow solid (1.95 g, 95%).

^{1}H-NMR (400 MHz, DMSO-d_6): δ 1.46 (s, 9H), 6.30 (d, *J*=12 Hz, 1H), 6.45 (d, *J*=12 Hz, 1H), 7.40 (d, *J*=8.5 Hz, 2H), 7.45-7.58 (d, 2H), 9.33 (brs, 1H), 10.40 (s, 1H), 13.37 (brs, 1H) ppm.

^{13}C-NMR (100 MHz, DMSO-d_6): δ 28.2 (CH_3), 79.0 (C), 118.4 (ArCH), 120.1 (ArCH), 131.0 (olefinic CH), 131.4 (olefinic CH), 132.8 (ArC), 135.8 (ArC), 152.8 (CO), 162.9 (CO), 166.8 (CO) ppm.

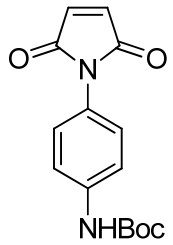
mp: 173-175 ºC (lit.[4] mp 165 ºC)

IR (KBr disk): 3364, 1701, 1521, 1403, 1314, 1239, 1164 cm^{-1}.

HR-MS (ESI^-): calcd. for $C_{15}H_{17}N_2O_5$ ($M-H^-$), 305.1143; found, 305.1142.

Elemental analysis: calcd. for $C_{15}H_{18}N_2O_5$, C, 58.82; H, 5.92; N 9.15; found, C, 58.91; H, 5.82; N, 9.06.

***tert*-Butyl 4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenylcarbamate (4)**



A stirred suspension of (*Z*)-4-(4-(*tert*-butoxycarbonylamino)phenylamino)-4-oxobut-2-enoic acid (1.80 g, 5.88 mmol, 1 eq.) in acetic anhydride (10 mL) was heated to 95 °C. Then, anhydrous sodium acetate (1.56 g, 5.88 mmol, 1 eq.) was added and the reaction was stirred for 2 hrs. The solution was allowed to stand overnight to give yellow, crystalline precipitate. Water (50 mL) was added and the mixture stirred until most acetic anhydride had hydrolyzed. The precipitate was filtered off and washed with satd. $NaHCO_3$ and water. The resultant solid was redissolved in CH_2Cl_2, dried (Na_2SO_4) and concentrated *in vacuo* to afford the compound **4** (1.30 g, 77%) as a pale yellow solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.52 (s, 9H), 6.62 (s, 1H), 6.83 (s, 2H), 7.25 (d, *J*=9.0 Hz, 2H), 7.46 (d, *J*=9.0 Hz, 2H) ppm.

^{13}C-NMR (100 MHz, $CDCl_3$): δ 28.3 (CH_3), 80.8 (C), 118.7 (ArCH), 125.7 (ArC), 126.8 (ArCH), 134.1 (olefinic CH), 138.1 (ArC), 152.5 (CO), 169.6 (CO) ppm.

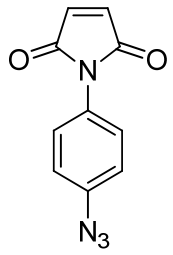
mp: 182-184 ºC (lit.[4] mp 179 ºC)

IR (KBr disk): 3368, 1707, 1529, 1416, 1401, 1314, 1238, 1157 cm^{-1}.

HR-MS (ESI^-): calcd. for $C_{15}H_{15}N_2O_4$ ($M-H^-$), 287.1037; found, 287.1042.

Elemental analysis: calcd. for $C_{15}H_{16}N_2O_4$, C, 62.49; H, 5.59; N 9.72; found, C, 62.46; H, 5.56; N, 9.63.

1-(4-Azidophenyl)-1*H*-pyrrole-2,5-dione (5)



To a stirred suspension of *tert*-butyl 4-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)phenylcarbamate (1.00 g, 3.5 mmol, 1 eq.) in dry CH_2Cl_2 (10 mL) was added dropwise CF_3COOH (5 mL) at 0 °C. The resulting clear solution was allowed to warm to room temperature and stirred for 2 hrs. Volatiles were removed *in vacuo* and the remainder taken up in a mixture of conc. acetic acid (10 mL) and water (10 mL). The clear solution was cooled to 0 °C in an ice bath, and sodium nitrite (340 mg, 4.9 mmol, 1.4 eq.) was added in one portion. After stirring for 1.5 hrs at 0 °C, sodium azide (340 mg, 5.2 mmol, 1.5 eq.) was added slowly. When the evolution of nitrogen gas had ceased, the reaction mixture was extracted with CH_2Cl_2 (20 mL). The organic phase was washed with sat. $NaHCO_3$ (20 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give the crude product. This was purified by column chromatography (Biotage SNAP 50g cartridge, eluting with hexane/ethyl acetate) to afford the title compound (470 mg, 63%) as yellow needles.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.78 (s, 2H), 7.05 (d, *J*=9.0 Hz, 2H), 7.28 (d, *J*=9.0 Hz, 2H) ppm.

^{13}C-NMR (100 MHz, $CDCl_3$): δ 119.6 (ArCH), 127.4 (ArCH), 127.9 (ArC), 134.2 (olefinic CH), 139.6 (ArC), 169.4 (CO) ppm.

mp: 116-118 ºC (lit.[1] mp 107 ºC)

IR (KBr disk): 3107, 2139, 2096, 1719, 1510, 1391, 1299, 1286, 1144 cm^{-1}.

HR-MS (CI+): calcd. for $C_{10}H_{10}N_5O_2$ ($M+NH_4^+$), 232.0835; found, 232.0844.

Elemental analysis: calcd. for $C_{10}H_6N_4O_2$, C, 56.08; H, 2.82; N 26.16; found, C, 56.00; H, 2.91; N, 26.05.

Expression and purification of proteins used in the study

Recombinant human $PHD2_{181–426}$ (*i.e.* the catalytic domain) was cloned, expressed in *E. coli* BL21(DE3), and purified by cation exchange and size exclusion chromatography, as described.[5] Apo-PHD2 was obtained by incubation with EDTA after cation exchange chromatography. Protein purity (> 95%) was assessed by SDS-PAGE.

The ternary GST-VCB complex was produced as described,[6] and purified by successive GST and gel filtration chromatography, thrombin cleavage, and further GST and gel filtration chromatography steps.

PHD2 hydroxylation assay

PHD2 substrate hydroxylation was measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Standard assay mixtures contained: 100 μM peptides (**CP** and **HCP**), 300 μM 2-oxogluatarate (2-OG), 4 mM ascorbic acid, 50 μM $FeSO_4 \cdot 7H_2O$ (dissolved first to 250 mM with 20 mM HCl, then 2.5 mM with Milli-Q water) and $PHD2_{181-426}$ (4 μM). Assay mixtures were made up to 50 μL with 50 mM Tris (pH 7.5). Reagents were pipetted into the base of a 5 ml tube as three separate spots: enzyme, substrate, and all the other reagents prepared as a master mixture. Assays were incubated at 37 °C for 30 minutes in a shaking incubator then quenched by 1:1 addition of 0.1% formic acid and stored at -20 °C for later MALDI-TOF analysis. For comparison, negative controls were without PHD2. Assays were carried out in triplicate. MALDI-TOF MS was performed on a MALDI-TOF microMX™ mass spectrometer (Waters Micromass™) in positive ion reflectron mode with the following parameters: laser energy 165, pulse 2050, detector 2700 and suppression 700. Calibration was carried out using 1 μl PepMix4 (Trypsinogen, Enolase, BSA) in 9 μl CHCA (recrystallized α-cyano-4-hydroxy-cinnamic acid) matrix (both Laser BioLabs). Sample were prepared by mixing 1 μL of quenched assay solution with CHCA MALDI matrix (1 μl) and spotting all together onto a MALDI 96 well sample plate and allowing to air dry before measurements. Data were analyzed using MassLynx v4.1.

FRET-based VCB binding assay

In order to analyze the binding of the probes (**CP** and **HCP**) to the VCB complex, a homogeneous fluorescence resonance energy transfer (FRET) assay was carried out essentially as described, using the analysis method reported for activity assays,[3,7] but without the addition of enzyme and cofactors. The prepared VCB complex was labelled with a Eu^{III}-cryptate linker (LANCE Eu-W1024-ITC, PerkinElmer; ~6 Eu per VCB), and the assay was performed in 384-well clear-bottom microplates (μclear, Greiner Bio-One) at a final probe concentration of 50 nM. The solution was allowed to reach binding equilibrium by shaking for 5 min at 25 ºC before detection on an Envision plate reader (Perkin Elmer). The data output ("FRET signal") is the ratio of the 665 nm and 615 nm emission signals resulting from the FRET excitation of streptavidin-allophycocyanin at 665 nm and the laser excitation of Eu^{III} at 615 nm, respectively, multiplied by 10,000.

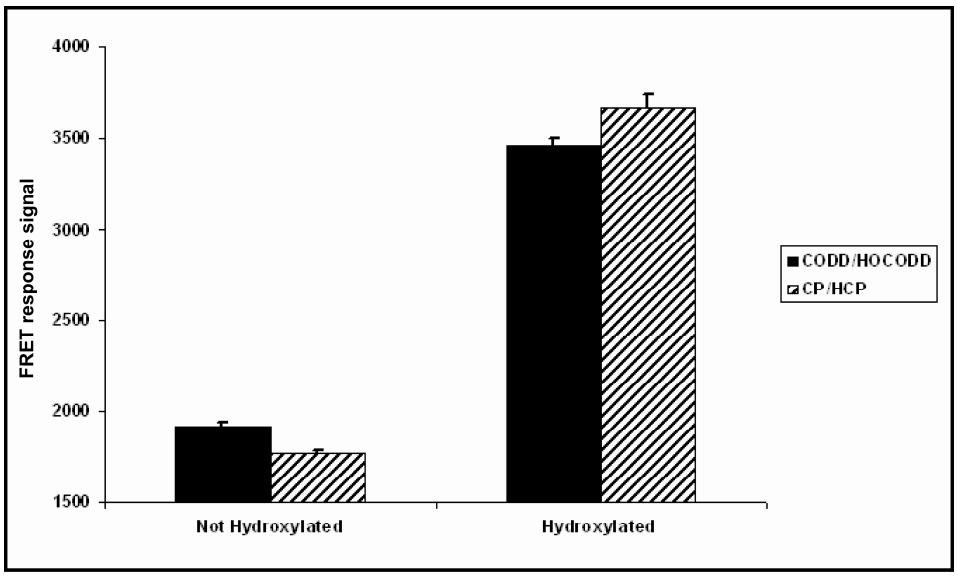


Fig. S2 Binding of the peptide probes (**CP** and **HCP**) to the VCB complex as analyzed by a FRET assay.[3,5] The readout was performed in triplicate and the standard deviations are given. The binding of biotinylated CODD and HO-CODD peptides is shown for comparison.

Cell cultures and preparation of whole cell lysates

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS,

Sigma-Aldrich, St Louis, Missouri, USA), 2mM *L*-glutamine (Sigma-Aldrich, St Louis, Missouri, USA), penicillin (100 UmL^{-1}) and streptomycin (100 μgmL^{-1}) at 37 ºC and 5% CO_2. HEK293T cells were transfected with the plasmid Flag-VHL[8] (pcDNA3) by a CaPO4 method[9] for the over-expression of human flag-tag VHL. After harvesting, cell pellets were washed twice with PBS and frozen with liquid nitrogen and stored at -80 ºC until use.

To obtain HEK293T cell lysates, cells were lysed for 30 min on ice in 0.125% NP40 PBS (pH 7.0) supplemented with complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation at 1000g for 5 min at 4 ºC, the supernatant was decanted and further centrifuged at 5000g for 5 min always at 4 ºC. After a further decantation, a resin slurry of avidin-coated agarose beads (Pierce, Thermo Scientific, Rockford, USA) was added to the lysate in order to remove from it the biotin-containing proteins (*e.g.* carboxylases), the resulting mixture was then rotated at 4 ºC for 30 min, centrifuged at 5000g (5 min, 4 ºC) and finally decanted. The total protein concentration was always determined according to the BCA™ Protein Assay Kit (Pierce, Thermo Scientific, Rockford, USA).

Photo cross-linking experiments

The photo cross-linking procedure is described here for the reaction between purified $PHD2_{181-426}$ and **CP**. Reaction with the purified VCB complex, (including with HEK293T cell lysates) were performed in an analogous manner; details are given in the respective Figure legends and/or in the next section.

$PHD2_{181-426}$ (5 µL of 40 µM Tris buffer solution, final concentration 4 µM, 5.6 µg), $MnCl_2$ (1 µL of 2.5 mM water solution, final concentration 50 µM) and *N*-oxalylglycine (NOG, 2.5 µL of 6 mM Tris buffer solution, final concentration 300 µM) were incubated with **CP** (10 µL of 20 µM Tris buffer solution, final concentration 4 µM) in Tris buffer (31.5 µL, 50 mM Tris, pH 7.5) in Eppendorf tubes (0.5 mL) with shaking at room temperature (45 min). Samples were then irradiated for 15 min (on ice) with UV light at 365 nm at an irradiance of 5 mW/cm^2 (Spectrolinker XL-1500).

After exposure to UV light, part of the samples were directly analyzed by MALDI-TOF MS and part was subjected to the affinity purification using avidin-coated agarose beads (see below).

Affinity enrichment experiments

The affinity enrichment procedure is described here for the reaction between the purified VCB complex and **HCP** in the presence of HEK293T cell lysates as background. For analyses with $PHD2_{181-426}$ and HEK293T cell lysates, the affinity enrichment processes were performed in an analogous way; details are given in the corresponding Figure legends and/or below in this section. The VCB complex (20 μL of 10 μM Tris buffer solution, final concentration 5 μM, ~ 3.6 μg) and **HCP** (4 μL of 50 μM Tris buffer solution, final concentration 5 μM) were incubated in the presence of 8 μL of HEK293T whole cell lysates (~ 5.0 μg/μL total protein concentration, ~ 40.0 μg) and of 8 μL of Tris buffer (50 mM Tris, pH 7.5) in an Eppendorf tube (0.5 mL) with shaking at room temperature for 45 min. The "competition control" sample was prepared in the same way by substituting the 8 μL of Tris buffer with 8 μL of a 500 μM Tris buffer solution of the HO-CODD peptide (final concentration 100 μM). The mixtures were then irradiated 20 min on ice with UV light at 365 nm at an irradiance of 5 mW/cm^2 (Spectrolinker XL-1500), and finally incubated with streptavidin-coated magnetic beads (50 μL, Dynabeads® MyOne™ Streptavidin C1, Invitrogen, Karlsruhe, Germany) for 30 min at room temperature on a shaker incubator. The beads were collected using a magnetic device (Dynal® DynaMag™ Spin, Invitrogen, Karlsruhe, Germany) and washed first five times with washing buffer (10mM HEPES, pH 7.2, 1M NaCl, 1% Triton X-100, 2mM EDTA, 4mM dithiotreitol), then three times with MS grade water. In other experiments (purified $PHD2_{181-426}$ alone and added into HEK293T cell lysates), after the photo cross-linking, the mixtures were incubated with avidin-coated agarose beads (Pierce® Monomeric Avidin Agarose, Thermo Scientific, Rockford, USA). In these cases washing was performed with the same buffer but without any device with careful removal of buffer by pipetting. The beads were stored at -20 ºC until further analysis by MALDI-TOF and/or SDS-PAGE followed by Western blotting or by silver staining, protein digestion and LC-MS/MS analysis. In the experiments aimed to label and affinity

enrich endogenous levels of (full length) PHD2 or the Flag-tagged VHL[8] the final concentrations of the probes (**CP** and **HCP**) were always 10 μM, the final concentrations of the competitors CODD and HO-CODD were 100 μM, the total protein amounts in the whole HEK293T cell lysates were ~ 200 μg for every single sample, and the affinity enrichment was always carried out with streptavidin-coated magnetic beads (see above). The "capture" of endogenous levels of (full length) PHD2 was performed in the presence of $MnCl_2$ (50 μM) and NOG (300 μM). Control experiments were always carried out under the same conditions as the "capture" reactions without irradiation.

MALDI-TOF analysis of photo-affinity tagging

The photo-affinity labelling experiments of $PHD2_{181-426}$ and VCB complex before and after the affinity enrichment were analysed by MS analyses using a MALDI-TOF microMX™ mass spectrometer (Waters Micromass™) in positive ion linear mode with the following parameters: laser energy 170, pulse 2050, detector 2700 and suppression 4500. Calibration was carried out using 1 μl ProteinMix (Cythochrome C, Myoglobin, Trypsinogen) in 9 μl SA (recrystallized sinapinic acid) matrix (both Laser BioLabs). Sample were prepared by mixing 1 μL of the irradiated mixture or of a water suspension of the beads with the SA matrix (1 μl) and spotting all together onto a MALDI 96 well sample plate and allowing to air dry before measurements. Data were analyzed using MassLynx v4.1. The yield of cross-linking was evaluated from the ratio between the area of the MALDI peak of the covalent adducts **CP**-PHD2 and **HCP**-VHL and the total area of the two peaks of the uncaptured and captured protein. For yield purposes the photo cross-linking experiments were carried out in triplicate (Fig. S5).

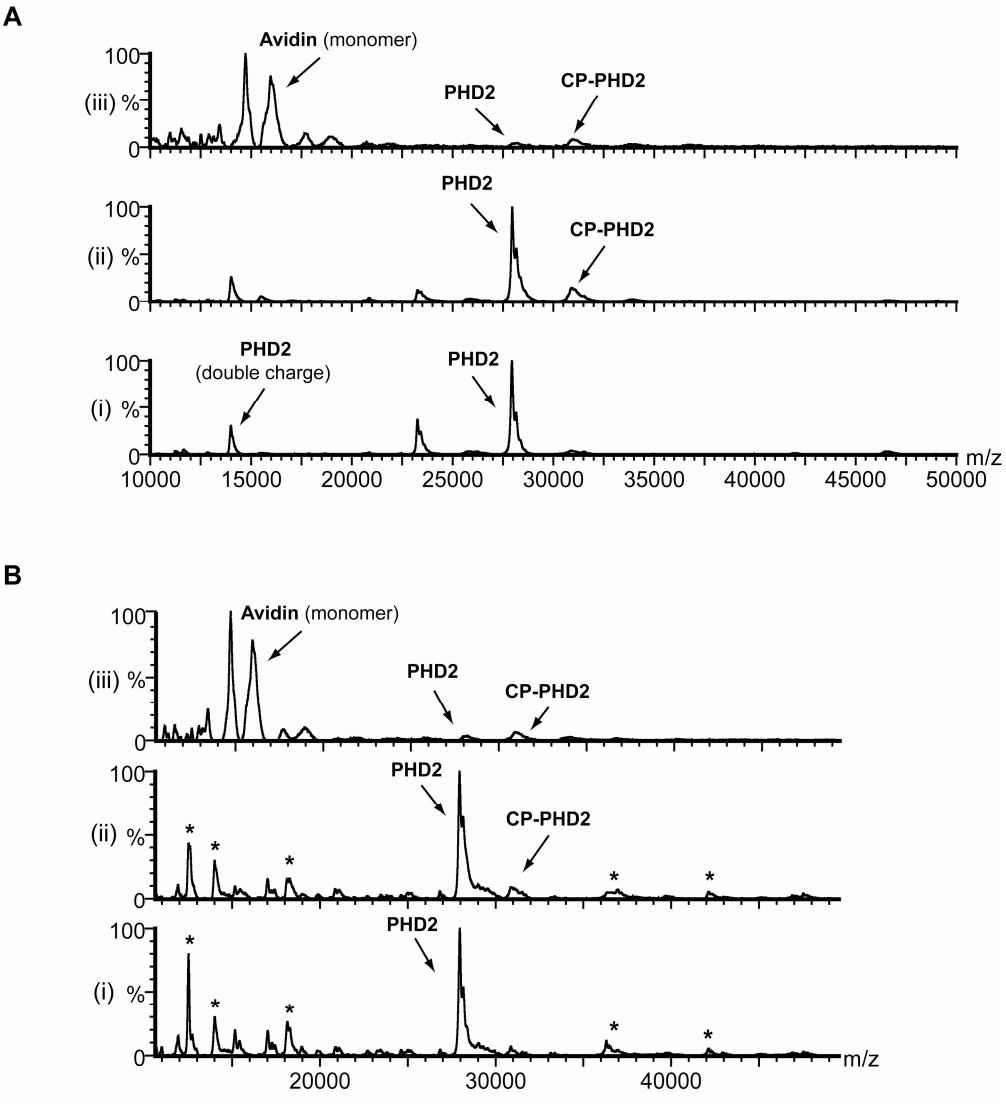


Fig. S3 Photo cross-linking to purified $PHD2_{181-426}$. (A) MALDI analysis of the cross-linking of $PHD2_{181-426}$ (4 μM) by **CP** (4 μM) in the presence of $MnCl_2$ (50 μM) and NOG (300 μM): (i), before irradiation; (ii), after irradiation; (iii) after irradiation and affinity purification. (B) MALDI analysis of the cross-linking of $PHD2_{181-426}$ (4 μM, 5.6 μg) by **CP** (4 μM) in the presence of $MnCl_2$ (50 μM), NOG (300 μM) and HEK293T cell lysates (60 μg): same legend as (A); background proteins are highlighted with an asterisk.

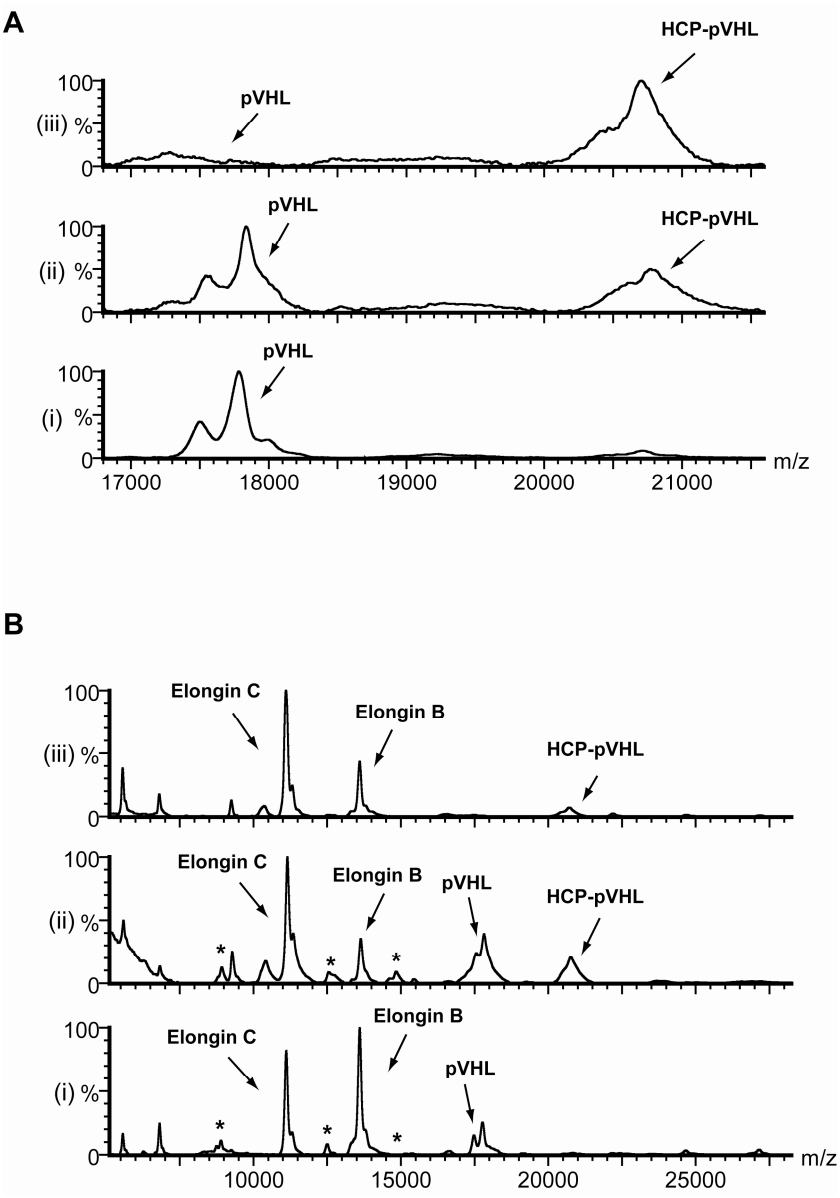


Fig. S4 Photo cross-linking to purified VCB. (A) Magnification of the pVHL region of the MALDI spectra in Fig. 3A: (i), before irradiation; (ii), after irradiation; (iii) after irradiation and affinity purification. (B) MALDI analysis of the cross-linking of pVHL (5 μM, 3.6 μg) by **HCP** (5 μM) in the presence of HEK293T cell lysates (40 μg): same legend as (A); background proteins are highlighted with an asterisk.

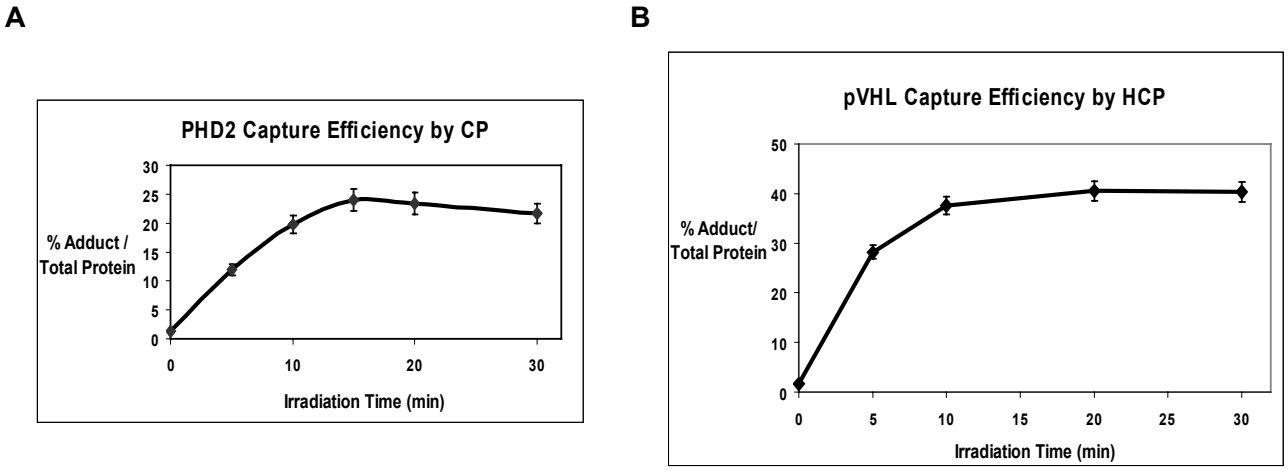


Fig. S5 Efficiency of protein "capture" by the peptide probes. The yield of cross-linking was evaluated from the ratio between the area of the MALDI peak of the covalent adduct protein-probe and the total area of the two peaks corresponding to the un-captured and captured protein. All experiments were carried out in triplicate. (A) $PHD2_{181-426}$ (4 μM) and **CP** (4 μM) were incubated (45 min, room temperature) in Tris buffer in the presence of Mn(II) (50 μM) and NOG (300 μM) then the mixture was analysed by MALDI. (B) VCB complex (5 μM) and **HCP** (5 μM) were incubated (45 min, room temperature) in Tris buffer and then the mixture was analysed by MALDI. Errors are given as standard deviations from the mean.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For analysis of photo-affinity tagging experiments by SDS-PAGE followed by silver staining or by Western blotting the beads with "captured" proteins were resuspended in an appropriate volume (depending on the amount of the beads) of reducing Laemmli[10] buffer and heated on an heating block at 95 °C for 10 min. Subsequently, the beads were separated from the released proteins using a magnet and/or carefully loading with a pipette the supernatant directly onto the gel. Gels were stained using the Pierce® Silver Stain for Mass Spectrometry (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions.

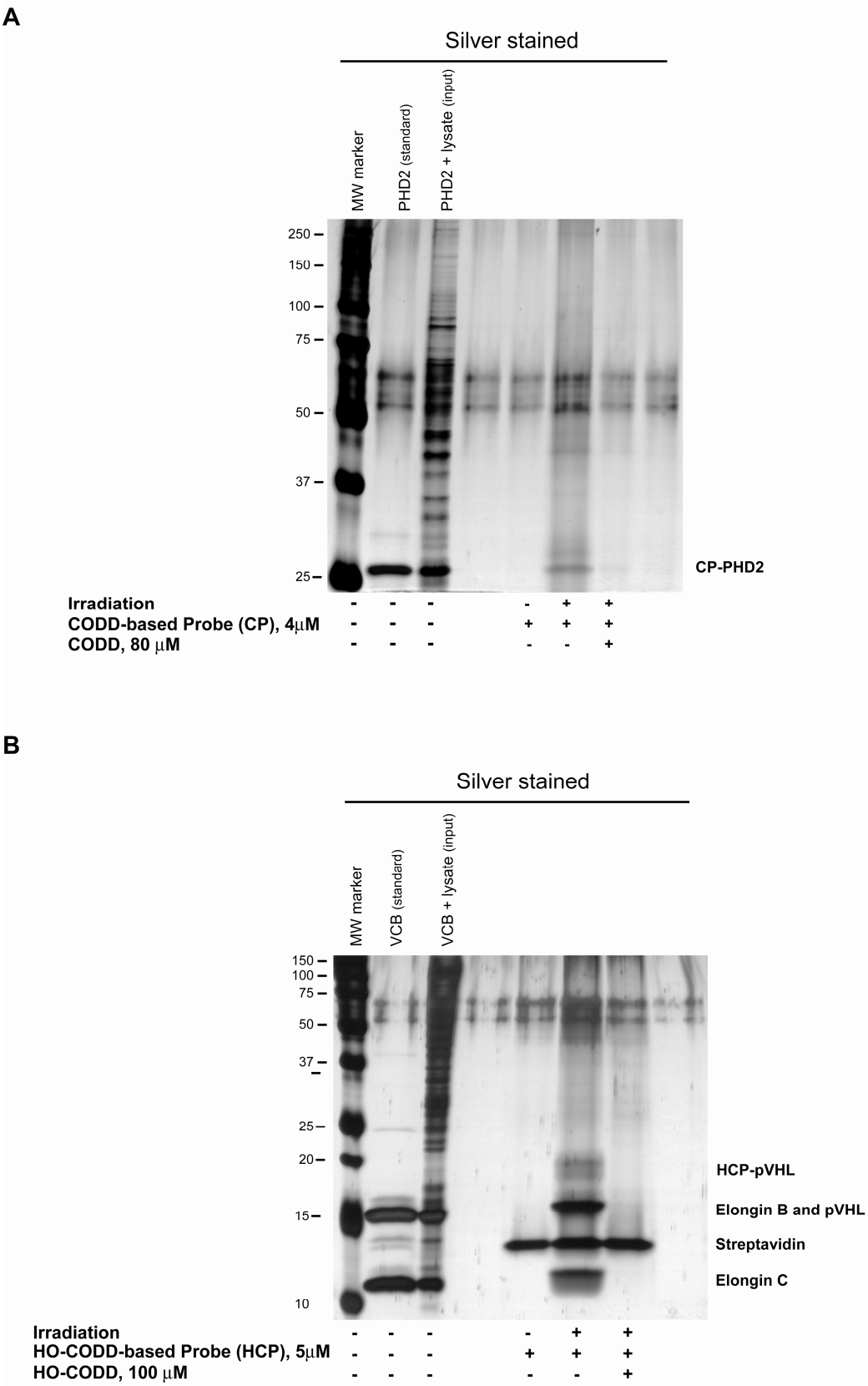


Fig. S6 Entire silver stained SDS-PAGE gel images of affinity enrichment of target proteins by the peptide probes. (A) Silver stained SDS-PAGE gel for the "capture" of $PHD2_{181-426}$ (4 μM, 5.6 μg) by **CP** (4 μM) in the presence of $MnCl_2$ (50 μM), NOG (300 μM) and HEK293T cell lysates (60

μg): $PHD2_{181-426}$ as standard was ~ 3% of the protein corresponding to the other lanes; the input lysate supplemented with $PHD2_{181-426}$ was ~ 4% of the total protein concentration corresponding to the other lanes.

(B) Silver stained SDS-PAGE gel for the "capture" of pVHL (5 μM, 3.6 μg) by **HCP** (5 μM) in the presence of HEK293T cell lysates (40 μg): the VCB complex as standard was ~ 3,5% of the protein concentration corresponding to the other lanes; the input lysate supplemented with VCB was ~ 4.5% of the total protein concentration corresponding to the other lanes.

Western blot analysis of photo-affinity tagging

Western blots were carried out according to standard laboratory procedures. After separation by SDS-PAGE, the proteins were transferred to PVDF membranes (Immobilon-P^{TM} Transfer Membrane, Millipore, Billerica, MA, USA). The membranes were blocked for 1 h at room temperature with a solution of 5% (wt/vol) skimmed milk powder in PBS buffer supplemented with 0.1% [vol/vol] Tween 20 [PBS-T]. Incubation with the primary antibody was performed for 1 h at room temperature or overnight at 4 ºC, followed by three wash steps in PBS-T and incubation with the secondary antibody for 1 h at room temperature. Antibodies were diluted in 5% skimmed milk powder in PBS-T as follows: polyclonal anti-PHD2 from rabbit (Abcam plc, Cambridge, UK) 1:1000, secondary anti-rabbit antibody conjugated to horseradish peroxidise (DakoCytomation, Glastrup, Denmark) 1:10000; monoclonal anti-PHD2 (clone 76A, prepared as reported previously)[11] from mouse 1:50, secondary anti-mouse antibody conjugated to horseradish peroxidise (DakoCytomation, Glastrup, Denmark) 1:10000; monoclonal anti-VHL from mouse (clone 32, BD Biosciences, BD Pharmingen^{TM}) 1:250, secondary anti-mouse antibody conjugated to horseradish peroxidise (DakoCytomation, Glastrup, Denmark) 1:10000; monoclonal anti-flag-HRP antibody from mouse (ANTI-FLAG® M2-Peroxidase, Sigma, Saint Louis, Missouri, USA) 1:10000. After three washes in PBS-T and one in PBS, membranes were usually treated with ECL Plus Western Blotting Detection System (Amersham^{TM}, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. In the Western blot detection of endogenous levels of

human PDH2 and of the Flag-tagged VHL the membranes were treated with the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, USA) always according to the manufacturer's instructions. Kodak X-Omat LS films (Sigma-Aldrich, St. Louis, Missouri, USA) were used to detect the chemiluminescence. In case of blots for the detection of biotinylated proteins, Amersham ECL Streptavidin Horseradish Peroxidase Conjiugate (GE Healthcare, Piscataway, USA) was used instead of a first antibody at a dilution of 1:500 in 5% skimmed milk powder in PBS-T and blots were developed directly after washing three times with PBS-T and once with PBS.

Protein digestion

The in-gel digestion for the identification of the captured $PHD2_{181-426}$ (Fig. 2F and Fig. S5A) and VCB (Fig. 3F and Fig. S5B) in the photo-affinity labelling tests in the presence of HEK293T cell lysates as background was carried out essentially as reported.[12]

Nano LC-MS/MS

The LC-MS/MS experiments for the identification after in-gel digestion of $PHD2_{181-426}$ and VCB complex photo labelled in the presence of HEK293T cell lysates as background, were carried out using an Ultimate™ HPLC (LC-Packings, Dionex, Amsterdam, The Netherlands) system directly coupled to a 3D high-capacity ion trap (HCTplus™, Bruker Daltonics, Bremen, Germany) essentially as reported.[12]

Proteins were identified by automated database searching against the human UniProtKB/Swiss-Prot using Mascot (Matrix Science) database essentially as reported.[13]

Table S1. Proteins identified from SDS-PAGE gel.

SDS-PAGE Band	Protein name	UniProt/ Swiss-Prot	Protein score[a]	Coverage rate (%)[b]	Peptide Matches
Band at ~28-31 kDa (Fig. 2F and S6A)	**PHD2**	Q9GZT9	677	62%	ITWIEGK LLFFWSDR AQFADIEPK CVTCIYYLNK FTDGQLVSQK HVDNPNGDGR ETGQQIGDEVR LALEYIVPCMNK HGICVVDDFLGK VELNKPSDSVGK NPHEVQPAYATR RNPHEVQPAYATR YAITVWYFDADER VELNKPSDSVGKDVF AMVACYPGNGTGYVR ALHDTGKFTDGQLVSQK HGICVVDDFLGKETGQQIGDEVR
Upper band at ~18-21 kDa (Fig. 3F and S6B)	**pVHL**	P40337	110	28%	LDIVR RLDIVR CLQVVR SLVKPENYR EPSQVIFCNR SLYEDLEDHPNVQK
Lower band at ~18-21 kDa (Fig. 3F and S6B)	**pVHL**	P40337	239	46%	LDIVR CLQVVR SLVKPENYR SLVKPENYR EPSQVIFCNR SLYEDLEDHPNVQKDLER SLYEDLEDHPNVQK VVLPVWLNFDGEPQPYPTLPPGTGR
Broad band at ~13-16 kDa (Fig. 3F and S6B)	**Elongin-B**	Q15370	210	59%	IVEGILK TTIFTDAK DDQLLDDGK ESSTVFELK MDVFLMIR ESSTVFELKR LYKDDQLLDDGK TLGECGFTSQTARPQAPATVGLAFR
Broad band at ~13-16 kDa (Fig. 3F and S6B)	**pVHL**	P40337	151	27%	LDIVR CLQVVR SLVKPENYR EPSQVIFCNR SLYEDLEDHPNVQK
Band at ~13 kDa (Fig. 3F and S6B)	**Streptavidin**	P22629	400	27%	STLVGHDTFTK INTQWLLTSGTTEANAWK YDSAPATDGSGTALGWTVAWK
Band at ~11-12 kDa (Fig. 3F and S6B)	**Elongin-C**	Q2KII4	261	63%	EIPSHVLSK EHALTSGTIK VCMYFTYK LISSDGHEFIV LISSDGHEFIVKR AMLSGPGQFAENETNEVNFR

[a] Protein scores were determined using Mascot version 2.3 (Matrix Science). The values represent – log of the probability that this identification is a random match.
[b] The coverage rate percentages are relative to the truncated proteins $PHD2_{181-426}$, $pVHL_{54-213}$ and Elongin C_{17-112} and to the full length Elongin B.

Supplemental notes and references

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